

LABELING



PATHWAY® Anti-c-KIT (9.7) Primary Antibody

Catalog number 790-2951

Caution: Federal law restricts this device to sale by or on the order of a physician, or to a clinical laboratory, and its use is restricted to, by, or on the order of a physician.

INDICATIONS AND USE

Intended Use

This antibody is intended for *in vitro* diagnostic (IVD) use.

Ventana® Medical Systems' PATHWAY Anti-c-KIT (9.7) Primary Antibody is intended for laboratory use, via light microscopy, for the qualitative detection of KIT protein in formalin-fixed, paraffin-embedded gastrointestinal stromal tumors (GISTs) using either an automated immunohistochemistry staining system or a manual assay. It is indicated as an aid in the diagnosis of GIST within the context of the patient's clinical history, tumor morphology, and other diagnostic tests evaluated by a qualified pathologist. It may be used after the diagnosis of GIST as an aid in the selection of GIST patients who may qualify for imatinib mesylate (Gleevec®/Glivec®) therapy.

PATHWAY Anti-c-KIT (9.7) Primary Antibody is optimized for use on Ventana Automated Slide Stainer and for manual application in combination with Ventana Medical Systems' *VIEW™* DAB Detection Kit and accessories. The clinical interpretation of any staining, or the absence of staining, must be complemented by morphological studies and evaluation of proper controls. Evaluation must be made by a qualified pathologist within the context of the patient's clinical history and other diagnostic tests.

Note:

The test is not intended as the sole basis for making the diagnosis of GIST and is not intended as the sole basis for selecting Gleevec/Glivec therapy. The proportion of c-KIT negative GIST patients who can respond to Gleevec/Glivec has not been established. A negative result would not necessarily exclude the diagnosis of GIST, nor should it preclude treatment with Gleevec/Glivec (14, 34, 39).

All of the subjects in the Novartis Gleevec/Glivec clinical trials were selected using an investigational immunocytochemical antibody (ICA). None of the subjects in those trials were selected using the Ventana PATHWAY Anti-c-KIT (9.7) Primary Antibody. 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.

Summary and Explanation

The c-KIT gene was cloned and characterized by Yarden *et al.* in 1987 (38). Its oncoprotein product is an approximately 145 kD to 125 kD transmembrane glycoprotein which 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 to the transforming proteins of the *src* family. The coding sequence is consistent with an extracellular ligand 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, and as reviewed in reference 11).

Normal cellular elements that stain positively for c-KIT include interstitial cells of Cajal (ICC), mast cells, breast epithelium, and skin basal cells (35). In addition to GISTs, other neoplasms that may express c-KIT include seminoma, melanoma, a small subset of breast carcinomas, and small cell lung carcinoma (10, 18, 35). Unexpected positive and negative staining results are discussed in **The Purpose of Daily Quality Control** section of this insert.

PATHWAY Anti-c-KIT (9.7) Primary Antibody contains a purified rabbit monoclonal antibody directed against an intracellular domain of the c-KIT oncoprotein. The immunogen is a synthetic peptide from the intracellular C-terminal domain of human CD117/c-KIT protein. The molecular weight of the antigen recognized by the antibody on Western blots is approximately 145 kD.

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 occurs in exon 11 (65-70% 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 13

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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, 31). It should be noted that activating mutations of KIT also occur in other human malignancies, most commonly in mast cell tumors and seminomas (11).

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 worldwide 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.

Imatinib mesylate is not perfectly specific and inhibits tyrosine kinases that are closely related to ABL, including ARG (ABL-related kinase), PDGFR α and PDGFR β (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* (13). Importantly, the drug was equally effective against wild type KIT and a mutant isoform commonly found in GISTs (point mutation in exon 11). During the same year, Dr. Jonathan Fletcher (Brigham & Women's Hospital/DFCI) showed that the drug could inhibit the growth of a GIST cell line (36). Based in part on these pre-clinical findings, a patient with GIST metastatic to the liver was granted compassionate use of imatinib mesylate in March, 2000. Within a matter of weeks the metastases showed an overall size decrease of 75%, with 6 of 28 hepatic lesions no longer detectable on follow up MRI scans after 8 months of therapy. This clinical response correlated with near complete inhibition of [18F]fluorodeoxyglucose uptake on PET scan. Post-treatment biopsy showed a marked decrease in tumor cellularity and extensive myxoid degeneration. Imatinib mesylate was well tolerated in this patient and all cancer-related symptoms disappeared (19).

The success in treating the first GIST patient with imatinib mesylate quickly led to a multi-center trial (CSTIB2222) that included the Dana Farber Cancer Institute, Fox-Chase Cancer Center, Oregon Health & Science University, and the University of Helsinki. Results of this phase II trial were reported at the 2001 and 2002 meetings of the American Society of Clinical Oncology (2, 23), and recently published (6). With a minimum follow-up of six months, partial responses (50% or more tumor shrinkage) were observed in 54% of patients, and an additional 28% had stable disease. Disease progression was seen in only 14% of patients (6).

Similar results were reported for the EORTC Soft Tissue and Sarcoma Group phase I study of imatinib mesylate for patients with advanced soft tissue sarcomas, including GISTs (37). Forty patients, of whom 36 had a GIST, were treated with dose levels from 400 mg to 1000 mg daily, with therapy continuing until progression, unacceptable toxicity, or patient refusal to proceed. Substantial activity was seen only in the GIST patients, with 19 of 36 patients having a partial response and only 7 failing therapy during 9 months of follow-up. Non-GIST patients did dramatically worse, with no documented responses in that treatment population. Based on the results of the CSTIB2222 trial and the EORTC trial, imatinib mesylate was approved by the FDA for the treatment of unresectable and metastatic GIST on February 1, 2002.

Clinical Significance

GISTs arise predominantly in the stomach (60%) and small intestine (25%), but also occur in the rectum (5%), esophagus (5%) and a variety of other locations (5%), including appendix, gallbladder, mesentery and omentum (reviewed in 11 & 24). Affected patients range in age from the teens to the 90's, but the majority are older and the peak is around age 60. A slight male predilection has been observed in most studies. There are no solid figures on the true incidence of GISTs, but it is estimated that ~4,500 new cases are diagnosed each year in the United States.

Most GISTs are comprised of a fairly uniform population of spindle cells (70% of cases), but some are dominated by epithelioid cells (20% of cases) and most of the remainder have a mixture of these two morphologies (9, 24). The spindle cells are usually arranged in short fascicles, but can be aligned in a strikingly schwannian pattern with prominent nuclear palisading. Approximately 5% of cases have prominent myxoid stroma. Occasional tumors have a neuroendocrine look that resembles either paraganglioma or carcinoid. Significant nuclear atypia is uncommon.

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 the cell surface and/or cytoplasm of GIST 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. Desmin expression in true KIT-positive GISTs is extremely uncommon (1-2% of cases) and is usually focal (9, 24, 25)

PRINCIPLES AND PROCEDURES

PATHWAY Anti-c-KIT (9.7) Primary Antibody is a rabbit monoclonal antibody which binds a site on 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 aid 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 c-KIT primary antibody (rabbit monoclonal) and contains approximately 5 ml (50 test) of prediluted reagent. The dispenser contains 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 immunoinhibition 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 VUEW 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 for 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, silanized or polylysine-coated
5. Drying oven capable of maintaining a temperature of 70°C ± 5°C.
6. Bar code labels for Automated Slide Stainer use (Ventana Medical Systems, Inc. Cat. No. 451-801 for negative control and any one of Cat. No. 451-001 through 451-451 for PATHWAY Anti-c-KIT (9.7) Primary Antibody)
7. Xylene (histological grade)
8. Ethanol or reagent alcohol (histological grade)
9. Deionized/distilled water
10. Ventana Automated Slide Stainer:
 - Ventana NexES® Slide Staining System, or
 - Ventana BenchMark™ Slide Staining System, or
 - Ventana BenchMark™ XT Slide Staining System
11. Ventana Medical Systems VUEW DAB Detection Kit
12. Ventana APK Wash Solution Concentrate (10X) (NexES IHC automated slide stainers)
13. Ventana EZ Prep™ Solution Concentrate (10X)
14. Ventana Cell Conditioning 1 (CC1) Solution Pre-dilute (BenchMark and BenchMark XT automated slide stainers)
15. 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)
16. CONFIRM™ Negative Control Rabbit Ig
17. Mounting Medium: Permanent Mounting Medium for use with DAB
18. Cover Glass
19. Light microscope (20-80X)
20. Staining jars or baths
21. Timer (capable of 2-10 minute intervals)
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 VIEW 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 dried by placing the slides in a 70°C (+/- 5°C) oven for at least two hours, but not longer than 24 hours (33). Studies at Ventana Medical Systems, Inc. indicate unbaked cut tissue and cell line sections affixed to glass slides and stored at room temperature are stable for 12 months. Each laboratory should validate the cut slide stability for their own laboratory use.

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. For instructions on when to label slides with bar code label, refer to the Instructions for Use section of the specific automated slide stainer.
2. Immerse the slides sequentially in 3 xylene baths for 5 ± 1 minutes each.
3. Transfer the slides to 100 % ethanol and immerse sequentially in 2 baths for 3 ± 1 minutes each.
4. Transfer the slides to 95% ethanol and immerse them in a bath of this solution for 3 ± 1 minutes
5. Transfer the slides to 80% ethanol and immerse them in this solution for 3 ± 1 minutes.
6. Transfer the slides to a bath of deionized or distilled water and dip a minimum of 10 times
7. Transfer slides to APK Wash (1X) solution or buffer solution as appropriate. For APK Wash solution, the slides should remain until you are ready to perform the staining run. For buffer solution, the slides should remain until you are ready to perform the antigen unmasking procedure. Do not allow the slides to dry.

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 Antigen Unmasking Procedure

Antigen enhancement (cell conditioning) procedure (for tissue slides to be stained on NexES IHC, or manual methods).

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 cracking 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 deionized water and place the heat shield, (circular 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 pan handle. Grip the lid handle, and rotate 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 rheostat 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 slide 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 with 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 large 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:

- Slides are deparaffinized through a series of xylene and gradient alcohols to water 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)
- 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.
- 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.
- Load the deparaffinized, antigen unmasked labeled slides from the APK Wash (1X). Avoid tissue drying.
- Initiate the staining run.
- If counterstain is selected, it will be applied to the slide and incubated with mixing for 2 minutes at 37°C.
- Mount and coverslip per standard laboratory practice for DAB.

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

Step		Platform / Method		
		NexES®	BenchMark™ or BenchMark XT	Manual Method
1.	Antigen Enhancement/Cell	CC1 sofn, 2 min, Decloaking Chamber, 120°C	CC1 sofn, 60 min, online (std.), 100°C	CC1 sofn, 2 min, Decloaking Chamber, 120°C
2.	Endogenous peroxidase block	Online, 4 min, 37°C	Online, 4 min, 42°C	3% H ₂ O ₂ , 10 min, Room Temperature (RT, 20-28°C)
3.	PATHWAY Anti-c-KIT (9.7) Primary	1 dispense (drop) antibody, 32 min, 37°C	1 dispense (drop) antibody, 32 min, 42°C	1 dispense (drop) of antibody, 10 min, RT
4.	Biotinylated goat anti-rabbit IgG	1 dispense (drop) reagent, 8 min, 37°C	1 dispense (drop) reagent, 8 min, 42°C	1 dispense (drop) of reagent, 10 min, RT
5.	Streptavidin-horseradish peroxidase conjugate	1 dispense (drop) conjugate, 8 min, 37°C	1 dispense (drop) conjugate, 8 min, 42°C	1 dispense (drop) of conjugate, 10 min, RT
6.	Substrate/DAB chromogen	1 dispense (drop) reagents, 8 min, 37°C	1 dispense (drop) reagents, 8 min, 42°C	1 dispense (drop) of reagents, 4 min, RT
7.	Copper enhancer	1 dispense (drop) enhancer, 4 min, 37°C	1 dispense (drop) enhancer, 4 min, 42°C	1 dispense (drop) of enhancer, 4 min, RT
8.	Hematoxylin counterstain	1 dispense (drop) stain, 2 min, 37°C	1 dispense (drop) stain, 2 min (BenchMark) or 4 min (BenchMark XT), 42°C	1 dispense (drop) of stain, 8 min, RT

BenchMark or BenchMark XT Automated Slide Stainers

- 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.
- Each slide must be labeled with the appropriate bar code specifying the staining procedure and PATHWAY Anti-c-KIT (9.7) Primary Antibody.
- Initiate the staining run.
- 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.
- Mount and coverslip per standard laboratory practice for DAB.

For All Instruments

- Start the staining run
- At the completion of the run, remove the slides from the automated slide stainer
- For MIEW 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 application using PATHWAY 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.

- Slides are chemically deparaffinized through a series of xylene cleaning reagents, then through a series of gradient alcohols to water (see section Manual Deparaffinization Procedure, page 3).
- Note: Slides must be horizontal, on a flat surface, when applying the reagents.
- 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.
- Apply enough volume of the endogenous peroxidase block (inhibitor reagent) to cover the specimen and incubate for 10 minutes at room temperature (20-28°C).
- Slides are then rinsed in deionized water and placed in a Decloaking Chamber for the antigen enhancement procedure using Ventana's CC1 cell conditioning solution as described in section II. F., Specimen Collection and Preparation for analysis.
- Rinse the slides in APK Wash Solution.
- Tap off excess buffer and wipe slides as before.
- Apply enough volume of PATHWAY Anti-c-KIT (9.7) Primary Antibody to cover the specimen and incubate for 10 minutes at room temperature.
- Rinse the slides in APK Wash Solution.
- Tap off excess buffer and wipe slides as before.
- Apply enough volume of the MIEW Biotinylated Ig reagent to cover the specimen and incubate for 10 minutes at room temperature.
- Rinse the slides in APK Wash Solution.
- Tap off excess buffer and wipe slides as before.
- Apply enough volume of the MIEW SA-HRP to cover the specimen and incubate for 10 minutes at room temperature.
- Rinse the slides in APK Wash Solution.
- Tap off excess buffer and wipe slides as before.
- Mix equal volumes of the MIEW DAB and the MIEW DAB H₂O₂ reagents together in a clean tube.
- Apply enough volume of the DAB/H₂O₂ mixture to cover the specimen and incubate for 4 minutes at room temperature.
- Rinse the slides in APK Wash Solution.
- Tap off excess buffer and wipe slides as before.
- Apply 1 drop of the MIEW Copper reagent and incubate the slides for 4 minutes at room temperature.
- Rinse the slides in APK Wash Solution to complete the manual staining protocol.
- Counterstain with hematoxylin.
- 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 procedure 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 autopsy/biopsy/surgical 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 weak, but positive staining 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 positively staining ICC.

The positive staining cells/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 few mast cells and weakly positive ICC 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 laboratorian 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 Ig, 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 negatively 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 (4) and/or the NCCLS IHC guideline (29). These quality control tests should be repeated for each new 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 kit 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 MIEW 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 Interstitial Cells of Cajal (ICC) in normal gastrointestinal mucosa. The presence of reddish-brown (3,3'-diaminobenzidine tetrachloride, 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 specimens 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 MIEW DAB detection, the negative control allows for detection of unintended antibody cross-reactivity to cells/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 cells/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 specimen 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. Interstitial 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 MIEW DAB detection, the patient sample allows for detection of specific c-KIT 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 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 mast 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

* Same source and type as the specific antibody but not directed against any human antigen. To detect non-specific antibody binding, e.g., binding of Fc portion of antibody by the tissue.

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 (26).
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(s) coding the antigen as a tumor dedifferentiates. Unexpected positive staining in tumors may be from 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 histopathological criteria. The clinical interpretation of any positive or negative staining should be complemented by morphological 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 horseradish peroxidase (28).
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 (15). Contact Ventana Medical Systems Customer Care (800-227-2155) with documented unexpected reaction(s).
8. Normal/nonimmune sera from the same animal source as secondary antisera 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 substrate 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 (26).
10. 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 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. 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 artifactual change during fixation or processing. As with any immunohistochemical test, a negative result means that the antigen was not detected, not that the antigen was absent in the cells/tissues assayed
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 denoted as positive mast 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, pituitary, 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, cervix, colon, esophagus, breast, kidney, tonsil, pancreas, skin, thyroid, small intestine, adrenal, uterus, heart, cerebellum, cerebellum, lung, testis, stomach, prostate, salivary gland, peripheral nerve, thymus, and placenta. With the exception of stromal mast cells and breast ductal epithelium, no positive staining was observed in any of the normal tissues studied. Normal bone marrow, pituitary, mesothelium, and parathyroid were not studied. Forty-nine neoplastic tissues were studied and included breast carcinoma, carcinoid, colon carcinoma, renal carcinoma, leiomyoma, liver carcinoma, lung carcinoma, 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 (PDGFR α), FMS-like tyrosine kinase 3 (FIt-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 kD 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 PDGFR α positive 3T3/A31 cell lysates, FIt-3 positive THP-1 cells lysates, and c-FMS positive RAW 264.7 cell lysates. Probing the same lysates with antibodies specific to PDGFR α , FIt-3, and c-FMS demonstrated that these antigens were present in the lysates.

3. 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 Micro array with cases of known mutational status

A total of 129 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

		ICA Polyclonal		
		Positive	Negative	Total
PATHWAY Anti-c-KIT (9.7)	Positive	86	0	86
	Negative	1	9	10
	Total	87	9	96

Positive Agreement = 99% (86/87) with 95% Clopper-Pearson Lower Confidence Bound (LCB) = 95%

Negative Agreement = 100% (9/9) with 95% LCB = 72%

Qualified Reader #2

		ICA Polyclonal		
		Positive	Negative	Total
PATHWAY Anti-c-KIT (9.7)	Positive	67	0	67
	Negative	15	9	24
	Total	82	9	91

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

Negative Agreement = 100% (9/9) with 95% LCB = 72%

Qualified Reader #3

		ICA Polyclonal		
		Positive	Negative	Total
PATHWAY Anti-c-KIT (9.7)	Positive	85	0	85
	Negative	1	9	10
	Total	86	9	95

Positive Agreement = 99% (85/86) with 95% LCB = 95%
 Negative Agreement = 100% (9/9) with 95% LCB = 72%

Results and Conclusions

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 GIST, and reinforce the importance of considering the patient's complete histopathologic profile and clinical history before making a diagnosis.

STUDY 2. PATHWAY Anti-c-KIT (9.7) Primary Antibody vs. ICA Using GIST/Non-GIST Sarcoma Tissue Micro array

Micro arrays comprised of 63 non-GIST sarcoma cases and 13 GIST cases were also studied by 3 qualified readers. However, about one-fifth of cores was variably lost to the readers or contained no tumor. Two GIST and two non-GIST tumors had no evaluable cores. These results are presented separately by pathologist, also.

Qualified Reader #1

		ICA Polyclonal		
		Positive	Negative	Total
PATHWAY Anti-c-KIT (9.7)	Positive	11	0	11
	Negative	0	61	61
	Total	11	61	72

Positive Agreement = 100% (11/11) with 95% LCB = 76%
 Negative Agreement = 100% (61/61) with 95% LCB = 95%

Qualified Reader #2

		ICA Polyclonal		
		Positive	Negative	Total
PATHWAY Anti-c-KIT (9.7)	Positive	10	0	10
	Negative	4	54	58
	Total	14	54	68

Positive Agreement = 71% (10/14) with LCB = 46%
 Negative Agreement = 100% (54/54) with LCB = 95%

Qualified Reader #3

		ICA Polyclonal		
		Positive	Negative	Total
PATHWAY Anti-c-KIT (9.7)	Positive	11	0	11
	Negative	2	58	60
	Total	13	58	71

Positive Agreement = 85% (11/13) with 95% LCB = 59%
 Negative agreement = 100% (58/58) with 95% LCB = 95%

Results and Conclusions

Two of the concordant positive specimens consisted of one spindle cell melanoma specimen and one synovial sarcoma specimen. C-KIT expression has been reported in approximately 20% of malignant melanomas (18). There is controversy in the literature regarding c-KIT expression in synovial sarcoma (32).

Four cases, all GISTs, had discrepant results according to at least one reader. For two of these cases, the discrepancy was noted with two of the readers. One reader had no discrepant results. These results also demonstrate comparable performance between the PATHWAY Anti-c-KIT (9.7) Primary Antibody and the ICA.

Reproducibility

Automated Reproducibility:

1. Intra-run reproducibility of staining was determined by staining with PATHWAY Anti-c-KIT (9.7) Primary Antibody on a NexES and BenchMark automated slide staining platforms and manual protocol by staining 9 slides each from a neutral

buffered formalin (NBF) fixed block containing 5 GIST cases and a NBF fixed leiomyosarcoma. The GIST cases all stained with similar intensity (± 0.5) across all instruments and the leiomyosarcoma case was negative across all instruments. Users should verify intra-run reproducibility results by staining several sets of serial sections

2. Inter-run reproducibility of staining was determined by staining on 3 different instruments and by manual protocol on 3 different days using PATHWAY Anti-c-KIT (9.7) Primary Antibody on paraffin sections from the same NBF fixed block containing 5 GIST cases and on a NBF fixed leiomyosarcoma. All GIST cases stained with similar intensity (± 0.5) across all instruments and days, and the leiomyosarcoma case was negative across all instruments and days. Users should verify between run reproducibility results by staining several sets of serial sections.

Lot-to-lot reproducibility:

Three lots of the PATHWAY Anti-c-KIT (9.7) Primary Antibody were compared by staining c-KIT positive GIST cases using the recommended protocol for the BenchMark™ automated slide stainer and VIEW DAB detection kit. The slides were read by a qualified Ventana Medical Systems' reader. The slides stained with the three lots of antibody were within a 0.5 staining intensity grade for both specific and non-specific (background) staining, demonstrating reproducible staining performance between production lots of the PATHWAY Anti-c-KIT (9.7) Primary Antibody. The sensitivity of PATHWAY Anti-c-KIT (9.7) Primary Antibody immunohistochemistry is dependent upon the preservation of the antigen. Any improper tissue handling during fixation, sectioning, embedding, or storage which alters the antigenicity weakens the KIT oncoprotein detection by Ventana's PATHWAY Anti-c-KIT (9.7) Primary Antibody and may generate false negative results.

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. Call Ventana Medical Systems Customer Care (800-227-2155).
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 instrument run to determine if it is due to the primary antibody or one of the common secondary reagents. Tissues may have been improperly collected, fixed, or deparaffinized. Follow proper procedure for collection, storage, and fixation. Call Ventana Medical Systems Customer Care (800-227-2155).
3. If excessive background staining occurs, it may be due to residual paraffin. 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 be sure slides are silanized or coated with polylysine or equivalent material. Refer to the Ventana Automated Slide Stainer Operator's Manual for corrective action or contact Customer Care (800-227-2155).
5. If specific staining is too dark then 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.

REFERENCES

1. Berman, J. and O'Leary, T. J. Gastrointestinal Stromal Tumor Workshop, Perspectives in Pathology, 32:578-582, 2001
2. Blanke, C. D., von Mehren, M., Joensuu, H., Roberts, P. J., Esienberg, B., Heinrich, M., Druker, B., Tuveson, D., Dimitrijevic, S., S.L., S., and Demetri, G. D. Evaluation of the safety and efficacy of an oral molecularly-targeted therapy, ST1571, in patients with unresectable or metastatic gastrointestinal stromal tumors (GISTs) expressing c-KIT (CD117)., Proceedings of ASCO. 20: 1a (abstr.), 2001.
3. Blechman, J, Lev, S., Brizzi, M., Leitnert, O., Pegoraro, L., Givol, D., and Yarden, Y. Soluble c-KIT proteins and Antireceptor Monoclonal Antibodies Confine the Binding Site of the Stem Cell Factor. J. Biol. Chem. 228:6 4399 – 4406, 1993.
4. Clinical Laboratory Improvement Amendments of 1988: Final Rule. 57 FR 7163, February 28, 1992
5. College of American Pathologists (CAP) Certification Program for Immunohistochemistry. Northfield, IL <http://www.cap.org>. (800) 323-4040.

6. Demetri GD, von Mehren M, Blanke CD, Van den Abbeele A, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman S, Silberman S, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CDM, Joensuu H. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *New Eng J Med*. 347: 472-480., 2002.
7. Druker, B. J., Talpaz, M., Resta, D. J., Peng, B., Buchdunger, E., Ford, J. M., Lydon, N. B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., and Sawyers, C. L. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia, *N Engl J Med*. 344: 1031-7., 2001.
8. Elias, J. M. *et al* Special Report: Quality control in immunohistochemistry. *Am. J. Clin. Pathol.* 92: 836, 1989.
9. Fletcher C., Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW. Diagnosis of gastrointestinal stromal tumors: a consensus approach. *Human Pathology* 33:459-465, 2002.
10. Gown, A.M., Bacchi, 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. Pathol.* 12:22a, 1999
11. Heinrich, M. C., Blanke, C. D., Druker, B. J., and Corless, C. L. Inhibition of KIT tyrosine kinase activity: a novel molecular approach to the treatment of KIT-positive malignancies, *J Clin Oncol*. 20 1692-703., 2002.
12. Heinrich, M., Corless, C., Blanke, C., Demetri, G., Joensuu, H., Mehren, M. v., McGreevey, L., Wait, C., Griffith, D., Chen, C.-J., Haley, A., Kiese, B., Druker, B., Roberts, P., Eisenberg, B., Singer, S., Silberman, S., Dimitrijevic, S., Fletcher, C., and Fletcher, J. KIT mutational status predicts clinical response to STI571 in patients with metastatic gastrointestinal stromal tumors (GISTs), *Proceedings of ASCO*. 21: 2a (abstract #6), 2002.
13. Heinrich, M., Wait, C. L., Yee, K. W. H., and Griffith, D. J. STI571 inhibits the kinase activity of wild type and juxtamembrane c-KIT mutants but not the exon 17 D816V mutation associated with mastocytosis., *Blood*. 96: 173b (Abstr.), 2001.
14. Heinrich, M., Corless, C., Duensing, A., McGreevey, L., Chen, C.-J., Joseph, N., Singer, S., Griffith, D., Haley, A., Town, A., Demetri, G., Fletcher, C., and Fletcher, J. PDGFRA Activating Mutations in Gastrointestinal Stromal Tumors. *Science*. 229:708-710, 2003
15. Herman, G. F. and Elfont, E. A. The taming of immunohistochemistry: the new era of quality control. *Biotech & Histochem*. 66: 194, 1991.
16. Hirota, S. Gastrointestinal stromal tumors: their origin and cause, *Int J Clin Oncol* 6: 1-5, 2001.
17. Hirota, S., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Ishiguro, S., Kawano, K., Hanada, M., Kurata, A., Takeda, M., Muhammad Tunio, G., Matsuzawa, Y., Kanakura, Y., Shinomura, Y., and Kitamura, Y. Gain-of-function mutations of c-KIT in human gastrointestinal stromal tumors. *Science*. 279: 577-80, 1998.
18. Hornick, J. L. and Fletcher, C. D. Immunohistochemical staining for KIT (CD117) in soft tissue sarcomas is very limited in distribution, *Am J Clin Pathol*. 117: 188-93, 2002
19. Joensuu, H., Roberts, P. J., Sarlomo-Rikala, M., Andersson, L. C., Tervahartala, P., Tuveson, D., Silberman, S., Capdeville, R., Dimitrijevic, S., Druker, B., and Demetri, G. D. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor, *N Engl J Med*. 344: 1052-6., 2001.
20. Kantarjian, H., Sawyers, C., Hochhaus, A., Guilhot, F., Schiffer, C., Gambacorti-Passerini, C., Niederwieser, D., Resta, D., Capdeville, R., Zoellner, U., Talpaz, M., and Druker, B. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia, *N Engl J Med*. 346: 645-52., 2002.
21. Kindblom, L. G., Remotti, H. E., Aldenborg, F., and Meis-Kindblom, J. M. Gastrointestinal pacemaker cell tumor (GIPACT). gastrointestinal stromal tumors show phenotypic characteristics of the interstitial cells of Cajal, *Am J Pathol* 152: 1259-69, 1998
22. Mauro, M. J and Druker, B. J. Chronic myelogenous leukemia, *Curr Opin Oncol* 13: 3-7, 2001.
23. Mehren, M v., Blanke, C., Joensuu, H., Heinrich, M., Roberts, P., Eisenberg, B., Silberman, S., Dimitrijevic, S., Kiese, B., Fletcher, J., Fletcher, C., and Demetri, G. High incidence of durable responses induced by imatinib mesylate (Gleevec) in patients with unresectable and metastatic gastrointestinal stromal tumors (GISTs), *Proceedings of ASCO*. 21: 403a (abstract #1608), 2002
24. Miettinen, M and Lasota, J. Gastrointestinal stromal tumors—definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis, *Virchows Arch*. 438: 1-12, 2001
25. Miettinen, M. *et al*. Immunohistochemical spectrum of GISTs at different sites and their differential diagnosis with a reference to CD117 (KIT) *Mod. Pathol.*, 13 1134-1142, 2000
26. Nadj, M and Morales, A. R. Immunoperoxidase, part I: the techniques and its pitfalls *Lab Med*. 14 767, 1983.
27. O'Dwyer, M. E., Mauro, M. J., and Druker, B. J. Recent advancements in the treatment of chronic myelogenous leukemia, *Annu Rev Med*. 53: 369-81, 2002.
28. Omata, M. *et al*. Nonimmunologic binding of horse radish peroxidase to hepatitis B surface antigen: a possible source of error in immunohistochemistry. *Am. J. Clin. Pathol.* 73: 626, 1980.
29. O'Leary, T. J. *et al*. Quality assurance for immunohistochemistry, approved guideline. MM4-A National committee for clinical laboratory standards (NCCLS). Wayne, PA. 1999; 1-46.
30. Rubin, B. P., Fletcher, J. A., and Fletcher, C. D. Molecular Insights into the Histogenesis and Pathogenesis of Gastrointestinal Stromal Tumors, *Int J Surg Pathol*. 8 5-10, 2000.
31. Rubin, B. P., Singer, S., Tsao, C., Duensing, A., Lux, M. L., Ruiz, R., Hibbard, M. K., Chen, C. J., Xiao, S., Tuveson, D. A., Demetri, G. D., Fletcher, C. D., and Fletcher, J. A. KIT Activation Is a Ubiquitous Feature of Gastrointestinal Stromal Tumors, *Cancer Res*. 61: 8118-8121, 2001.
32. Sabah, M., Leader, M., and Kay, E. The Problem with KIT: Clinical Implications and Practical Difficulties with CD117 Immunostaining, *Appl. Immunochem. & Mol. Morph.*, 11:56-61, 2003.
33. Sheehan, D. C., and B.B. Hrapchak. *Theory and Practice of Histotechnology*. 1980. The C.V. Mosby Company, St. Louis.
34. Taniguchi, M., Nishida, T., Hirota, S., Isozaki, K., Ito, T., Nomura, T., Matsuda, H., and Kitamura, Y. Effect of c-KIT mutation on prognosis of gastrointestinal stromal tumors, *Cancer Res*. 59: 4297-300, 1999.
35. Tsuura, Y.; Hiraki, H; Watanabe, K; *et al*. Preferential localization of c-KIT product in tissue mast cells, basal cells of skin, epithelial cells of breast, small lung carcinoma and seminoma/dysgerminoma in human: immunohistochemical study on formalin-fixed, paraffin-embedded tissues, *Virchows Archiv* (1994) 424: 135-141.
36. Tuveson, D. A., Willis, N. A., Jacks, T., Griffin, J. D., Singer, S., Fletcher, C. D., Fletcher, J. A., and Demetri, G. D. STI571 inactivation of the gastrointestinal stromal tumor c-KIT oncoprotein: biological and clinical implications, *Oncogene*. 20: 5054-8., 2001.
37. van Oosterom, A. T., Judson, I., Verweij, J., Stroobants, S., Donato di Paola, E., Dimitrijevic, S., Martens, M., Webb, A., Sciot, R., Van Glabbeke, M., Silberman, S., and Nielsen, O. S. Safety and efficacy of imatinib (STI571) in metastatic gastrointestinal stromal tumours: a phase I study, *Lancet*. 358: 1421-3., 2001.
38. Yarden, Y., Kuang, W., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T., Chen, E., Schlessenger, J., Franke, U., and Ullrich, A. Human prot-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand, *EMBO Journal*, 6:3341-3351, 1987.
39. Medeiros, F., Corless, C.L., Duensing, A., Hornick, J.L., Oliveira, A.M., Heinrich, M.C., Fletcher, J.A., Fletcher, C.D. KIT-negative gastrointestinal stromal tumors. Proof of concept and therapeutic implications. *Am J Surg Pathol Jul* 28(7): 889-894 2004

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