This antibody is intended for use in diagnostic and research applications. It is important to consult the package insert for complete instructions and precautions. Indications and use:

1. **Note:** The c-KIT gene encodes a receptor tyrosine kinase that is expressed in the gastrointestinal stromal tumor (GIST) cell line. It is also expressed in other malignancies, including seminomas, melanomas, and breast and skin basal cells. The c-KIT protein is involved in signal transduction pathways, and its activation is associated with tumorigenesis.

2. **Summary and Explanation:** The c-KIT gene was cloned and characterized by Yarden et al. in 1987. Its oncoprotein product is an approximately 145 kD to 125 kD transmembrane glycoprotein that is structurally similar to platelet-derived growth factor receptor (PDGFR). The protein has associated tyrosine kinase activity similar to that of several growth factor receptors and is involved in the signaling pathways of the c-kit family. Conjugation of the extracellular ligand-binding domain and an intracellular domain is consistent with the extracellular ligand and binding domain and an intracellular kinase domain. This suggests that c-KIT may be involved in signal transduction when bound by its ligand, stem cell factor, and may stimulate mitogenic activity (3).

3. **Normal cellular elements that stain positively for c-KIT include intestinal cells of Cajal (ICC), mast cells, breast epithelium, and small bowel cells (3).** Additional antibodies, such as anti-CD34 and PDGF-R, can be used to distinguish c-KIT from other cell types, and this antibody provides an independent and concordant result.

4. **The Ventana PATHWAY Anti-c-KIT (9.7) Primary Antibody was compared to the ICA on independent sets of samples and found to provide acceptably concordant results.**

5. **Targeted Therapy of GISTs with the Tyrosine Kinase Inhibitor Imatinib Mesylate (Gleevec/Glivec):** Imatinib mesylate (STI571; Gleevec, Glivec) is a 2-phenylpyrimidine derivative that blocks the binding of ATP to ABL kinase. Developed by Novartis Pharmaceutical Corporation in collaboration with Dr. Brian Druker (Oregon Health & Science University), this drug has received widespread attention for its effectiveness against chronic myelogenous leukemia (CML). The BCR-ABL fusion gene product of the Philadelphia chromosome in CML is responsible for driving tumor proliferation. More than 85% of chronic phase CML patients taking one oral dose per day achieve a complete hematologic response and many reach a complete cytogenetic remission (7, 20). Several excellent reviews on this topic have been published (22, 27). Imatinib mesylate received FDA approval for the treatment of CML in May, 2001, and received approval for its second indication, GIST, in February 2002.

6. **Imatinib mesylate is not perfectly specific and inhibits various kinases that are closely related to ABL, including PDGFR (PDGF-related), PDGFR (Gleevec/Glivec package insert).** In 1999, Dr. Michael Heinrich (Oregon Health & Science University) demonstrated that imatinib mesylate is a potent inhibitor of KIT in vitro (9). It should be noted that activating mutations of c-KIT are also found in other human malignancies, most commonly in normal cell tumors and seminomas (11).

7. **The Role of KIT Gene Mutations in the Development of GISTs:** In their landmark 1998 publication, Dr. Hirota and his colleagues established not only that GISTs express KIT, but that KIT gene mutations are present in these tumors. Furthermore, they showed that the resulting mutant KIT isoforms demonstrate kinase activity in the absence of stem cell factor, the natural ligand for KIT (17). These observations have been confirmed by a growing number of groups and it is now established that KIT mutations are present in >85% of GISTs (11, 16, 24, 30, 31). The majority of mutations occur in exon 11 (50-70% of GISTs) and includes a wide range of deletions, insertions, point mutations, or combinations thereof. An insertion/duplication of six base pairs in exon 9 is found in ~15% of GISTs, almost exclusively in those arising in the small intestine. Mutations also occur in exons 7, 9, 6, 10, 11, and 17, but are much rarer (11, 12). Regardless of the exon involved, KIT gene mutations in GISTs are invariably in frame and, when cloned and expressed in vitro, have constitutive kinase activation. Moreover, phosphorylation of KIT (a marker of kinase activation) is consistently detectable in GIST tumor extracts, supporting a direct role for KIT in intracellular signaling (31). There are GISTs, however, that do not have detectable KIT gene mutations and may therefore be driven by activation of other signaling pathways (14, 15). It should be noted that activating mutations of c-KIT also occur in other human malignancies, most commonly in normal cell tumors and seminomas (11).
Because GISTs have a relatively broad morphologic spectrum, the differential diagnosis includes a number of mesenchymal, neural, and neuroendocrine neoplasms that occur in the abdomen. These include leiomyoma, leiomyosarcoma, schwannoma, malignant peripheral nerve sheath tumor, inflammatory myofibroblastic tumor, fibromatosis, neuroendocrine tumors (carcinoid and islet cell), malignant mesothelioma, angiosarcoma, and sarcomatoid carcinoma. Recent success in treating GISTs with imatinib mesylate has placed new emphasis on the importance of making this diagnosis accurately. Fibromatosis and leiomyosarcoma are two tumors that are not infrequently mistaken for GIST.

In 1998, Drs. Seichi Hirota (Osaka University) and Lars-Gunnar Kindblom (University of Gothenburg) each independently observed that GISTs express the receptor tyrosine kinase KIT (CD117) (17, 21). Their observations provided a clue to the possible cell of origin for GISTs, namely the interstitial cells of Cajal (ICC). These inconspicuous, dendritic-like cells are widely distributed throughout the muscularis propria of the esophagus, stomach, small and large bowel. They play an important role in gut motility by regulating slow-wave contractions. Like GISTs, ICC express KIT and the majority are also positive for CD34. The hypothesis that GISTs are pathogenetically related to ICC in the gut wall, as proposed by both Dr. Hirota and Dr. Kindblom, is now widely accepted (17, 21).

Subsequent studies from a large number of different laboratories have confirmed that KIT is the single most specific marker of GISTs. Immunodetectable KIT is present on tumor cells in approximately 90% of cases. In the vast majority of tumors KIT expression is strong and uniform, but some cases show only focal positivity, and KIT is absent in a small subset (~5%) of tumors that are otherwise morphologically and immunophenotypically consistent with GIST. Among KIT-positive GISTs, CD34 expression is detectable in 60-70% of cases, while 30-40% are positive for smooth muscle actin (SMA), and 5% for S-100 protein. None of these antigens are specific for GISTs. Dermal expression in true KIT-positive GISTs is extremely uncommon (1-2% of cases) and is usually focal (8, 24, 25).

**PRINCIPLES AND PROCEDURES**

**PATHWAY Anti-c-KIT (9.7) Primary Antibody** is a rabbit monoclonal antibody which binds to the internal domain of the KIT oncoprotein in paraffin-embedded tissue sections. The specific antibody is localized by a biotin-conjugated secondary antibody formulation that recognizes rabbit and mouse immunoglobulins. This step is followed by the addition of a streptavidin-enzyme conjugate that binds to the biotin present on the secondary antibody. The specific antibody-secondary antibody-streptavidin enzyme complex is then visualized with a precipitating enzyme reaction product. Each step is incubated for a precise time and temperature. At the end of each incubation step, sections are washed to stop the reaction and remove unbound material that would hinder the desired reaction in subsequent steps. Results are interpreted using a light microscope and in the differential diagnosis of pathophysiological processes, which may or may not be associated with a particular antigen.

The use of Ventana Medical Systems’ prediluted PATHWAY Anti-c-KIT (9.7) Primary Antibody and ready-to-use detection kits, in combination with a Ventana Automated Slide Stainer, reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting, and manual reagent application. For further information refer to the Ventana Automated Slide Stainer Operator’s Manual.

Histological tissue preparations have the advantage of intact tissue morphology to aid in the interpretation of the c-KIT positivity of the sample. All histological tests should be interpreted by a specialist in gastrointestinal tumor morphology, and/or pathology, and the results should be used in conjunction with other clinical and laboratory data.

**MATERIALS AND METHODS**

**A. Reagents Provided**

**PATHWAY Anti-c-KIT (9.7) Primary Antibody (clone 9.7) consists of one dispenser of 5 ml (50 test) of prediluted reagent. The dispensers contain approximately 25 µg of antibody in Tris buffer, pH 7.5, with carrier protein, non-ionic detergent, and 0.09% sodium azide as a preservative. The antibody is directed against a synthetic peptide from the C-terminal (cytoplasmic) domain of the KIT oncoprotein.**

**PATHWAY Anti-c-KIT (9.7) Primary Antibody is obtained from cell culture supernatant. Total protein concentration is approximately 14 mg/ml. Specific antibody concentration is approximately 5 µg/ml. PATHWAY Anti-c-KIT (9.7) Primary Antibody is rabbit immunoglobulin class IgG. There is no known irrelevant antibody in the preparation. The specificity of the antibody was demonstrated by Western blot analysis and by immune inhibition assay where the immunostain was inhibited with the immunizing c-KIT peptide.**

**B. Reconstitution, Mixing, Dilution, Titration**

Ventana Medical Systems’ PATHWAY Anti-c-KIT (9.7) Primary Antibody is optimized for use on Ventana Automated Slide Stainers, and for manual application in combination with Ventana VIEW DAB Detection Kit and accessories. No reconstitution, mixing, dilution, or titration is required. Further dilution may result in loss of antigen staining. Any such change must be validated by the user. Differences in tissue processing and technical procedure in the user’s laboratory may produce significant variability of results, necessitating regular performance of "in-house" controls (see Quality Control Procedures, page 4).

**C. Materials and Reagents Needed But Not Provided**

The following reagents and materials are required (or staining but not provided with PATHWAY Anti-c-KIT (9.7) Primary Antibody:

1. Negative tissue control slide (normal colon tissue)
2. Positive tissue control slide (GIST tissue)
3. Microtome
4. Microscope slides, stained or polystyrene-coated
5. Drying oven capable of maintaining a temperature of 60°C ± 5°C
7. Xylene (histological grade)
8. Ethanol or reagent alcohol (histological grade)
9. Deionized/distilled water
10. Ventana Automated Slide Stainer
11. Ventana NexES® Slide Staining System, or
12. Ventana BenchMark™ Slide Staining System, or
13. Ventana BenchMark™ XT Slide Staining System
14. Ventana Medical Systems’ VIEW DAB Detection Kit
15. Ventana APK Wash Solution Concentrate (10X) (NexES IHC automated slide stainers)
16. Ventana EZ Prep™ Solution Concentrate (10X)
17. Ventana Cell Conditioning 1 (CC1) Solution Pre-dilute (BenchMark and BenchMark XT automated slide stainers)
18. Ventana Low Temperature Liquid Coverslip™ Solution Pre-dilute (NexES IHC automated slide stainers, or Ventana High Temperature Liquid Coverslip Solution Pre-dilute (BenchMark and BenchMark XT automated slide stainers)
19. COVER GLASS
20. LIGHT MICROSCOPE (20X-80X)
21. Staining jars or baths
22. WASH BOTTLES
23. Absorbent wipes
24. Ventana Hematoxylin or Nuclear Fast Red Counterstain
25. Ventana Bluing Reagent
26. Decloaking Chamber, Digital Pressure Cooker (Biocare Medical) (NexES IHC automated slide stainers)
27. Tissue Tek™ slide rack (Biocare Medical)

**Storage and Handling**

Store PATHWAY Anti-c-KIT (9.7) Primary Antibody at 2° to 8°C. Do not freeze.

Replace the cap and store dispenser in an upright position when not in use on the instrument. This will insure proper reagent delivery. Use care to avoid damaging dispensers.

PATHWAY Anti-c-KIT (9.7) Primary Antibody should be allowed to stand at least 30 minutes at room temperature prior to use. PATHWAY Anti-c-KIT (9.7) Primary Antibody must be returned to storage conditions identified above immediately after use.

Every PATHWAY Anti-c-KIT (9.7) Primary Antibody dispenser is expiration dated. Do not use reagent beyond expiration date listed on the antibody dispenser label for the prescribed storage method. Any storage conditions other than those specified in this product information sheet must be validated by the user.

**Indications of Instability**

When properly stored, the reagent is stable through dating indicated on the label. There are no obvious signs to indicate instability of this product. However, positive and negative controls should be run simultaneously with unknown specimens. Positive controls assure that the specimen staining was carried out correctly. Negative reagent controls are used to assess non-specific staining which must be taken into consideration when interpreting results. Decrease in staining intensity of positive control material may indicate reagent instability. If this is observed, contact Ventana Medical Systems Customer Care (800-227-2155).
Specimen Collection and Preparation for Analysis

Formalin-fixed, paraffin-embedded tissues are suitable for use with PATHWAY Anti-c-KIT (9.7) Primary Antibody when used with Ventana AWE DAB Detection and a Ventana Automated Slide Stainer, or manual application.

The recommended fixative is 10% neutral buffered formalin. The amount recommended is 15 to 20 times the volume of tissue. No fixative will penetrate more than 2 to 3 mm of solid tissue or 5 mm of porous tissue in a 24 hour period. A 3 mm or smaller section of tissue should be fixed no less than 12 hours and no more than 24 hours.

Fixation can be performed at room temperature (15-25°C) (37)

Properly fixed and embedded tissues expressing the antigen will keep at least 2 years if stored in a cool place (15-25°C). The Clinical Laboratory Improvement Act (CLIA) of 1988 requires in 42 CFR 493.1259(b) that "The laboratory must retain stained slides at least ten years from the date of examination and retain specimen blocks at least two years from the date of examination" (4).

Approximately 5 μm thick sections should be cut and picked up on glass slides. The slides should either be silanized or coated with a polylysine compound. Tissue should be deparaffinized on the instrument if the option is selected, barcode slides and place of antigen staining. Any such change must be validated by the user.

Slides stained on the BenchMark or BenchMark XT automated slide stainers can be deparaffinized on the instrument. If this option is selected, barcode slides and place them on the instrument. If the option is not selected follow the Manual Deparaffinization Procedure above.

Manual Deparaffinization Procedure

Required when using the NexES IHC automated slide stainers, manual staining methods, or if deparaffinization is not selected on the BenchMark or the BenchMark XT automated slide stainer:

1. Prepare the Decloaking Chamber for use.
2. Place the pan into the chamber. NOTE: Make sure that the outside of the pan is completely dry prior to placing it in the chamber. If the outside of the pan is wet, the pressure cooker will make a croaking noise and any water in the chamber will cause a malfunction.
3. Align the handles of the pot with the handles of the chamber.
4. Fill the pan with 500 ml of distilled water and place the heat shield, (Masonic screen), in the center of the pot (the heat shield keeps the plastic containers from warping).
5. Place each Tissue Tek staining dish, filled with 250 ml of Cell Conditioning Solution 1 Pre-Dilute, CC1 (pH 8.5), and the appropriate slides on the heat shield which is placed in the center of the pan. Up to 2 containers may be placed in the chamber, but make sure both are touching the heat shield.
6. Put the Decloaking Chamber lid on and secure. (Align the open arrow with the white dot on the lid handle. Gently rotate the lid clockwise to the closed position. When the lid is locked in the proper position, the Vent Lever will lower the weight on the vent nozzle.)
7. Turn the heat to 10 and lock into place (approximately 120°C).
8. Turn on the Decloaking Chamber and monitor until the pressure reaches 17-25 psi and the temperature is 120° - 125° C. Once the Decloaking Chamber reaches the desired temperature, time for 2 minutes using a calibrated manual timer, as the Decloaking Chamber timer is not "real time" consistent. When the manual timer goes off, turn the Decloaker timer to the off position. The heat will turn off and the light will turn from "heat on" to "keep warm". NOTE: Technician must monitor temperature and pressure conditions to confirm desired specifications are met.
9. Once the cell conditioning procedure is completed, turn off the Decloaking Chamber.
10. The technician can monitor the declining pressure by periodically checking the pressure gauge. When pressure reaches 0 psi, the Decloaking chamber can now be opened safely. Rotate the lid counterclockwise and remove it slowly, allowing steam to escape away from your hand. NOTE: Be very careful when opening lid, as surface and liquid temperatures remain high.
11. Remove the container of slides from the pan and place slide holders containing processed slides in a container of room temperature deionized water.
12. Once rinsing is complete, place the slides in a Tissue Tek slide rack filled with deionized water for maintaining hydration while slides are barcoded. One by one, remove the slides from the slide rack, blot the frosted end dry, ensuring the tissue sections do not dry during the process. Label the slides with the appropriate barcode label, and return it to the slide container. Repeat this process for all slides.
13. Once all slides have been barcoded, empty the deionized water from the slide container and refill it with 1X APK Wash. Slides should remain in this solution until ready to perform staining run.

NOTE: Slides must be stained within 4 hours of being cell conditioned. They may be left in wash solution for up to 2 hours if necessary, as long as tissue is not allowed to dry. Blot-dry frosted end of processed tissue slides, ensuring that the tissue sections do not dry. Properly label processed slides with bar codes and place in Wash Solution until ready to load on Ventana NexES IHC automated slide stainer or manual staining application.

WARNINGS AND PRECAUTIONS

1. This antibody is intended for in vitro diagnostic use.
2. Take reasonable precautions when handling reagents. Use disposable gloves when handling suspected carcinogens or toxic materials, for example xylene, formaldehyde, or DAB.
3. Do not smoke, eat or drink in areas where specimens or reagents are being handled.
4. Avoid contact of eyes and mucous membranes with reagents. If reagents come in contact with sensitive areas, wash with copious amounts of water.
5. Patient specimens and all materials coming into contact with them should be handled as if capable of transmitting infection and disposed of with proper precautions. Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
6. Avoid microbial contamination of reagents as this could produce incorrect results.
7. Incubation times and temperatures other than those specified may give erroneous results. Any such change must be validated by the user.
8. The reagents have been optimally diluted and further dilution may result in loss of antigen staining. Any such change must be validated by the user.
9. When used according to instructions, this product is not classified as a hazardous substance. The preservative in the reagent is sodium azide (NaN3). Symptoms of overexposure to NaN3 include skin and eye irritation, and irritation of mucous membranes and upper respiratory tract. The concentration of sodium azide in this product is less than 0.1% and does not meet the OSHA criteria for a hazardous substance. Build-up of NaN3 may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with larger volumes of water to prevent azide accumulation in plumbing.
10. Consult local or state authorities with regard to recommended method of disposal.

INSTRUCTIONS FOR USE

Step by Step Procedure

PATHWAY Anti-c-KIT (9.7) Primary Antibody was developed for use on a Ventana Automated Slide Stainer and for manual application in combination with Ventana Medical Systems' Detection Kits and accessories. Recommended staining protocols for the Ventana Automated Slide Stainer are listed in Table 1 below. The parameters for the automated procedures can be displayed, printed, and edited according to the procedure in the Ventana Automated Slide Stainer Operator's Manual. Other operating parameters for the Automated Slide Stainers have been preset at the factory. Manual application must follow the prescribed protocol for optimal staining results.

The procedure for staining PATHWAY Anti-c-KIT (9.7) Primary Antibody on the Ventana Automated Slide Stainer is as follows. (Refer to the Operator's Manual for specific details on the operation of the Ventana Automated Slide Stainer)

NexES IHC Automated Slide Stainers

Antigen Unmasking Required:
1. Slides are deparaffinized through a series of xylene and gradient alcohols to wash and appropriate buffer (see Manual Deparaffin Procedure, above). Perform antigen unmasking procedure (see Manual Antigen Unmasking Procedure, above) and transfer slides to APK Wash (1X).
2. Load the PATHWAY Anti-c-KIT (9.7) Primary Antibody dispenser, appropriate detection kit dispensers, and required accessory reagents onto the reagent tray and place them on the NexES IHC automated slide stainer. Check bulk fluids and waste.
3. Each slide must be labeled with the appropriate bar code specifying the staining procedure and PATHWAY Anti-c-KIT (9.7) Primary Antibody. The slide bar codes should be applied after the antigen enhancement procedure. Dry the painted end of the slide and then apply the PATHWAY Anti-c-KIT (9.7) Primary Antibody slide bar code.
4. Load the deparaffinized, antigen unmasked labeled slides from the APK Wash (1X). Avoid tissue drying.
5. Initiate the staining run.
6. If counterstain is selected, it will be applied to the slide and incubated with mixing for 2 minutes at 37°C.
7. Mount and coverslip per standard laboratory practice for DAB.

Table 1. Recommended Protocols for PATHWAY Anti-c-KIT (9.7) Primary Antibody

<table>
<thead>
<tr>
<th>Step</th>
<th>NexES®</th>
<th>Platform / Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Antigen Enhancement/Cell</td>
<td>CC1 sofn, 2 min, Decloaking Chamber, 120°C</td>
</tr>
<tr>
<td>2.</td>
<td>Endogenous peroxidase block</td>
<td>Online, 4 min, 37°C</td>
</tr>
<tr>
<td>3.</td>
<td>PATHWAY Anti-c-KIT (9.7) Primary</td>
<td>1 dispense (drop) reagent, 8 min, 37°C</td>
</tr>
<tr>
<td>4.</td>
<td>Biotinylated goat anti-rabbit IgG</td>
<td>1 dispense (drop) conjugate, 8 min, 37°C</td>
</tr>
<tr>
<td>5.</td>
<td>Streptavidin-horseradish peroxidase conjugate</td>
<td>1 dispense (drop) reagent, 8 min, 37°C</td>
</tr>
<tr>
<td>6.</td>
<td>Substrate/DAB chromogen</td>
<td>1 dispense (drop) reagent, 8 min, 37°C</td>
</tr>
<tr>
<td>7.</td>
<td>Copper enhancer</td>
<td>1 dispense (drop) enhancer, 4 min, 37°C</td>
</tr>
<tr>
<td>8.</td>
<td>Hematoxylin counterstain</td>
<td>1 dispense (drop) stain, 2 min, 37°C</td>
</tr>
</tbody>
</table>

**BenchMark or BenchMark XT Automated Slide Stainers**

1. The PATHWAY Anti-c-KIT (9.7) Primary Antibody dispenser, appropriate detection kit dispensers, and required accessory reagents are loaded onto the reagent tray and placed on the Ventana BenchMark or BenchMark XT. Check bulk fluids and waste.
2. Each slide must be labeled with the appropriate bar code specifying the staining procedure and PATHWAY Anti-c-KIT (9.7) Primary Antibody.
3. Initiate the staining run.
4. If counterstain is selected, it will be applied to the slide and incubated with mixing for 2 minutes (Benchmark) or 4 minutes (BenchMark XT) at 42°C.
5. Mount and coverslip per standard laboratory practice for DAB.

**For All Instruments**

1. Start the staining run.
2. At the completion of the run, remove the slides from the automated slide stainer.
3. For IVIEW DAB detection, wash in a mild dishwashing detergent or alcohol to remove the coverslip solution; dehydrate, clear, and coverslip with permanent mounting media in the usual manner.

**PATHWAY Anti-c-KIT (9.7) Primary Antibody Manual Assay Procedure:**

For the manual applicationpathway Anti-c-KIT (9.7) Primary Antibody and detection reagents the reagent dispensers are manually dispensed to give a reproducible drop volume.

**Note:** Each drop from the dispenser delivers ~100 μL. An average tissue section on a slide should require two drops for adequate coverage.

1. Slides are chemically deparaffinized through a series of xylene clearing reagents, then through a series of gradient alcohols to water (see section Manual Deparaffinization Procedure, page 3).
2. Note: Slides must be horizontal, on a flat surface, when applying the reagents.
3. Tap off excess liquid. Using a lint-free tissue (such as Kimwipe or gauze pad), carefully wipe around the specimen to remove any remaining liquid and to keep reagents within the prescribed area.
4. Apply enough volume of the endogenous peroxidase block (inhibitor reagent) to cover the specimen and incubate for 10 minutes at room temperature (20-26°C).
5. Slides are then rinsed in deionized water and placed in a Decloaking Chamber for the antigen enhancement process using Ventana’s CC1 cell conditioning solution as described in section II. F., Specimen Collection and Preparation for analysis.
6. Rinse the slides in APK Wash Solution.
7. Tap off excess buffer and wipe slides as before.
8. Apply enough volume of the AMEW Bioевloluted Ig reagent to cover the specimen and incubate for 10 minutes at room temperature.
9. Rinse the slides in APK Wash Solution.
10. Tap off excess buffer and wipe slides as before.
11. Apply enough volume of the AMEW SA-IRP to cover the specimen and incubate for 4 minutes at room temperature.
12. Rinse the slides in APK Wash Solution.
13. Tap off excess buffer and wipe slides as before.
14. Apply enough volume of the AMEW SA-HRP to cover the specimen and incubate for 10 minutes at room temperature.
15. Rinse the slides in APK Wash Solution.
16. Tap off excess buffer and wipe slides as before.
17. Mix equal volumes of the DABH2O2 mixture to cover the specimen and incubate for 4 minutes at room temperature.
18. Rinse the slides in APK Wash Solution.
19. Tap off excess buffer and wipe slides as before.
20. Apply 1 drop of the AMEWA Copper reagent and incubate the slides for 4 minutes at room temperature.
21. Rinse the slides in APK Wash Solution to complete the manual staining protocol.
22. Counters with hematoxylin.
23. Mount and coverslip per standard laboratory practice for DAB.

**Quality Control Procedures**

Differences in tissue processing and technical procedures in the user’s laboratory may produce significant variability in results, necessitating regular performance of in-house controls in addition to the following procedures. Consult the quality control guidelines of “Special report: Quality control in Immunohistochemistry” (8) and/or the Proposed NCCLS guideline for IHC (29).

**Positive Tissue Control**

A positive control tissue fixed and processed in the same manner as the patient specimens must be run for each set of test conditions and with every PATHWAY Anti-c-KIT (9.7) Primary Antibody staining protocol performed by the instrument. This tissue should contain both positive staining cell/tissue components and negative cell/tissue components and serve as both the positive and negative control tissue. Control tissues should be fresh autopsychi/biopsychi specimens prepared and fixed as soon as possible in a manner identical to test sections. Such tissues may monitor all steps of the analysis, from tissue preparation through staining.

A tissue with weak positive staining is more suitable than strong positive staining for optimal quality control and to detect minor levels of reagent degradation. Ideally, a tissue which is known to have low expression of the reagent in question should be chosen to ensure that the system is sensitive to small amounts of reagent degradation or problems with the IHC methodology. Generally, however, neoplastic tissue that is positive for c-KIT is strongly positive due to the nature of the pathology. An example of tissue to use as a positive control with PATHWAY Anti-c-KIT (9.7) Primary Antibody is GIST tumor demonstrating positivity for c-KIT and containing normal colon tissues with weakly positive staining IHC.

The positive staining cell/tissue components (cell membrane and/or cytoplasmic staining of neoplastic cells) are used to confirm that PATHWAY Anti-c-KIT (9.7) Primary Antibody was applied and the instrument or manual assay functioned properly. It is beneficial to perform c-KIT staining on tumor sections that include normal mucosa because the low mast cells and weakly positive IHC that may be present will also stain positively and serve as internal positive controls (1).
Known positive tissue controls 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 tissue controls fail to demonstrate positive staining, results with the test specimens should be considered invalid.

Negative Tissue Control

Use a tissue control known to be fixed, processed and embedded in a manner identical to the patient sample(s) with each staining run to verify the specificity of PATHWAY Anti-c-KIT (9.7) Primary Antibody for demonstration of c-KIT, and to provide an indication of specific background staining (false positive staining). The variety of different cell types in most tissue sections can also be used by the laboratory as internal negative control sites to verify PATHWAY Anti-c-KIT (9.7) Primary Antibody performance specifications. However, this should be verified by the user. For example, the same tissue used for the positive tissue control (GIST tumors) may be used as the negative tissue control. The non-staining components (surrounding stroma, blood vessels, and epithelium) should demonstrate absence of specific staining, and provide an indication of specific background staining. Alternatively, normal colon is an adequate negative control tissue. If specific staining occurs in the negative tissue control (other than mast cells and ICC), results with the patient specimens should be considered invalid.

Nonspecific Negative Reagent Control

A negative reagent control must be run for every tissue block stained to aid in the interpretation of each patient result. A negative reagent control is used in place of the primary antibody to evaluate nonspecific staining and allow better interpretation of specific staining at the antigen site. This provides an indication of nonspecific background staining for each slide. In place of the primary antibody, stain the slide with CONFIRM Negative Control Rabbit IgG, a rabbit negative control IgG. Rabbit negative control IgG is the ideal negative control because it is nonimmune, and processed in the same way as the primary antibody. Buffers could also be used as a nonspecific negative reagent control. Buffer alone is a less desirable alternative to the previously described negative reagent control. The dilution factor and incubation period for the negative reagent control should correspond to that of the primary antibody.

When panels of several antibodies are used on serial sections, the nonspecific staining areas of one slide may serve as a negative/non-specific binding background control for other antibodies.

Unexplained Discrepancies

Unexplained discrepancies in control results should be referred to Ventana Medical Systems Customer Care (800-227-2155). If quality control results do not meet specifications, patient results are invalid. See the Troubleshooting section of this insert for additional information.

Assay Verification

Prior to initial use of this antibody in the user's laboratory or if there is a change of lot number, the specificity of the antibody should be verified by staining a number of positive and negative tissues with known performance characteristics. Refer to the quality control procedures previously outlined in this section of the product insert and to the quality control recommendations of the CAP certification program for immunohistochemistry (1) and/or the NCCLS/HEP guideline (23). These quality control tests should be repeated for each lot or whenever there is a change of lot number of one of the reagents in a matched set or a change in assay parameters. Quality control cannot be meaningfully performed on an individual reagent in isolation since the matched reagents, along with a defined assay protocol, must be tested in unison before using a lot for clinical purposes. Tissues listed in the Performance Characteristics section of this package insert are suitable for assay verification.

Assay verification on a daily basis may be accomplished through the proper use of the above-mentioned positive and negative controls, as described in this section (Quality Control Procedures). In addition, it is recommended that, on a monthly basis, the c-KIT positive tissue control be stained and compared to the same tissue control stained the previous month. Comparison of controls stained at monthly intervals serves to monitor the assay stability, sensitivity, specificity, and reproducibility.

All quality control requirements should be performed in conformance with local, state and/or federal regulations or accreditation requirements.

Interpretation of Staining within the Context of Controls

The Ventana immunostaining procedure produces a reddish-brown colored DAB reaction product to precipitate at the antigen sites of localized PATHWAY Anti-c-KIT (9.7) Primary Antibody. A qualified pathologist who is experienced in immunohistochemical procedures must evaluate positive and negative controls and qualify the stained product before interpreting patient results.

The Purpose of Daily Quality Control

Positive Tissue Control: When used with PATHWAY Anti-c-KIT (9.7) Primary Antibody and MEW DAB detection, the positive control allows for the control for all steps of the analysis, validating the reagent and procedures used for staining. When used with a Nonspecific Antibody* or Ventana buffer plus same detection system as used with PATHWAY Anti-c-KIT (9.7) Primary Antibody, the positive control allows for the detection of non-specific background staining.

The positive tissue control stained with PATHWAY Anti-c-KIT (9.7) Primary Antibody should be examined first to ascertain that all reagents are functioning properly. The positive tissue control is tissue or cells expressing c-KIT, and could be located in patient tissue, e.g., mast cells. The ideal control is weakly positive staining tissue like Intestinal Cells of Cajal (ICC) in normal gastrointestinal mucosa. The presence of redish-brown (3,3'-diaminobenzidine tetrahydrochloride, DAB) reaction product with the target cells' cytoplasm and/or plasma membrane is indicative of positive reactivity. Strong cytoplasmic, membrane, and occasional dot-like perinuclear Golgi staining are reliable indicators of c-KIT expression. The surrounding stroma, lymphoid cells, and blood vessels should be negative. Staining of c-KIT expression in GIST is often heterogeneous and not all neoplastic cells display positivity. Therefore, specific staining in any neoplastic cells should be considered a positive result. If the positive tissue control fails to demonstrate positive staining, any results with the test specimen should be considered invalid.

Counterstaining with hematoxylin will result in a pale to dark blue coloration of the cell nuclei. Excessive or incomplete counterstaining may compromise proper interpretation of results.

Negative Tissue Control: When used with PATHWAY Anti-c-KIT (9.7) Primary Antibody and MEW DAB detection, the negative control allows for detection of unbound antibody cross-reactivity to cellular components. When used with a Nonspecific Antibody* or Ventana buffer plus same detection system as used with PATHWAY Anti-c-KIT (9.7) Primary Antibody, the negative control allows for the detection of non-specific background staining.

The negative tissue control should be examined after the positive tissue control to verify the specific labeling of the target antigen by the primary antibody. The absence of specific staining in the negative tissue control confirms the lack of antibody cross-reactivity to cellular components. The same tissue used for the positive tissue control may be used as the negative tissue control. The variety of cell types present in most tissue sections offers internal negative control sites, but this should be verified by the user. Alternately, normal colon is an adequate negative control tissue. Intact stromal, smooth muscle, and epithelial elements should show no staining. If inappropriate staining occurs in the negative tissue control, results with the patient specimens should be considered invalid.

Nonspecific staining, if present, will have a diffuse appearance. Sporadic light 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 will often stain nonspecifically (26). The absence of specific membrane or cytoplasmic staining should be interpreted as being negative for c-KIT expression.

Internal Control: Staining of normal colon tissue can provide internal positive controls. Intestinal cells of Cajal (ICC) and mast cells should stain positively, and may be used as an internal positive control with ICC demonstrating weakly positive cell membrane staining and mast cells demonstrating strongly positive staining of the cell membrane and the cytoplasm.

Interpretation of Staining of Patient Tissue

Patient Tissue: When used with PATHWAY Anti-c-KIT (9.7) Primary Antibody and MEW DAB detection, the patient sample allows for detection of specific c-KIT staining. When used with a Nonspecific Antibody* or Ventana buffer plus some detection system as used with PATHWAY Anti-c-KIT (9.7) Primary Antibody, the patient sample allows for the detection of non-specific background staining.

Patient specimens should be examined last. Positive staining intensity should be assessed within the context of any nonspecific background staining of the negative reagent control. GIST tumors are considered positive for c-KIT protein expression if any neoplastic cells demonstrate specific cytoplasmic and/or cell membrane staining. Strong cytoplasmic, membrane, and occasional dot-like perinuclear Golgi staining are reliable indicators of c-KIT expression. Staining of c-KIT in GIST is often heterogeneous and not all neoplastic cells display positivity. Rare focal positivity should be interpreted with caution. A positive result confirms the diagnosis of GIST when morphologic and clinical features are consistent with GIST (1, 32).

The absence of specific membrane or cytoplasmic staining should be interpreted as being negative for c-KIT expression. As with any immunohistochemical test, a negative result means that the antigen in question was not detected, not that the antigen is absent in the cells/tissue assayed. It is beneficial to perform c-KIT staining on tumor sections that include normal mucosa because the few most cells and ICC that may be present will also stain positively and serve as an internal positive control (1). If necessary use a
panel of antibodies to aid in the identification of false negative reactions. (See Quality Control Procedures, page 4.)

The morphology of each tissue sample should also be examined utilizing a hematoxylin and eosin stained section when interpreting any immunohistochemical result. The patient's morphologic findings and pertinent clinical data must be interpreted by a qualified pathologist. Refer to the sections Summary and Explanation, Limitations, and Performance Characteristics for specific information regarding immunoreactivity.

**Limitations**

General Limitations:

1. Immunohistochemistry (IHC) is a multistep diagnostic process that consists of specialized training in the selection of the appropriate reagents; tissue selection, fixation, processing, 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. Unexpected negative staining in tumors may be due to loss of expression of antigen or loss of the gene coding the antigen as a tumor dedifferentiates. Unexpected positive staining in tumors may be form expression of an antigen not usually expressed in normal cells or persistence or acquisition of an antigen in a dedifferentiated tumor that develops morphologic and immunohistochemical markers associated with another cell lineage. Histopathologic classification of tumors is not an exact science and some literature reports of unexpected staining are controversial.

5. The clinical interpretation of any positive or negative staining should be evaluated within the context of clinical presentation, morphology, and other histopathologic criteria. The clinical interpretation of any positive or negative staining should be complemented by morphologic studies using proper positive and negative internal and external controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist who is familiar with the proper use of IHC antibodies, reagents, and methods, to interpret all of the steps used to prepare and interpret the final IHC preparation.

6. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HbsAg) may exhibit nonspecific staining with horse-radish peroxidase (29).

7. Reagents may demonstrate unexpected reactions in previously untreated 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 pathologic tissues (15). Contact Ventana Medical Systems Customer Care (800-227-2195) with documented unexpected reaction(s).

8. Normal neoplastic sera from the same animal source as secondary antibodies used in blocking steps may cause false negative or positive results due to auto-antibodies or natural antibodies.

9. False positive results may be seen due to non-immunological binding of proteins or substrates reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes), endogenous peroxidase activity (cytochrome C), or endogenous biotin (e.g. liver, brain, breast, kidney) depending on the type of immunostain used (25).

10. The PATHWAY Anti-c-KIT (9.7) Primary Antibody is not intended for use in flow cytometry. Performance characteristics have not been determined for flow cytometry.

**Specific Limitations:**

1. Ventana Medical Systems' PATHWAY Anti-c-KIT (9.7) Primary Antibody has been optimized for a 32 minute incubation time with Ventana Automated Slide Stainers, or a 10 minute incubation with the manual assay protocol. Users who deviate from recommended test procedures must accept responsibility for interpretation of patient results under these circumstances.

2. The PATHWAY Anti-c-KIT (9.7) Primary Antibody demonstrates c-KIT antigen that survives routine tissue fixation with neutral buffered formalin, processing and sectioning.

3. False negative cases may result from various factors, including true antigen decrease; loss or structural change during tumor "dedifferentiation" or terminal differentiation, or antifactual change during fixation or processing. As with an immunohistochemical test, a negative result means that the antigen was not detected, not that the antigen was absent in the cell/tissue samples.

4. Neoplastic tissue is the recommended positive control tissue. While many normal human tissues react positively with PATHWAY Anti-c-KIT (9.7) Primary Antibody, the staining pattern in normal tissues is generally detected as positive mass cell staining.

5. Not all GIST tumors are positive for c-KIT protein expression; 5-10% may be negative (34).

6. The following normal tissues were not tested for specificity: bone marrow, pluriy, mesothelium, and parathyroid.

**Performance Characteristics**

**Specificity**

1. Specificity of PATHWAY Anti-c-KIT (9.7) Primary Antibody was determined by a study that showed appropriate staining of a variety of formalin fixed, paraffin embedded normal and neoplastic tissues. Normal tissues studied included spleen, skeletal muscle, ovary, liver, ovary, colon, esophagus, breast, kidney, tonsil, pancreas, skin, thyroid, small intestine, adrenal, uterus, heart, cerebrum, cerebellum, lung, testis, stomach, prostate, salivary gland, peripheral nerve, thymus, and placenta. With the exception of stomal mast cells and breast ductal epithelium, no positive staining was observed in any of the normal tissues studied. Normal bone marrow, pluriy, mesothelium, and parathyroid were not studied. Forty-nine neoplastic tissues were studied and included breast carcinoma, carcinoid, colon carcinoma, renal carcinoma, leukemia, liver carcinoma, lung cancer, lymphoma, melanoma, ovarian carcinoma, pancreatic carcinoma, prostate carcinoma, sarcoma, stomach carcinoma, teratoma, thyroid carcinoma, vascular tumor, and undifferentiated carcinoma. With the exception of stromal mast cells, no positive staining was observed in any of the neoplastic tissues studied.

2. The PATHWAY Anti-c-KIT (9.7) Primary Antibody was tested in Western blotting experiments for reactivity against cell lysates containing c-KIT, and the following proteins that are structurally related to c-KIT: Platelet-derived growth factor receptor α (PDGFRα), FMS-like tyrosine kinase 3 (Flt-3), and macrophage colony stimulating factor receptor (c-FMS). In Western blots of c-KIT positive GIST 822 cell lysates, PATHWAY Anti-c-KIT (9.7) recognized a doublet band of 140-145 kDa which is consistent with the known molecular weight of c-KIT protein. PATHWAY Anti-c-KIT (9.7) was unreactive in Western blotting experiments with PDGFRα positive 3T3/31 cell lysates, Flt-3 positive THP-1 cells lysates, and c-FMS positive RAW 264.7 cell lysates. Probing the same lysates with antibodies specific to PDGFRα, Flt 3, and c-FMS demonstrated that these antigens were present in the lysates.

**Agreement Studies**

Two studies were conducted to determine the agreement between PATHWAY Anti-c-KIT (9.7) Primary Antibody vs. the investigational immunocytochemical antibody (ICA) used in the Gleevec/Glivec clinical trial using clinical cases with known diagnoses.

**STUDY 1. PATHWAY Anti-c-KIT (9.7) Primary Antibody vs. ICA Using a GIST Tissue microarray with cases of known mutational status**

A total of 125 cases was a part of the original GIST micro array. However, about one-fourth of the cores was variably lost to each of the three qualified readers. Since one of the reader’s results was considerably different than the other two, the results are presented separately by qualified reader.

Qualified Reader #1

<table>
<thead>
<tr>
<th>ICA Polyclonal</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATHWAY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-c-KIT (9.7)</td>
<td>Positive</td>
<td>86 0 86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>1 9 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>87 9 96</td>
<td></td>
</tr>
</tbody>
</table>

Positive Agreement = 95% (86/87) with 95% Clopper-Pearson Lower Confidence Bound (LCB) = 95%

Negative Agreement = 100% (95) with 95% LCB = 72%

Qualified Reader #2

<table>
<thead>
<tr>
<th>ICA Polyclonal</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATHWAY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-c-KIT (9.7)</td>
<td>Positive</td>
<td>67 0 67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>15 9 24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>82 9 91</td>
<td></td>
</tr>
</tbody>
</table>

Positive Agreement = 82% (67/82) with 95% LCB = 73%

Negative Agreement = 100% (95) with 95% LCB = 72%
Ten (10) of the 95 cases with a core evaluated by at least 2 of the qualified readers, did not demonstrate over-expression of c-KIT protein. These results demonstrate that the absence of c-KIT over expression alone cannot be used to eliminate a diagnosis of undifferentiated sarcoma. Of these 10 cases, two were GISTs and two non-GIST tumors had no evaluable cores. These results are presented separately by pathologist, also.

**TROUBLESHOOTING**

1. If the c-KIT positive control tissue exhibits weaker staining than expected, check other positive controls run during the same run to determine if it is due to the primary antibody or one of the common secondary reagents.

2. If the c-KIT positive control tissue is negative, check to ensure that the slide has the proper bar code label. If the slide is labeled properly, check other positive controls run during the same run to determine if it is due to the primary antibody or one of the common secondary reagents.

3. If excessive background staining occurs, it may be due to paraffin artifact. If this is the case, repeat deparaffinization procedure. Alternatively, high levels of endogenous biotin may be present. Preincubate tissue with biotin blocking reagents (Ventana Endogenous Biotin Blocking Kit). If all of the paraffin has not been removed, the deparaffinization procedure should be repeated.

4. If tissue sections wash off slide, check to see sure slides are stained or coated with polylysine or equivalent material. Refer to the Ventana Automated Slide Stainer Operator’s Manual for corrective action or contact Customer Service (800-227-2155).

5. If specific staining is too dark than the PATHWAY Anti-c-KIT (9.7) Primary Antibody incubation time may be shortened from the recommended 32 minutes. Any such change must be validated by the user.

6. For corrective action, refer to the Step By Step Procedure section (page 3), the automated slide stainer Operator’s Manual or contact your local Ventana office.


10. Gown, A. M., Basch, C. E. C-KIT (CD117) is a uniform marker of non-neoplastic breast tissue and is also expressed in a unique subset of breast cancers, Mod. Path., 12:22a, 1999.


18. Homick, J. L. and Fletcher, C. D. Immunohistochemical staining for KIT (CD117) is a uniform marker of non-neoplastic breast tissue and is also expressed in a unique subset of breast cancers, Mod. Path., 12:22a, 1999.


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