For In Vitro Diagnostic Use. FDA approved as an aid in the differential diagnosis of gastrointestinal stromal tumors (GIST). After diagnosis of GIST, results from c-Kit pharmDx™ may be used as an aid in identifying those patients eligible for treatment with Gleevec™/Glivec® (imatinib mesylate).
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Introduction

Welcome to the c-Kit pharmDx Interpretation Manual

This guide for pathologists includes key technical histological staining interpretation tips when using the c-Kit pharmDx kit. Utilization of the following suggestions will ensure the quality results expected from c-Kit pharmDx.

The c-Kit pharmDx Interpretation Manual objectives are simple:

- To provide an understanding of c-kit/CD117 receptor biology.
- To ensure that the c-Kit pharmDx assay is performed consistent with Dako recommendations for optimal results.
- To encourage reproducible results by introducing a standard approach to staining and interpretation.
- To provide pathologists with a tool to allow for consistent interpretation of c-Kit pharmDx results.
- To facilitate troubleshooting of the c-Kit pharmDx assay if problems occur.

We hope this c-Kit pharmDx Interpretation Manual is useful and we encourage your feedback on how we can improve this tool. Contact your local Dako representative with feedback (see back panel for contact information).

The c-Kit pharmDx assay is a qualitative immunohistochemical (IHC) kit system used manually and on the Dako Autostainer, for the identification of c-kit protein/CD117 antigen (c-kit protein) expression in normal and neoplastic formalin-fixed paraffin-embedded tissues for histological evaluation. The c-Kit pharmDx rabbit polyclonal antibodies specifically detect the c-kit protein in CD117 antigen-expressing cells.

The c-Kit pharmDx is indicated as an aid in the differential diagnosis of gastrointestinal stromal tumors (GIST). After diagnosis of GIST, results from c-Kit pharmDx may be used as an aid in identifying those patients eligible for treatment with Gleevec™/Glivec® (imatinib mesylate).

Results from hematoxylin and eosin (H&E) stains and a panel of antibodies can aid in the differential diagnosis of GIST. Interpretation must be made by a qualified pathologist, within the context of a patient’s clinical history, proper controls, and other diagnostic tests.

Note This 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 outcome of c-kit negative GIST patients treated with 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.¹ ² ³

All subjects in the Novartis Gleevec/Glivec clinical trials were selected using an investigational Novartis Clinical Trial Protocol (NCTP). The primary anti-c-kit rabbit polyclonal antibody reagent used in the NCTP was purchased from Dako. The c-Kit pharmDx primary polyclonal antibody reagent underwent the same method of production, purification and quality control as did the NCTP polyclonal anti-c-kit reagent.

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**c-kit Overview**

The proto-oncogene c-kit, otherwise known as CD117 antigen or Stem Cell Factor Receptor, is a 145 kD type III transmembrane tyrosine kinase receptor. The c-kit gene encodes a transmembrane tyrosine kinase receptor, structurally similar to platelet derived growth factor receptors A and B, as well as the colony stimulating factor 1 receptor. It is thought to play an important role in hematopoiesis, spermatogenesis, and melanogenesis. The c-kit protein contains extracellular domains with 5 Ig-like loops, a highly hydrophobic transmembrane domain, and an intracellular domain with tyrosine kinase activity split by a kinase insert in an ATP-binding region and in a phosphotransferase domain.

Receptor activation is accompanied by receptor dimerization, substrate phosphorylation and autophosphorylation, receptor internalization, activation of protein kinases and phospholipases, and transcription of different proto-oncogenes. The c-kit tyrosine kinase receptor pathway has been shown to be important for tumor growth and progression in several cancers. Mutations in the c-kit gene may lead to ligand independent phosphorylation (activation) of the c-kit tyrosine kinase, which are believed to have a central pathogenic role in e.g. gastrointestinal stromal tumors.

![Figure 1 Schematic representation of c-kit structure and mechanism of action](image)

**c-Kit pharmDx Specificity**

Rabbit polyclonal c-kit antibodies were obtained by subcutaneous injection of a 14 mer peptide (positions 963-976 of the C' terminus of the c-kit protein) coupled to thyroglobulin. The antisera was fractionated through antigen bound affinity chromatography.

A number of soft tissue sarcoma specimens (e.g. leiomyosarcoma) were tested for c-kit expression. This study involved a comparison between the c-Kit pharmDx assay and the Novartis Clinical Trial Protocol (NCTP) as used for the selection of patients for treatment with Gleevec. Two of a total of twenty eight specimens tested displayed positive staining. The result was consistent using the two protocols. All positive immunostaining was abolished after absorption of the primary antibody with a synthetic peptide (16 amino acid C-terminal end of the c-kit protein). It was therefore concluded that the primary antibody used in the c-Kit pharmDx assay reacted specifically with the c-kit protein in the two positively stained soft tissue sarcomas.

The c-kit pharmDx assay has been tested for reactivity against cell lines expressing c-kit protein. In Western blotting of a lysate of the small cell lung carcinoma cell line SY that over-expresses c-kit mRNA, the antibody reagent labeled a band of 145 kD corresponding to the c-kit protein. The labeled band is rather broad, from 120 to 155 kD. An additional band of 100 kD was also labeled. Further, applying a second anti-c-kit antibody, a 100 kD protein was, likewise, labeled. This labeling was abolished when the antibody reagent was incubated with the synthetic c-kit peptide antigen used for immunization. In additional studies, the c-kit antibody reagent was tested using Western blotting for cross-reactivity to proteins that share structural homology, such as; Platelet Derived Growth Factor Receptor (PDGFRa), macrophage colony stimulating factor receptor (c-FMS); and FMS-like tyrosine kinase 3 (FLT-3). No cross-reactivity was observed with these homologous proteins. Furthermore, in Western blotting the Dako antibody reagent did not react with a lysate of the adenocarcinoma cell line HS, which does not express the c-kit gene.
c-kit Expression in Normal Tissues

The c-kit protein is also expressed on a variety of normal cells including many epithelial cell types. Non-epithelial cells that express c-kit protein include mast cells and macrophages.

Some examples of normal tissue stained with c-Kit pharmDx and recommended reagents are summarized in the table below. All tissues were formalin-fixed and paraffin-embedded. Staining was performed on the Dako Autostainer.

<table>
<thead>
<tr>
<th>TISSUE TYPE (# Tested)</th>
<th>POSITIVE TISSUE ELEMENT STAINING AND STAINING PATTERN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal (3)</td>
<td>None</td>
</tr>
<tr>
<td>Bone Marrow (3)</td>
<td>Rare mast cells (2+): cytoplasmic</td>
</tr>
<tr>
<td>Breast (3) +</td>
<td>Epithelial cells (3+): membrane and cytoplasmic</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain/Cerebellum (3)</td>
<td>Purkinje cell processes (1+): cytoplasmic</td>
</tr>
<tr>
<td>Brain/Cerebrum (3)</td>
<td>None</td>
</tr>
<tr>
<td>Cervix (3)</td>
<td>None</td>
</tr>
<tr>
<td>Colon (3) +</td>
<td>Lamina propria and submucosal mast cells (2+): cytoplasmic</td>
</tr>
<tr>
<td>Esophagus (3)</td>
<td>Submucosal mast cells (2+): cytoplasmic</td>
</tr>
<tr>
<td>Heart (3) –</td>
<td>None</td>
</tr>
<tr>
<td>Kidney (3)</td>
<td>Tubular epithelium (2+): cytoplasmic</td>
</tr>
<tr>
<td>Liver (3)</td>
<td>None</td>
</tr>
<tr>
<td>Lung (3)</td>
<td>Mast cells and macrophages (2+): cytoplasmic</td>
</tr>
<tr>
<td>Mesothelial Cells (3)</td>
<td>None</td>
</tr>
<tr>
<td>Ovary (3)</td>
<td>None</td>
</tr>
<tr>
<td>Pancreas (3)</td>
<td>Rare large duct epithelial cells (3+): Mast cells (2+): cytoplasmic</td>
</tr>
<tr>
<td>Parathyroid (3)</td>
<td>None</td>
</tr>
<tr>
<td>Peripheral Nerve (3)</td>
<td>Mast cells in soft tissue (2+): cytoplasmic</td>
</tr>
<tr>
<td>Pituitary (3)</td>
<td>None</td>
</tr>
<tr>
<td>Prostate (3)</td>
<td>None</td>
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<tr>
<td>Salivary Gland (3)</td>
<td>None</td>
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<tr>
<td>Skeletal Muscle (3) –</td>
<td>Mast cells (2+): cytoplasmic</td>
</tr>
<tr>
<td>Skin (3) +</td>
<td>Basal epidermal melanocytes (1+): cytoplasmic</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Small Intestine (3)</td>
<td>Neuronal cells (1+): cytoplasmic</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen (3)</td>
<td>Mast cells (2+): cytoplasmic</td>
</tr>
<tr>
<td>Stomach (3)</td>
<td>Mast cells in lamina propria (2+): cytoplasmic</td>
</tr>
<tr>
<td>Testis (3)</td>
<td>None</td>
</tr>
<tr>
<td>Thymus (3)</td>
<td>None</td>
</tr>
<tr>
<td>Thyroid (3)</td>
<td>None</td>
</tr>
<tr>
<td>Tonsil (3)</td>
<td>None</td>
</tr>
<tr>
<td>Uterus (3)</td>
<td>None</td>
</tr>
</tbody>
</table>

+ Recommended for **positive** control tissue
– Recommended for **negative** control tissue
The c-Kit pharmDx Kit

The c-Kit pharmDx kit contains polyclonal antibodies and control slides sufficient to complete an IHC staining procedure on formalin-fixed, paraffin-embedded specimens.

Following incubation with the primary polyclonal antibodies to human c-kit protein, this kit is optimized for use with a ready-to-use visualization reagent based on dextran technology. This reagent consists of both secondary goat anti-rabbit antibody molecules and horseradish peroxidase molecules linked to a common dextran polymer backbone, thus eliminating the need for sequential application of link antibody and peroxidase conjugate. The enzymatic conversion of the subsequently added chromogen results in formation of a visible reaction product at the antigen site. The specimen may then be counterstained and coverslipped. Results are interpreted using a light microscope. Control slides containing two formalin-fixed paraffin-embedded mouse (+) and human (–) cell lines with staining intensity scores of 2+ and 0 respectively are provided for quality control of the kit reagent performance.

Two c-Kit pharmDx kit configurations are available:

<table>
<thead>
<tr>
<th>Kit Code</th>
<th>Kit Description</th>
<th>Test Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1906</td>
<td>c-Kit pharmDx kit for manual use</td>
<td>25</td>
</tr>
<tr>
<td>K1907</td>
<td>c-Kit pharmDx kit for Dako Autostainer</td>
<td>35</td>
</tr>
</tbody>
</table>

The c-Kit pharmDx kits include:

- c-Kit pharmDx Polyclonal Rabbit IgG
- Rabbit IgG Negative Control Reagent
- c-Kit pharmDx Control Slides

Materials required, but not supplied include:

- Wash Buffer (code S3006)
- Dual Endogenous Enzyme Block (DEEB) (code S2003)
- Target Retrieval Solution (code S1699 or S1700)
- EnVision+ HRP, Rabbit (code K4002 or K4003)
- DAB+ (code K3467 or K3468)
- Hematoxylin (S3301 or S3302)

Figure 2 Kit Procedure

1. **STEP 1**
   - Target Retrieval Solution in either calibrated pressure cooker or water bath.

2. **STEP 2**
   - Application of DEEB. Incubate 5 minutes.

3. **STEP 3**
   - Application of Primary Antibody. Incubate 30 minutes.

4. **STEP 4**
   - Application of EnVision+ HRP, Rabbit. Incubate 30 minutes.

5. **STEP 5**
   - Application of Substrate-Chromogen. Incubate 10 minutes.
Efficacy and safety testing of imatinib mesylate (Gleevec) for anti-tumor activity in gastrointestinal stromal tumors (GISTs) was performed under the sponsorship of Novartis. Adults with a histologically confirmed, unresectable or metastatic GIST that expressed CD117 antigen (c-kit) as assessed with a home brew immunohistochemical assay using Dako c-kit antibody reagent were eligible for the study. Subjects were randomly assigned to a treatment regimen of 400 or 600 mg Gleevec in capsular form administered daily. A total of 147 subjects were enrolled in the trial at 4 study centers. Confirmation of CD117 antigen (c-kit) positive gastrointestinal stromal tumor was completed for 135/137 cases. In 10 cases, pathologic material was not available for central review.

With follow-up of more than 9 months, partial responses were documented for 53.7 percent of the subjects. An additional 27.9 percent of subjects had stable disease, while disease progression was found for 13.8 percent of patients within 3 months after study entry.

**Figure 3** Recommended Workflow
Technical Tips for Optimal c-Kit pharmDx Performance

For accurate and consistent results adherence to recommended c-Kit pharmDx procedure is essential. High quality results can be achieved in any laboratory by following these guidelines.

Technical problems may arise in two areas, those involving sample collection and preparation of tissue in the pre-analytical processing of the specimen and those involving the actual performance of the c-Kit pharmDx assay itself. Technical problems related to performance of the assay are generally caused by planned and unplanned procedural deviations from the c-Kit pharmDx protocol. Quality control slides are provided to detect false results from technical problems.

Tissue Fixation and Variables

Suboptimal sample handling and processing can affect the c-Kit pharmDx results.

Some of the variables that may affect results are as follows:

- Specimens drying prior to fixation.
- Fixation with alternative fixatives (neutral buffered formalin is recommended)
- Age, pH, and storage conditions of fixative.
- Length of fixation.

c-Kit pharmDx Protocol Recommendations

- Specimens preserved in neutral buffered formalin fixative are suitable for testing with c-Kit pharmDx. Alternative fixatives have not been validated and may give erroneous results.
- **Automated Staining** Dako recommends the use of c-Kit pharmDx on a Dako Autostainer or Autostainer Plus. Use of c-Kit pharmDx on alternative automated platforms has not been validated and may give erroneous results.
- **Wash Buffer** Use only wash buffer S3006 (TBST Wash Buffer). Dilute the recommended wash buffer 1:10 using distilled or deionized water. Store unused solution at 2-8°C for no more than 7 days. Discard diluted solution if cloudy in appearance.
- **Storage of Reagents** Reagents and control slides should be stored at 2-8 °C. Do not use the kit after the expiration date printed on the outside of the kit box.
- **Companion Reagents** To ensure consistent, reproducible results, use specified companion reagents to perform c-Kit pharmDx assay.
- **Proper Incubations** All incubation times must be performed according to the package insert. Stay within the tolerance indicated in the package insert for all incubation times to avoid erroneous false-negative or false-positive results.
- For high quality results, review the c-Kit pharmDx Training Checklist (Table 2) prior to beginning your staining run.
c-Kit pharmDx™ Interpretation Manual

Table 2: c-Kit pharmDx Training Checklist

Institution ________________________________ Date ________________________________
Trained by ________________________________ Person Trained/Title ________________________________
Manual or Automated Staining Run? ________________________________ Autostainer Software Version ________________________________ Serial Number ________________________________

**c-Kit pharmDx is a modular assay system requiring specified companion reagents and controls to ensure reproducible results.**

**COMPANION REAGENTS**

- Wash Buffer (S3006)  YES  NO
- Target Retrieval Solution (S1699 or S1700)  YES  NO
- Dual Endogenous Enzyme Block (S2003)  YES  NO
- EnVision+ HRP, Rabbit (K4002 or K4003)  YES  NO
- DAB+ (K3467 or K3468)  YES  NO

**MANUAL OR AUTOMATED PROCEDURE**

- Control slides and kit stored at 2-8°C?  YES  NO
- Cell Line control slides and all reagents warmed to room temperature (20-25°C) prior to starting assay?  YES  NO
- Tissues fixed in 10% neutral buffered formalin?  YES  NO
- Specimens stained within 2 months of tissue mounting on slides when stored at room temperature?  YES  NO
- Xylene and alcohol solutions changed after 40 slides?  YES  NO
- Deparaffinization and rehydration protocol followed?  YES  NO
- Wash buffer prepared properly?  YES  NO
  - Prepare sufficient quantity of wash buffer 10X, 1:10 in reagent-quality water, deionized or distilled water.
  - Distilled or deionized water (not tap water) used for water washes after last alcohol bath in deparaffinization?
  - Temperature and time incubations programmed for Target Retrieval Solution and specimens heated for 30 seconds at 125°C in pressure cooker or for 20 minutes at 95-99°C in the water bath?
  - Regressive hematoxylin counterstains are not used?

**MANUAL PROCEDURE continued**

- Specimen fully covered with Dual Endogenous Enzyme Block for 5 minutes?  YES  NO
- Specimen fully covered with Primary Antibody for 30 minutes?  YES  NO
- Specimen fully covered with EnVision+ HRP, Rabbit for 30 minutes?  YES  NO
- DAB+ Substrate-Chromogen prepared properly?  YES  NO
  - 1 drop DAB+ Chromogen to 1 mL DAB+ Substrate Buffer.
  - Specimen fully covered with Substrate-Chromogen solution for 10 minutes?  YES  NO

**AUTOMATED PROCEDURE**

- Slides placed in buffer bath 5 (+/-1) minutes before loading onto the Dako Autostainer?  YES  NO
- Appropriate protocol template used?  YES  NO
- Was the Autostainer programming reviewed for accuracy?  YES  NO
- Slides rinsed with buffer between steps and double rinsed after the EnVision+ HRP, Rabbit step with an additional 5-minute rinse hold?  YES  NO
- Specimen fully covered with Dual Endogenous Enzyme Block for 5 minutes?
- Specimen fully covered with Primary Antibody for 30 minutes?
- Specimen fully covered with EnVision+ HRP, Rabbit for 30 minutes?
- DAB+ Substrate-Chromogen prepared properly?
  - Add 1 drop of Liquid DAB+ Chromogen to one mL of DAB+ Substrate Buffer and mix.
  - Specimen fully covered with Substrate-Chromogen solution for 10 minutes?  YES  NO

**INSTRUMENTATION/EQUIPMENT**

- Is regular preventative maintenance performed on the pressure cooker or water bath and the Dako Autostainer?  YES  NO
- Is the necessary equipment available to perform the c-Kit pharmDx assay according to protocol?  YES  NO

**If you answered NO to any of the above, you have deviated from protocol and should consult with your Dako Technical Support Representative for assistance (800 424 0021).**

Additional observations or comments:

...
Quality Control

c-Kit pharmDx is an IHC assay system requiring controls to ensure reproducible results.

The first quality control step for interpretation is the evaluation of the control cell slides. Positive and negative cell lines are included in each c-Kit pharmDx kit (Figures 4 and 5) for the purpose of verification of staining runs, every time the assay is performed. Appropriate staining of the control cell lines provides evidence that the c-Kit pharmDx assay is functioning properly. They should not be used as an aid in interpretation of patient results. No staining of the HT-29 control cell line (0) and moderate brown cytoplasmic or paranuclear staining in the P815 control cell line (2+) indicates that the staining run is valid. If the intensity of the positive control cell line is too weak or too strong a false negative or false positive result can occur. **If either of the control cell lines have staining results outside of these criteria, results from all of the test slides stained simultaneously within the same run should be considered invalid and repeated.**

The second quality control step is the interpretation of positive control tissue (Figure 6). Use a positive control tissue (known to be c-kit positive) fixed, processed and embedded in a manner similar to the patient sample(s) with each staining run to verify the specificity of the primary antibody and in conjunction with the corresponding negative reagent control tissue provides an indication of specific background staining. The presence of a moderate brown reaction in the cell membrane or cytoplasm is indicative of positive reactivity. One positive tissue slide for each of the test conditions should be included in each staining run. Verify that the negative tissue control slide demonstrates no specific reactivity. Known positive tissue controls should be utilized for monitoring the correct performance of reagents and processed tissue. NOT as an aid in formulating a specific diagnosis of patient samples. There are also positive tissue elements that can very often serve as intrinsic controls in patient specimens in addition to positive and negative control tissues. Specifically, c-kit positive tissue elements such as Interstitial Cells of Cajal (ICC’s) are frequently present in GIST and adjacent normal tissues (Figure 7). Furthermore, mast cells (Figure 8) are commonly found in a variety of tissue types. **If the positive tissue controls fail to demonstrate appropriate positive staining, results with the test specimens should be considered invalid.**

The third quality control step involves the interpretation of the negative control tissue (Figure 9). Use a negative control tissue (known to be c-kit negative) fixed, processed and embedded in a manner similar to the patient sample(s) with each staining run to verify the specificity of the primary antibody and in conjunction with the corresponding negative reagent to provide an indication of specific background staining. The variety of different cell types present in most tissue sections offers an internal negative control. **If specific staining occurs in the negative control tissue, results with the test specimens should be considered invalid.**

---

**Figure 4** P815 positive cell line control stained with c-Kit pharmDx, 2+ moderate intensity, 40x.

**Figure 5** HT-29 negative control cell line stained with c-Kit pharmDx, 0 intensity, 40x.

**Figure 6** Breast positive control tissue. Ductal epithelial cells stained positively with c-Kit pharmDx, 2+ moderate intensity, 40x.

**Figure 7** Interstitial Cells of Cajal (ICC’s) staining in GIST adjacent normal tissue, 2+ moderate intensity, 20x.

**Figure 8** Mast cell staining, 2+ moderate intensity, 10x.

**Figure 9** Skeletal muscle negative control tissue stained with c-Kit pharmDx, 0 intensity, 20x.
c-Kit pharmDx Evaluation and Reporting

Slide evaluation should be performed by a pathologist using a light microscope.

The c-Kit pharmDx assay stains a variety of normal and neoplastic tissues. Observed c-kit staining patterns may be heterogeneous or homogeneous depending on the tissue and/or tumor type. Heterogeneity includes various staining intensities, ranging from 0–3+, within a single neoplasm. Positive cell staining patterns may also be heterogeneous including membrane, cytoplasmic staining, or both.

The performance characteristics of c-Kit pharmDx make possible the visualization of four intensity levels from 0–3+. c-kit staining at the cellular level has been observed on both the membrane and the cytoplasm (including paranuclear) at all intensity levels.

Dako recommends the following interpretation guideline in the assessment of c-kit immunostaining.

All assessments are to be made on the tumor region of the specimen.

Steps to c-Kit pharmDx Interpretation

1. Evaluate the control cell lines to validate the assay staining run.

**Examples of acceptable and unacceptable P815 positive cell line staining with c-Kit pharmDx.**

   ![Unacceptable weak staining intensity (too light); 40x. Weak staining intensity of cell line may result in false-negative results.](image1)

   ![Acceptable staining (moderate intensity); 40x.](image2)

   ![Unacceptable excessive staining intensity (too dark); 40x. Excessive staining intensity of cell line may result in false-positive results.](image3)

2. Evaluate the positive and negative tissue control slides to validate proper tissue preparation, staining techniques and assay performance.

3. An H&E stained slide of the test tissue is useful for comparison. The neoplasm may not be easily appreciable on the c-Kit pharmDx stained slide.

4. Evaluate the c-Kit pharmDx stained section for identification of neoplasm at low power, 4x magnification. 3+ intensity staining of the tumor cells is easily identified at 4x magnification.

5. Observe cells that stain brown, move to a higher power (10x magnification) to confirm the staining. In general, most cases should be observed at 10x magnification.

6. If the staining pattern is an artifact, it should be disregarded for evaluation. Find another representative area(s).

7. Use 20x or 40x magnification to assess staining pattern (cytoplasmic versus membrane, including paranuclear). Confirmation of c-Kit pharmDx staining at 20x magnification may be useful in those neoplasms with abundant cytoplasmic staining. Some tumor cells have several populations of cells with different intensities of c-kit membrane staining.

8. If there is no tumor staining and normal elements are not staining, review control slides of that staining run to confirm levels of c-kit expression that are observed. If positive elements in the control slides are negative, repeat the staining run.
Interpretation of c-kit protein expression must be made within the general morphological and clinical context of the tumor. There are three morphologic categories of GIST: spindle cell (70%), epithelioid (20%), or mixed types. Regardless of morphology, the majority of GISTs express c-kit protein in a significant proportion of the tumor cells. Positive homogeneous immunostaining with c-Kit pharmDx is primarily seen in the cytoplasm, with or without a golgi/paranuclear (dot-like) pattern, and in the cell membranes, with either complete or incomplete circumferential staining. Some cases show a heterogeneous staining pattern in combination with the staining patterns previously listed. Artifactual staining has also been observed in the extracellular spaces.

Positive and negative cell lines are included in each c-Kit pharmDx kit to validate staining runs, every time the assay is performed. Appropriate staining of the control slides provides evidence that the c-Kit pharmDx assay is functioning properly. No staining of the HT-29 control cell line (0) and moderate brown membrane, cytoplasmic and paranuclear staining in the P815 control cell line (2+) indicates that the staining run is valid. If the intensity of the positive control cell line is too weak or too strong a false negative or false positive result may be obtained and the test should be repeated.

Slide evaluation should be performed by a pathologist using a light microscope. All assessments are to be made on the tumor region of the specimen. For evaluation of the immunocytochemical staining and interpretation, an objective of 10x or 20x magnification is appropriate. Use intact cells for interpretation of staining results; necrotic or degenerated cells often stain non-specifically.

c-Kit pharmDx test results should be reported as positive or negative, using cytoplasmic and membrane staining as the evaluable structure (Table ). Positivity for c-kit protein expression is defined as any specific cytoplasmic and/or membrane staining in the tumor cells. It should also be noted that a small percentage of GISTs do not express c-kit protein. Therefore, a small percentage or absence of c-kit staining does not exclude the diagnosis of GIST.

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

**Diagnosis and Immunohistochemical Staining**

If necessary, use a panel of antibodies to aid in the identification of false negative reactions. When staining is focally positive, additional testing should be considered to confirm a GIST diagnosis. Genetic testing as well as the staining patterns specified in Table 3 aid in the differentiation between GIST and other mesenchymal sarcomas. Refer to c-Kit pharmDx Package Insert sections “Summary and Explanations” and “Limitations and Performance characteristics”, for specific information regarding immunoreactivity.

### Table 3  Immunohistochemical schema for the Differential Diagnosis of Spindle Cell Tumors of the GI Tract

<table>
<thead>
<tr>
<th></th>
<th>c-Kit</th>
<th>CD34</th>
<th>SMA</th>
<th>S-100</th>
<th>Desmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIST</td>
<td>≥95%</td>
<td>60–70%</td>
<td>30–40%</td>
<td>≤5%</td>
<td>≤2%</td>
</tr>
<tr>
<td>Smooth muscle tumor</td>
<td>–</td>
<td>10–15%</td>
<td>+</td>
<td>Rare</td>
<td>+</td>
</tr>
<tr>
<td>Schwannoma</td>
<td>–</td>
<td>Usually</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Fibromatosis</td>
<td>Disputed*</td>
<td>Rare</td>
<td>+</td>
<td>–</td>
<td>Rare</td>
</tr>
</tbody>
</table>

* Most, but not all authors report that fibromatoses are negative for c-kit.
Description
Deparaffinized tissue and appropriate control tissue sections are stained using the FDA-approved Dako c-Kit pharmDx immunohistochemistry kit.
A positive result is based on any specific cytoplasmic and/or membrane staining within the tumor cells.

<table>
<thead>
<tr>
<th>PATIENT RESULT</th>
<th>POSITIVE</th>
<th>NEGATIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-kit protein</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The c-Kit pharmDx is indicated as an aid in the differential diagnosis of gastrointestinal stromal tumors (GIST). After diagnosis of GIST, results from c-Kit pharmDx may be used as an aid in identifying those patients eligible for treatment with Gleevec™/Glivec® (imatinib mesylate).

All subjects in the Novartis Gleevec/Glivec clinical trials were selected using an investigational Novartis Clinical Trial Protocol (NCTP). The primary anti-c-kit rabbit polyclonal antibody reagent used in the NCTP was purchased from Dako. The c-Kit pharmDx primary polyclonal antibody reagent underwent the same method of production, purification and quality control as did the NCTP polyclonal anti-c-kit reagent.

<table>
<thead>
<tr>
<th>REPORT TO TREATING PHYSICIAN</th>
<th>DEFINITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-kit protein Negative</td>
<td>Absence of staining in tumor cells.</td>
</tr>
<tr>
<td>c-kit protein Positive</td>
<td>Positive staining is defined as any IHC staining of tumor cell cytoplasm and/or membranes above background level.</td>
</tr>
<tr>
<td>Staining Intensity</td>
<td>1+</td>
</tr>
</tbody>
</table>

Absence of staining in the cells of stromal tumors should be reported as negative for c-kit expression. A small percentage of GISTs do not express c-kit protein. Therefore, absence of c-kit staining does not exclude the diagnosis of GIST. Additional Immunohistochemical markers or genetic testing should be considered to aid in the differential diagnosis of these cases and other mesenchymal sarcomas.
Image Guide for Interpretation

c-Kit pharmDx Interpretation Guidelines

Dako emphasizes that interpretation of c-Kit pharmDx be performed within the context of the pathologist's past experience and best medical judgment. This guide will highlight examples of c-Kit pharmDx positivity and negativity, different staining intensities and areas of interpretation that are potentially problematic for c-Kit pharmDx users.

**Figure 10** Examples of tissues stained with c-Kit pharmDx.

- **GIST, no cytoplasmic or membrane staining (0) 20x.**
- **GIST, cytoplasmic staining (1+) 20x.**
- **GIST, cytoplasmic and membrane staining (2+) 20x.**
- **GIST, cytoplasmic and membrane staining (3+) 20x.**
Staining Patterns

The Dako c-Kit pharmDx stains a variety of normal and neoplastic tissues. Observed c-kit protein staining patterns are homogeneous or heterogeneous depending on the tissue and/or tumor type. The staining can show a range of 0-3+ staining intensity. Positive cell staining patterns can be membrane and / or cytoplasmic.

Heterogeneous Staining

Heterogeneity includes various staining intensities within a single neoplasm.

Homogeneous Staining

Homogeneous c-kit protein expression exhibits homogeneous staining patterns.

Dot-like Paranuclear Staining Pattern

Homogeneous c-kit protein expression can exhibit paranuclear staining patterns.
Membrane Staining

Cancers with c-kit protein expression can exhibit membrane staining patterns.

![Image of GIST with strong 3+ membrane staining; positive, 20x.](image)

**c-kit pharmDx Staining of Normal and Benign Tissues**

Normal and benign tissues, some of which are summarized in Table 1, exhibit specific c-kit staining. These can serve as useful internal controls. Staining of normal tissue elements should be excluded from the c-kit interpretation.

c-kit is expressed in a variety of normal cells. These cells include but are not limited to:

- Ductal and myoepithelial cells of the breast
- Purkinje cell processes
- Lamina propria cells of the colon
- Tubular epithelium of the kidney
- Melanocytes and glandular myoepithelial cells of the skin
- Interstitial Cells of Cajal of the small intestine
- Mast cells

Mast cells can be strongly reactive with the c-Kit pharmDx assay. Reactivity in granulocytes is caused by endogenous peroxidase activity. The staining pattern is usually cytoplasmic, not membrane.

![Image of strong 3+ mast cell staining; 20x](image)

**Figure 17** Strong 3+ mast cell staining, 20x (2+ staining was observed in the normal tissue testing).

**Non-evaluable Areas of Tissue**

Areas on stained slides that should not be evaluated include dissociated, free floating groups or aggregates of neoplastic cells, necrotic cells, and damaged areas of the tissue section (torn sections, folded or wrinkled areas, etc.)
Factors to Consider in Evaluating c-Kit pharmDx Stains

Non-Specific Background Staining

Non-specific background staining is defined as diffuse, non-specific staining of tissue elements. It may be caused by a variety of factors including both biologic activity and technological processes.

**Biologic Activity**

- Pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (granulocytes) may result in non-specific background staining due to decomposition of $\text{H}_2\text{O}_2$ within the substrate.

**Technological Processes**

- Use of fixatives other than the recommended 10% neutral buffered formalin
- Incomplete deparaffinization of tissue sections prior to staining
- Use of alternative buffers (recommended wash buffer (Code S3006))
- Incomplete rinsing of reagents from slides
- Inappropriate drying of slides during staining procedure (use a humid chamber when assay is performed manually)

The level of non-specific background staining may be ascertained by incubation of test specimen with the negative control reagent. If non-specific background staining is significant, the specific staining must be interpreted with caution.

Improper Target Retrieval

c-Kit pharmDx includes pretreatment by means of heat induced target retrieval. Tissue sections may be over retrieved, causing disruption of cell morphology, overall tissue architecture or tissue removal from the slide. Run the assay with careful attention to the duration of the target retrieval step.

**Note** If tissue removal from the slide is a persistent problem, tissue sections may be mounted on slides which provide better section adherence (charged, silanized, and poly-L-lysine coated slides).
Interpreting Artifacts

Edge Artifact

Frequently, increased staining is observed around the periphery of the tissue specimen. It is known as the “edge effect.” Edge artifacts are commonly the result of inappropriate pre-analytic handling of the tissue. The edge effect represents fixation artifact or tissue drying prior to fixation. Usually the staining artifact is limited to a thin rim of stained cells with an abrupt termination to the staining reaction. Often the method of surgical extraction is the cause (see Crush Artifact section).

Tissue section edge staining artifacts are common if there are significant tissue section irregularities. Thick tissue sections may mimic edge artifacts and can be corrected by recutting the tissue block to produce a uniform, thin, 3-5 μm thick section that is devoid of folds and wrinkles.

When the positive reaction is only at the edge of the tissue section, evaluation of c-kit staining should exclude tumor cells within the region’s edge artifact.

Crush Artifact

Compression of tissue around the periphery of specimens, especially biopsy specimens, can produce non-specific staining of the tissue components in addition to the membranes of neoplastic cells. The appearance of this staining artifact is similar to those produced by tissue edge-staining artifacts.

The staining intensity of cell membranes within compressed tissue is frequently greater than similar appearing cells in regions of normal architectural tissue arrangements.

Retraction Artifact

Stromal retraction around tumor cell glands can create clefts where pooled antibody can nonspecifically stain. Thorough washing after the primary antibody reagent incubation step may prevent this reaction. Retraction space staining usually is a hemi-circumferential reaction around the periphery of the gland.

Thermal Artifact

Thermal electrocautery may alter nuclei and cell membranes. Dako recommends that the evaluation of c-Kit pharmDx staining be performed on tissue with no or minimal thermal electrocautery artifacts.
Examples of GIST Stained with c-Kit pharmDx

**Negative c-Kit pharmDx Immunostaining**

*Figure 18* GIST, example of H&E; 10x.

*Figure 19* GIST, example of negative staining; 10x.

**Positive Homogeneous c-Kit pharmDx Immunostaining**

*Figure 20* GIST, example of H&E; 10x.

*Figure 21* GIST, example of weak 1+ positive homogeneous staining; 10x.

*Figure 22* GIST, example of weak 1+ positive homogeneous staining; 20x.
Positive Homogeneous c-Kit pharmDx Immunostaining (moderate)

Figure 23 GIST, example of moderate 2+ positive homogeneous staining; 20x.

Figure 24 GIST, example of moderate 2+ positive homogeneous staining; 20x.

Figure 25 GIST, example of moderate 2+ positive homogeneous staining; 20x.

Positive Homogeneous c-Kit pharmDx Immunostaining (strong)

Figure 26 GIST, example of strong 3+ positive homogeneous staining; 10x.

Figure 27 GIST, example of strong 3+ positive homogeneous staining; 10x.

Figure 28 GIST, example of strong 3+ positive homogeneous staining; 40x.
Differential Immunostaining of Leiomyoma

Figure 29 Leiomyoma, example of H&E; 10X.

Figure 30 Leiomyoma, example of CD34 strong 3+ staining; 10X.

Figure 31 Leiomyoma, example of c-kit negative 0 staining; 10X.

Figure 32 Leiomyoma, example of SMA moderate 2+ staining; 10X.
Differential Immunostaining of Leiomyosarcoma

Figure 33 Leiomyosarcoma, example of H&E; 10X.

Figure 34 Leiomyosarcoma, example of CD34 moderate 2+ staining; 10X.

Figure 35 Leiomyosarcoma, example of c-kit negative 0 staining; 10X.

Figure 36 Leiomyosarcoma, example of Desmin weak 1+ staining; 10X.

Figure 37 Leiomyosarcoma, example of SMA strong 3+ staining; 10X.
Differential Immunostaining of Schwanoma

Figure 38 Schwanoma, example of H&E; 10X.

Figure 39 Schwanoma, example of c-kit negative 0 staining; 10X

Figure 40 Schwanoma, example of CD34 moderate 2+ staining; 10X

Figure 41 Schwanoma, example of S100 moderate 2+ staining; 10X
References


4. Biosource


9. CLIS (formerly National Committee for Clinical Laboratory Standards, NCCLS). Quality Assurance for Immunocytochemistry; Approved guideline. Villanova, PA 1999. Order code MM4-A


Additional c-kit Resources


- Dematteo RP, Heinrich MC, El-Rifai WM, and Demetri G. Clinical Management of Gastrointestinal Stromal Tumors: Before and After STI-571. Human Pathology, May 2002; 33:5; 466


- Gleevec /Glivec® (imatinib mesylate) Package Insert

- c-Kit pharmDx Package Insert