

LABELING

c-Kit pharmDx™**Code K1907**

35 tests for use on the Dako Autostainer

Intended use

For In Vitro Diagnostic Use.

The c-Kit pharmDx™ assay is a qualitative immunohistochemical (IHC) kit system used 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 DakoCytomation. 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.

**Summary
and explanation****Introduction**

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 and 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.⁴ The c-kit tyrosine kinase receptor pathway has been shown to be important for tumor growth and progression in several cancers.⁵ 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.⁶

Gastrointestinal mesenchymal tumors have historically been difficult to differentiate and diagnose. In 2001, imatinib mesylate (Gleevec™, Novartis, Basel, Switzerland) was approved by the US Food and Drug Administration for the treatment of gastrointestinal stromal tumors (GIST). Approval was based on a clinical study in which adults with GIST that expressed c-kit protein demonstrated by immunohistochemistry (Novartis Clinical Trial Protocol) were enrolled and treated with imatinib mesylate.⁷

GIST cases are described as three morphologic categories: spindle cell, epithelioid and mixed types. Regardless of morphology, the majority of GIST's express c-kit protein in a significant proportion of the tumor cells.⁸⁻¹¹ It should be noted that a small percentage of GIST's do not express c-kit protein. Relatively few other tumors may be c-kit protein positive. These include metastatic melanoma, angiosarcoma, Ewing sarcoma, mastocytoma, seminoma, and pulmonary small cell carcinoma. GIST are usually also positive for heavy molecular-weight caldesmon, often positive for CD34 (60-80%), and generally negative for desmin and S100 protein.¹²

Specificity

Rabbit anti-c-kit polyclonal c-kit antibodies were obtained by subcutaneous injection of a 14 mer peptide (positions 963-976 of the intracellular C' terminus of the c-kit protein) coupled to thyroglobulin. The antiserum was specifically purified through antigen-bound, activated thiol AvidGel F affinity chromatography.

A small number of soft tissue sarcomas have also been found to be c-kit protein positive.⁶ Some of these specimens (e.g. leiomyosarcoma) were tested for c-kit expression. The 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™ in the clinical trials performed by Novartis. 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). Therefore the primary antibody used in the c-Kit pharmDx™ assay specifically reacted with the c-kit protein in the two positively stained soft tissue sarcomas.

In-house studies using c-Kit pharmDx demonstrated c-kit protein expression in a variety of normal cells. These cells include ductal and myoepithelial cells of the breast, purkinje cell processes, lamina propria cells of the colon, tubular epithelium of the kidney, melanocytes and myoepithelial cells of the skin, interstitial Cells of Cajal of the small intestine, and mast cells. The cytoplasmic reactivity in granulocytes was caused by endogenous peroxidase activity and was visible with the Negative Control Reagent.

Reagents

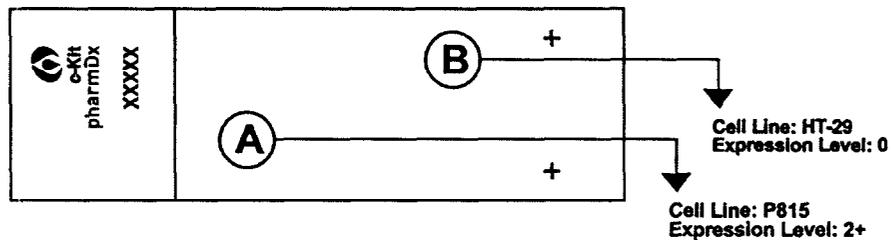
Code K1907 is for Automated Staining using the Dako Autostainer.

The materials listed are sufficient for 35 tests (35 patient slides and 10 control slides incubated with primary antibody reagent to c-kit protein and 35 slides incubated with the corresponding Negative Control Reagent). The number of tests is based on the use of the c-Kit pharmDx™ Autostainer protocol with ready-to-use reagents.

The kit provides materials sufficient for a maximum of 10 individual staining runs.

Materials provided

<i>Quantity</i>	<i>Description</i>
1x9 mL	<p>c-Kit pharmDx™ Polyclonal Rabbit IgG</p> <div style="border: 1px solid black; padding: 2px; width: fit-content;"> <p>c-Kit pharmDx™ RABBIT ANTI-HUMAN c-kit PROTEIN</p> </div> <p>Polyclonal rabbit anti-human c-kit IgG in a Tris-HCl buffer, containing stabilizing protein and 0.015 mol/L sodium azide.</p>
1x9 mL	<p>Rabbit IgG Negative Control Reagent</p> <div style="border: 1px solid black; padding: 2px; width: fit-content;"> <p>c-Kit pharmDx™ NEGATIVE CONTROL REAGENT</p> </div> <p>Polyclonal rabbit IgG at greater than or equal to the positive anti-c-kit concentration in a Tris-HCl buffer, containing stabilizing protein and 0.015 mol/L sodium azide.</p>
2x5 slides	<p>c-Kit pharmDx™ Control Slides</p> <div style="border: 1px solid black; padding: 2px; width: fit-content;"> <p>c-Kit pharmDx™ CONTROL SLIDES</p> </div> <p>Each slide contains sections of two pelleted, formalin-fixed, paraffin-embedded mouse (+) and human (-) cell lines, which represent both moderate and no expression levels of c-kit protein. The IHC staining scores of the cell pellets are 2+ and 0.</p>



Principle of procedure

The c-Kit pharmDx™ IHC kit contains polyclonal antibodies and control slides to complete an IHC staining procedure for formalin-fixed and 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.

Materials required, but not supplied

The c-Kit pharmDx™ IHC protocol was validated using the following DakoCytomation staining reagents:
 Wash Buffer (code S3006)
 Dual Endogenous Enzyme Block (code S2003)
 Target Retrieval Solution (code S1699 & S1700)
 EnVision+ HRP, Rabbit (code K4002 & K4003)
 DAB+ (code K3467 & K3468)

Note: To ensure proper staining results, only these staining reagents should be used with c-Kit pharmDx™. Deviations from this recommended protocol have not been validated.

Other materials required to perform c-Kit pharmDx™ include the following:

- Ammonium hydroxide, 15 mol/L diluted to 0.037 mol/L
- Hematoxylin, alcohol or water-based such as DakoCytomation's Hematoxylin (code S3301 or S3302)
- Coverslips
- Water bath, capable of maintaining 95–99°C or pressure cooker, capable of heating up to 121°C
- Distilled or deionized water (reagent-quality water)
- Drying oven, capable of maintaining 56–60°C
- Ethanol, absolute and 95%
- Light microscope (4x–40x objective magnification)
- Mounting medium, such as DakoCytomation's Faramount (code S3025)
 - or DakoCytomation's Glycergel (code C0563) or DakoCytomation's Ultramount (code S1964)
- Positive and negative tissues to use as process controls (see Quality control section)
- Slides, Fisher's SuperFrost Plus, poly-L-lysine-coated slides, charged slides,
 - or DakoCytomation's Silanized Slides (code S3003) (see Specimen preparation section)
- Staining jars or baths
- Timer (capable of 2–30 minute intervals)
- Xylene, toluene, or xylene substitutes
- 1 mL pipette
- Dako Autostainer Universal Staining System (code S3400) or Autostainer Plus (code S3800) with Dako Autostainer software version 3.0.0 or higher. (Please refer to the Dako Autostainer User Guide for necessary components.)

Note: All reagents included or available separately such as DakoCytomation's Wash Buffer (code S3006) are formulated specifically for use with this test. In order for the test to perform as specified, no substitutions can be made.

Precautions

1. For professional users. For In Vitro Diagnostic Use.
2. This product contains sodium azide (NaN₃), a chemical that is highly toxic in pure form. At product concentrations, though not classified as hazardous, build-ups of NaN₃ that may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.¹³
3. As with any product derived from biological sources, proper handling procedures should be used for reagents as well as specimens.¹⁴
4. Incubation times, temperatures, or methods other than those specified may give erroneous results.
5. Do not substitute primary antibodies or negative control reagents with primary antibodies and negative control reagents of different manufactured lots (lot numbers appear on vial labels) or with reagents from other manufacturers. This may give erroneous results.
6. The kit reagents are optimally diluted for use with the recommended reagents. Further dilution is not recommended and may result in loss of antigen staining. The user must verify any such changes.¹⁵
7. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.¹⁴
8. Unused solution should be disposed of according to local, State, and Federal regulations.

Risk and Safety Statements

DAB Chromogen

- R 40 Limited evidence of a carcinogenic effect.
- R43 May cause sensitization by skin contact.
- R68 Possible risk of irreversible effects.
- S35 This material and its container must be disposed of in a safe way.
- S 36/37 Wear suitable protective clothing and gloves.

Storage

Store c-Kit pharmDx™ kit at 2–8°C.

Control slides must also be stored at 2–8°C.

Do not use the kit after the expiration date printed on the outside of the kit box. There are no obvious signs to indicate instability of this product, therefore, positive and negative controls should be run simultaneously with patient specimens. If the reagents are stored under any conditions other than those specified in the package insert, they must be validated by the user.¹⁵

Specimen preparation

Biopsy specimens must be handled to preserve the tissue for IHC staining. Standard methods of tissue processing should be used for all specimens.¹⁶

Paraffin-embedded sections

Formalin-fixed and paraffin-embedded tissues are suitable for use. Alternative fixatives have not been validated and may give erroneous results. Specimens from the biopsy should be blocked into a thickness of 3 or 4 mm and fixed for the time period appropriate for the fixative. The tissues are then dehydrated and cleared in a series of alcohols and xylene, followed by infiltration by melted paraffin. The paraffin temperature should not exceed 60°C. Properly fixed and embedded tissue blocks expressing the c-kit protein will keep indefinitely prior to sectioning and slide mounting if stored in a cool place (15–25°C).^{16, 17}

Tissue specimens should be cut into sections of 3–5 µm. After sectioning, tissues should be mounted on Fisher's SuperFrost Plus, DakoCytomation's Silanized (code S3003), charged slides or poly-L-lysine coated slides and

placed in drying racks. The slide racks should be pounded on an absorbent towel to remove water trapped under paraffin and on glass and then dried at room temperature for one hour. The rack of slides should then be placed in a 56–60°C incubator for one hour. Any excess water remaining on slides after removal from the incubator should be removed by pounding slides on towels and drying for one additional hour in the incubator. After removal from the incubator, slides should be held at room temperature until cool and paraffin has hardened. To preserve antigenicity, tissue sections, mounted on slides, should be stained within 2 months of sectioning when held at room temperature (20–25°C). Consult the DakoCytomation Handbook: "Immunocytochemical Staining Methods"¹⁸ or References 16 and 17 for further details on specimen preparation.

The use of decalcified tissues has not been validated and is not recommended. The slides required for c-kit evaluation and verification of tumor presence should be prepared at the same time. A minimum of 5 slides should be prepared, 1 slide for tumor presence, 2 slides for c-kit protein evaluation, and 2 slides for back-up.

Reagent preparation The following reagents must be prepared prior to staining:

Target Retrieval Solution

Prepare a sufficient quantity of Target Retrieval Solution by diluting Target Retrieval Solution 10x 1:10 using distilled or deionized water (reagent-quality water) for the wash steps. Discard Target Retrieval Solution after use. **Note:** When using DakoCytomation's Target Retrieval Solution (Code S1700) no dilution is necessary.

Wash Buffer Solution

Prepare a sufficient quantity of Wash Buffer by diluting Wash Buffer 10x 1:10 using distilled or deionized water (reagent-quality water) for the wash steps. Store unused solution at 2–8°C for no more than 7 days. Discard buffer if cloudy in appearance.

Substrate-Chromogen Solution (DAB+)

This solution should be mixed thoroughly prior to use. Any precipitate developing in the solution does not affect staining quality.

To prepare DAB+ Substrate-Chromogen Solution, add 1 drop of Liquid DAB+ Chromogen to one mL of DAB+ Substrate Buffer and mix. Discard any unused solution.

Stability of prepared DAB+ is approximately 5 days when stored at 2–8°C.

Important Note: The color of the Liquid DAB+ Chromogen in the bottle may vary from clear to light lavender-brown. This will not affect the performance of this product. Dilute per the guidelines above. Addition of excess Liquid DAB+ Chromogen to the DAB+ Substrate Buffer will result in deterioration of the positive signal.

Counterstain

Prepare ammonia water for counterstain bluing if required.

Ammonia water (0.037 mol/L) is prepared by mixing 2.5 (±0.5) mL of 15 mol/L (concentrated) ammonium hydroxide with 1 liter of reagent quality water. Unused 0.037 mol/L ammonia water may be stored at room temperature (20–25°C) in a tightly capped bottle for up to 12 months.

Mounting Medium

Mounting media such as DakoCytomation's Faramount Aqueous Mounting Medium, Ready-to-use (code S3025) or DakoCytomation's Glycergel Mounting Medium (code C0563) is recommended for aqueous mounting. Liquify Glycergel by warming to approximately 40(±5)°C prior to use.

Non-aqueous, permanent mounting is also suitable, such as DakoCytomation's Ultramount (code S1964).

**Staining procedure
on the
Dako Autostainer**

Procedural Notes

The user should read these instructions carefully and become familiar with all components prior to use (see Precautions). Refer to "Dako Autostainer, Universal Staining System, User Guide".

All reagents should be equilibrated to room temperature (20–25°C) prior to immunostaining. Likewise, all incubations should be performed at room temperature.

Do not allow tissue sections to dry during the staining procedure. Dried tissue sections may display increased nonspecific staining.

Note: The reagents and instructions supplied in this system have been designed for optimal performance when used with the recommended reagents and materials. Further dilution of the reagents or alteration of incubation times or temperatures may give erroneous or discordant results.

Deparaffinization and Rehydration

Prior to staining, tissue slides must be deparaffinized to remove embedding medium and rehydrated. Avoid incomplete removal of paraffin. Residual embedding medium will result in increased nonspecific staining.

- STEP 1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
- STEP 2. Tap off excess liquid and place slides in absolute ethanol for 3 (±1) minutes. Change baths and repeat once.
- STEP 3. Tap off excess liquid and place slides in 95% ethanol for 3 (±1) minutes. Change baths and repeat once.
- STEP 4. Tap off excess liquid and place slides in reagent-quality water for 5 (±1) minutes.
- STEP 5. Tap off excess liquid and place slides in Wash Buffer. Begin assay as outlined in Staining Procedure.

Xylene and alcohol solutions should be changed after 40 slides.

Toluene or xylene substitutes, such as Histoclear™, may be used in place of xylene.

Pretreatment

Recommended procedure: Water Bath

- STEP 1. Fill staining jars, e.g. Coplin jars, with the diluted Target Retrieval Solution (see Reagent preparation). Place staining jars containing Target Retrieval Solution in water bath. Heat water bath and the Target Retrieval Solution to 95–99°C (do not boil). Cover jars with lids to stabilize the temperature and avoid evaporation.
- STEP 2. Immerse room temperature deparaffinized sections in the preheated Target Retrieval Solution in the staining jars. Re-equilibrate temperature of the water bath and the Target Retrieval Solution back to 95–99°C. Incubate for 20 (±1) minutes at 95–99°C.
- STEP 3. Remove the entire jar with slides from the water bath. Allow the slides to cool in the Target Retrieval Solution for 20 (±1) minutes at room temperature.
- STEP 4. Decant Target Retrieval Solution and rinse sections in Wash Buffer (see Reagent preparation).
- STEP 5. For optimal performance, soak sections in Wash Buffer for 5 (±1) minutes after target retrieval and prior to staining.

Recommended procedure: Pressure Cooker (121°C)

- STEP 1. Fill staining jars, e.g. Coplin jars, with the diluted Target Retrieval Solution (see Reagent Preparation).
- STEP 2. Immerse room temperature deparaffinized sections into Target Retrieval Solution in the staining jars.
- STEP 3. Place staining jars into the pressure cooker and tighten the lid.
- STEP 4. Incubate in a pressure cooker set at 121°C for 5 minutes. Allow to cool without venting.
- STEP 5. When pressure has released remove the entire jar with slides from the pressure cooker.
- STEP 6. Decant Target Retrieval Solution and rinse sections in Wash Buffer (see Reagent Preparation).
- STEP 7. For optimal performance, soak sections in Wash Buffer for 5 (±1) minutes after target retrieval and prior to staining.

Note: The Target Retrieval Solution is designed for single use application only. Do not re-use.

Automated Staining Protocol

Refer to "Dako Autostainer, Universal Staining System, User Guide".

- STEP 1. Select protocol and program staining run (Figure 1).
- STEP 2. Use Auto programs to set up program and begin the c-Kit pharmDx™ program.
- STEP 3. Place the reagent vials in the Dako Autostainer reagent rack according to the computer generated reagent map.
- STEP 4. Load the slides onto the Dako Autostainer according to the computer generated slide map.
- STEP 5. Begin the run.
- STEP 6. Remove slides from the Dako Autostainer.

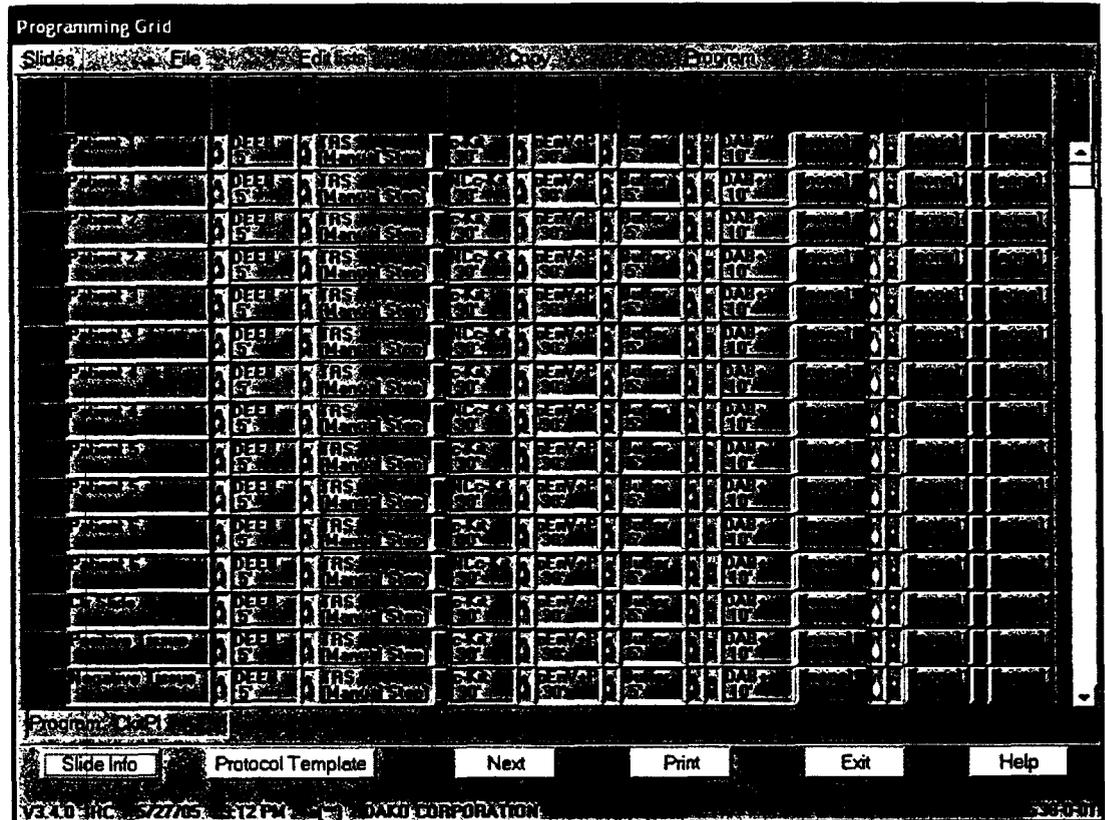
Collect DAB+ Substrate-Chromogen Solution waste in a hazardous materials container for proper disposal.

Proceed to **Counterstain and Mounting**.

Note: Rinse slides in reagent-quality water after the DAB+ Substrate-Chromogen solution step. (Dako Autostainer hardware versions 02 and 03 rinse the slides in reagent-quality water after the substrate-chromogen step. The 01 hardware version of the Dako Autostainer rinses slides in buffer. Therefore, slides that are stained on Dako Autostainer version 01 hardware must be rinsed with the reagent-quality water after they have been removed from the Autostainer).

Figure 1. Autostainer Programming Grid

Representation of the program run on the Dako Autostainer.



The programming grid (above) is a pharmDx™ Master Protocol Template containing protocol elements needed to run with the c-Kit pharmDx™ kit. Only c-Kit reagents are shown in this example grid.

**Counterstain
(instructions for
Hematoxylin)**

The colored end-product of the staining reaction is alcohol and water insoluble. Hematoxylin, either alcohol or water-based such as DakoCytomation's Hematoxylin (code S3301, automated; or S3302, manual), may be used. Do not use regressive counterstains.

- STEP 1. Remove slides from the Dako Autostainer and counterstain in hematoxylin as described below.
- STEP 2. Immerse slides in a bath of hematoxylin. Incubate for 2–5 minutes, depending on the strength of hematoxylin used.
- STEP 3. Rinse gently in a reagent-quality water bath. Ensure that all residual hematoxylin has been cleared.
Optional: Dip slides 10 times into a bath of 0.037 mol/L ammonia water, however this is not required (see Reagent preparation section).

Note: The use of DakoCytomation's Hematoxylin (code S3301) is strongly recommended. Using a 3-minute incubation, this counterstain produces a mild purple/blue end product that does not obscure specific immunostaining. Strong counterstaining may mask weak c-kit protein expression.

- STEP 4. Rinse gently in a reagent-quality water bath for 2–5 minutes.

Mounting

Mounting media such as DakoCytomation Faramount Aqueous Mounting Medium, Ready-to-use (code S3025) or DakoCytomation GlycerGel Mounting Medium (code C0563) is recommended for aqueous mounting. Liquify GlycerGel by warming to approximately 40(±5)°C prior to use.

Non-aqueous, permanent mounting is also suitable such as DakoCytomation Ultramount (code S1964).

Note: It is recommended that slides are read within six weeks of staining. However, some fading may occur, depending on several factors including, but not limited to; counterstaining, mounting materials and methods and slide storage conditions. To minimize fading, store slides in the dark at room temperature (20–25°C).

Quality control

Deviations in the recommended procedures for tissue fixation, processing, and embedding in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls in addition to the DakoCytomation-supplied Control Slides. In the USA, consult the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry. See also CLIS Quality Assurance for Immunocytochemistry Approved ¹⁹Guideline¹⁹ and References 20-23 for additional information.

c-Kit pharmDx™ Control Slides (provided)

Each of the supplied Control Slides contains two pelleted, formalin-fixed, paraffin-embedded mouse (+) and human (-) cell lines with staining intensity scores of 2+ and 0. One slide should be stained in each staining procedure. The evaluation of the DakoCytomation-supplied Control Slide cell lines indicates the validity of the staining run. They should not be used as an aid in interpretation of patient results.

Positive Control Tissue

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

The tissues used for the positive tissue controls should give weak positive staining so they can detect subtle changes in the primary antibody sensitivity. The Control Slides supplied with this system or specimens processed differently from the patient sample(s) validate reagent performance only and do not verify tissue preparation.

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 appropriate positive staining, results with the test specimens should be considered invalid.

Normal cell types that may be used as a weak c-kit positive control include basal epidermal melanocytes and glandular myoepithelial cells of the skin, as well as epithelial and myoepithelial cells of the breast. Strong internal controls can be neuronal (Interstitial Cells of Cajal) and mast cells in the intestine.

Negative Control Tissue

Use a negative control tissue (known to be c-kit protein negative) fixed, processed, and embedded in a manner identical to the patient sample(s) with each staining run to verify the specificity of the primary antibody and to provide an indication of specific background staining. The variety of different cell types present in most tissue sections offers internal negative control sites (this should be verified by the user).

If specific staining occurs in the negative control tissue, results with the patient specimens should be considered invalid. Tissue elements which may be used as negative controls include cardiac myocytes of the heart. Pancreatic acinar cells are also c-kit negative, whereas pancreatic bile duct epithelium stains positively.

Non-specific Negative Control Reagent

Use the supplied Negative Control Reagent in place of the primary antibody with a section of each patient specimen to evaluate nonspecific staining and allow better interpretation of specific staining at the antigen site. The incubation period for the Negative Control Reagent should correspond to that of the primary antibody.

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

Assay Verification

Prior to initial use of an antibody or staining system in a diagnostic procedure, the user should verify the antibody's specificity by testing it on a series of in-house tissues with known IHC performance characteristics representing known positive and negative tissues. Refer to the Quality control procedures previously outlined in this section of the product insert and to the Quality control requirements of the CAP Certification Program for Immunohistochemistry and/or CLIS Quality Assurance for Immunocytochemistry, Approved Guideline.¹⁹ These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay

parameters. Gastrointestinal Stromal Tumors (GIST) with known c-kit protein staining intensities and negative tissues are suitable for assay verification.

Table 1: The Purpose of Daily Quality control

<i>Tissue: Fixed and Processed Like Patient Sample</i>	<i>Specific Antibody & Detection System</i>	<i>Background: Non-specific Antibody (Rabbit Negative Control Reagent) provided</i>
DakoCytomation-supplied Control Slide.	Controls staining procedure only. (The evaluation of the DakoCytomation-supplied Control Slide cell lines indicates the validity of the staining run.)	
Positive Control: Tissue or cells containing target antigen to be detected (could be located in patient tissue). The ideal control is weakly positive staining tissue to be most sensitive to antibody or antigen degradation.	Controls all steps of the analysis. Validates reagents and procedures used for c-kit protein staining.	Detection of non-specific background staining.
Negative Control: Tissues or cells expected to be negative (could be located in patient tissue or positive control tissue).	Detection of unintended antibody cross-reactivity to cells/cellular components.	Detection of non-specific background staining.
Patient Tissue.	Detection of specific staining.	Detection of non-specific background staining.

Interpretation of staining procedure

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.¹⁹

Positive and negative staining cell lines are included in each c-Kit pharmDx™ kit to validate assay performance. Appropriate staining of the control slides provides evidence that the c-Kit pharmDx™ reagents are functioning properly and the protocol has been performed accurately. No cytoplasmic 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. Deviation from the optimal staining intensity of the positive control cell line (too weak or too strong) may result in a false negative or a false positive result and indicate that the test should be repeated.

Evaluation of the positive tissue control should be performed. Weak staining of skin myoepithelial cells indicates that the reagents are working properly. If the positive tissue controls fail to demonstrate positive staining, any results with the test specimens should be considered invalid.

The negative tissue control should be examined after the positive tissue control to verify the specificity of the labeling of the antigen by the primary antibody. The absence of specific staining in the negative tissue control confirms lack of antibody cross-reactivity to cells/cellular components. If specific staining occurs in the negative tissue control, results with the patient specimen should be considered invalid. Nonspecific staining, if present, usually has a diffuse appearance. Sporadic staining of connective tissue may also be observed in sections from excessively formalin-fixed tissues. Use intact cells for interpretation of staining results. Necrotic or degenerated cells often stain nonspecifically.²⁰

Examine patient specimens last. Positive staining intensity should be assessed within the context of any non-specific background staining of the negative reagent control. 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.²⁴ Regardless of morphology, the majority of GISTs express c-kit protein in a significant proportion of the tumor cells. 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 (a mixture of strong or weak cytoplasmic and/or membranous) staining pattern. Staining has also been observed in the extracellular spaces.

c-Kit pharmDx™ test results should be reported as positive or negative, using cytoplasmic and membrane staining as the evaluable structure (Table 2). Positivity for c-kit protein expression is defined as any specific cytoplasmic

and/or membrane staining in the tumor cells. Focal positivity or staining in less than 10% of tumor cells should be interpreted with caution.²⁵ As with any immunohistochemistry test, a negative result means that the antigen was not detected, not that the antigen was absent in the cells/tissues assayed. It should also be noted that a small percentage of GISTs do not express c-kit protein. Therefore, absence of c-kit protein staining does not necessarily exclude the diagnosis of GIST.

Table 2: c-Kit pharmDx™ Staining Results

Report to treating physician	Definition
c-kit protein negative	Absence of staining in the tumor cells.
c-kit protein positive	Positive staining is defined as any IHC staining of tumor cell cytoplasm and/or membranes above background level.
	Staining Intensity: 1+, 2+, or 3+

If necessary, use a panel of antibodies to aid in the identification of false negative reactions. When staining is focally positive or too weak, 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 "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.⁸

	c-kit	CD34	SMA Smooth Muscle Actin	S-100	Desmin
GIST	≥95%	60-70%	30-40%	≤5%	≤2%
Smooth muscle tumor	-	10-15%	+	Rare	+
Schwannoma	-	Usually	-	+	-
Fibromatosis	Disputed*	Rare	+	-	Rare

* Most, but not all authors report that fibromatoses are negative for c-kit.

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 counterstaining may compromise interpretation of results.

Limitations

General limitations

IHC is a multistep diagnostic process that requires specialized training in the selection of the appropriate reagents; tissue selection, fixation, and processing; preparation of the IHC slide; and interpretation of the staining results.

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.

The clinical interpretation of any positive staining or its absence must be evaluated within the context of clinical presentation, morphology and other histopathological criteria. The clinical interpretation of any staining, or its absence must be complemented by morphological studies and proper controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist who is familiar with the antibodies, reagents and methods used to interpret the stained preparation. Staining must be performed in a certified licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.

This product is not intended for flow cytometry. Performance characteristics have not been determined for flow cytometry.

Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.²⁶

Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues.¹⁹ Contact DakoCytomation technical support with

documented unexpected reactions.

Product specific limitations

A small percentage of GISTs do not express c-kit protein. Therefore, the absence of c-kit staining does not exclude the diagnosis of GIST. 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) and endogenous peroxidase activity (cytochrome C).²¹

For optimal and reproducible results, the c-kit protein requires target retrieval when tissues are routinely fixed (neutral buffered formalin) and paraffin embedded.

DakoCytomation 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.

Stained control cell lines should be used only for validation of the staining run and should not be used to score the staining reaction in tissue sections.

Specimens preserved by routine processing in neutral buffered formalin are suitable for testing with c-Kit pharmDx™. Tissues fixed in alternative fixatives have not been validated.

Performance characteristics

Specificity

The c-kit antibody reagent 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 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 labelled.²⁷ Further, applying a second anti-c-kit antibody, a 100 kD protein was, likewise, labelled. This labelling was abolished when the antibody reagent was incubated with the synthetic c-kit peptide antigen used for immunization.²⁸ 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 DakoCytomation antibody reagent did not react with a lysate of the adenocarcinoma cell line HS, which does not express the c-kit gene.²⁷

Comparison Studies

Immunostaining using the c-Kit pharmDx™ assay was performed on formalin-fixed paraffin-embedded sarcomas and multi-tissue arrays (MTAs) containing a selection of sarcomas that included positive and negative c-kit protein expression. The tissues were tested using the c-Kit pharmDx™ assay after the use of the two recommended methods of heat induced epitope retrieval (HIER). Briefly, water bath (95–99°C) for 20 minutes and pressure cooker (121°C) for 5 minutes of heating, both followed by 20 minutes of cool down, were used as heat sources. The remaining steps of the staining procedure were identical. These assays were compared to a concurrently run simulation of the Novartis Clinical Trial Protocol (NCTP) that had been used previously to select patients for the clinical trial that obtained FDA approval for the use of Gleevec/Glivec with diagnosed GIST patients. The NCTP used a 2X higher concentration of DakoCytomation primary polyclonal antibody against c-kit (A4502) (the same reagent used in c-Kit pharmDx) and no HIER. Results from the concurrent comparison of the NCTP and the c-Kit pharmDx™ assay using pressure cooker as the heat source are listed in Table 4 and similar results (overall agreement =98.7%), were seen when a water bath was used as a heat source. Percent positive and negative agreements were similar for the two comparisons.

Table 4. 2x2 table of c-Kit pharmDx™ protocol with pressure cooker test results compared to “NCTP” test result

c-Kit pharmDx™ Pressure Cooker TRTest Result	Concurrent NCTP Assay Test Results		
	Positive n (%)	Negative n (%)	Total
Positive	188 (60.8)	3 (0.9)	191
Negative	1 (0.3)	117 (37.9)	118
Total	189	120	309

Percent Positive Agreement = 188/(188 +1); = 0.995 x 100 = 99.5% (95% exact CI: 97.1% - 100%)

Percent Negative Agreement = $117/(117+3)$, = 0.975 x 100 = 97.5% (95% exact CI: 92.9% - 99.5%)

Overall Agreement = $(188+117)/308$ = 0.987 x 100 = 98.7% (95% exact CI: 96.7% - 99.7%)

A subset of these specimens (35 cases) represented tissues from patients participating in the Novartis Gleevec clinical trial. Of these patients 21 were treated with Gleevec™ (58.3%). When the same specimens were retested with the simulated NCTP and c-Kit pharmDx™ 20 were found to be positive with all three procedures, whereas 1 specimen was found to be negative. Among the 147 patients treated with Gleevec™ in the Novartis Gleevec Clinical Trial, it was reported that 54% had a partial response, and 28% had stable disease.⁷

For a subset of 259 specimens presented in Table 4, the CD117 test result was determined at the time the specimens were put into the MTAs. Thirty-five of the cases were enrolled in the Gleevec clinical trials (n=35), and the remainder were tested in the same laboratories that had participated in the Gleevec trials. Results from 179 specimens (the total was less than 259 due to specimen loss or lack of tumor in the MTAs) are presented in Table 5 below. These specimens demonstrated an overall agreement to the original CD117 status of 94.9% (95% exact CI: 90.7% - 97.7%)

Table 5: 2x2 table of pressure cooker test results compared to Original test result

Pressure cooker test result	Original tissue CD117 status		
	Positive n	Negative n	Total n (%)
Positive	136	3	139 (78%)
Negative	6	34	40 (22%)
Total	142	37	179

Percent positive agreement = $136 / (136+6)$; = 0.957 x 100 = 95.7% (95% exact CI: 91.0% - 98.4%)

Percent negative agreement = $34 / (34+3)$; = 0.919 x 100 = 91.9% (95% exact CI: 78.1% - 98.3%)

Overall agreement = $(136+34) / (179)$; = 0.949 x 100 = 94.9% (95% exact CI: 90.7% - 97.7%)

Reproducibility

Inter-run reproducibility (Manual Staining)

Inter-run reproducibility was tested manually at three laboratories by two technicians in each laboratory over 3 days with 5 different specimens (4 positive, 1 negative in each laboratory). In the 30 tissues tested, the staining intensity varied by no more than +/- one staining intensity grade with the following exceptions: At sites 1 and 3, one slide (each) varied by two intensity grades. This was a result of tissue disruption at site 1. Overall excellent reproducibility (100%) was seen for positive versus negative results.

Inter-laboratory reproducibility (Manual and Automated Staining using the Dako Autostainer)

Immunohistochemical staining of 30 randomized and masked specimens of various expression levels of c-kit (10 negative and 20 positive), was performed at three geographically separated laboratories. Cut slides were forwarded to each testing laboratory for manual and automated staining and evaluation by a pathologist. At two sites, positive/negative determination of c-kit protein expression was perfect (100%) within and between laboratories in both manual and automated testing procedures. At site 2, duplicate slides of two tissues, designated before the study to be negative were found to be, in actuality, positive. Follow-up analysis revealed that a small area (approximately 20% of the tumor cells) was stained positively. This section of tumor was not seen in the specimens tested at the other laboratories.

Intra-run reproducibility (Manual Staining)

At each of the study sites, 5 slides of a same positive specimen were included in the set of 30 specimens stained manually. Staining intensity and percent of tumor staining were assessed. The tissue result remained positive at each study site, and the staining intensity remained within +/- 1 intensity grade variation.

Immunoreactivity

A summary of the c-Kit pharmDx™ assay immunoreactivity on the recommended panel of normal tissues is presented in Table 6. All tissues were formalin-fixed and paraffin-embedded. The instructions provided and the reagents recommended in this package insert were used to perform 30 Normal tissue testing on the DAKO Autostainer

Table 6: Evaluation of Normal Tissue Staining by c-Kit pharmDx™*

Tissue Type (# tested)	Staining Pattern
Adrenal (3)	None
Bone Marrow (3)	Rare mast cells (2+): cytoplasmic
Breast (3)	Epithelial cells (3+): membrane and cytoplasmic Myoepithelial cells: (1+): cytoplasmic

Brain/Cerebellum (3)	Purkinje cell processes (1+): cytoplasmic
Brain/Cerebrum (3)	None
Cervix (3)	None
Colon (3)	Submucosal mast cells (2+); and Lamina propria (2+) cytoplasmic
Esophagus (3)	Submucosal mast cells (2+): cytoplasmic
Heart (3)	None
Kidney (3)	Tubular epithelium (2+): cytoplasmic
Liver (3)	None
Lung (3)	Mast cells and macrophages (2+): cytoplasmic
Mesothelial Cells (3)	None
Ovary (3)	None
Pancreas (3)	Rare large duct epithelial cells (3+); Mast cells (2+): cytoplasmic
Parathyroid (3)	None
Peripheral Nerve (3)	Mast cells in soft tissue (2+): cytoplasmic
Pituitary (3)	None
Prostate (3)	None
Salivary Gland (3)	None
Skeletal Muscle (3)	Mast cells (2+): cytoplasmic
Skin (3)	Basal epidermal melanocytes (1+): cytoplasmic Glandular myoepithelial cells (1+): cytoplasmic
Small Intestine (3)	Neuronal cells (1+): cytoplasmic Mast cells (2+): cytoplasmic
Spleen (3)	Mast cells (2+): cytoplasmic
Stomach (3)	Mast cells in lamina propria (2+): cytoplasmic
Testis (3)	None
Thymus (3)	None
Thyroid (3)	None
Tonsil (3)	None
Uterus (3)	None

*The majority of tissues tested had positive staining of fibroblasts in stromal tissue (1+, fibrous) as well as mast cells and macrophages. Endogenous peroxidase-induced staining of eosinophils has been observed occasionally.

In-house studies using c-Kit pharmDx demonstrated c-kit protein expression in a variety of normal cells. These cells include ductal and myoepithelial cells of the breast, purkinje cell processes, lamina propria cells of the colon, tubular epithelium of the kidney, melanocytes and myoepithelial cells of the skin, interstitial Cells of Cajal of the small intestine, and mast cells. The cytoplasmic reactivity in granulocytes was caused by endogenous peroxidase activity and was visible with the Negative Control Reagent.

Troubleshooting

Table 7: Troubleshooting

<i>Problem</i>	<i>Probable Cause</i>	<i>Suggested Action</i>
1. No staining of slides.	1a. Programming error. Reagents not used in proper order.	1a. Check programming grid to verify that the staining run was programmed correctly.
	1b. Reagent vials were not loaded in the correct locations in reagent racks.	1b. Check the Reagent Map to verify the proper location of reagent vials.
	1c. Insufficient reagent in reagent vial.	1c. Ensure that enough reagent is loaded into the reagent vials prior to beginning the run. Refer to Reagent Map for volumes required.
	1d. Sodium azide in wash buffer bath.	1d. Use fresh, azide-free wash buffer, materials not supplied.
2. Weak staining of slides.	2a. Inadequate reagent incubation times.	2a. Review Staining Procedure instructions.
	2b. Inappropriate fixation method used.	2b. Ensure that patient tissue is not over-fixed or that non-approved fixative is not being used.
	2c. Inadequate target retrieval with Target Retrieval Solution.	2c. Verify that the Target Retrieval Solution is incubated at 95–99°C for 20 minutes or that the water bath or pressure cooker is functioning properly.
3. Excessive background staining of slides.	3a. Paraffin incompletely removed.	3a. Use fresh clearing solutions and follow procedure outlined in the Deparaffinization and Rehydration section.
	3b. Starch additives used in mounting sections to slides.	3b. Avoid using any additives for adhering sections to glass slides. Many of these are immunoreactive.
	3c. Slides not thoroughly rinsed.	3c. Ensure that the Autostainer is properly primed prior to running. Check to make sure that adequate buffer is provided for entire run. Additional rinse may be applied after the Visualization step, if desired.
	3d. Sections dried during staining procedure.	3d. Verify that the appropriate volume of reagent is being applied to slides. Make sure the Autostainer is run with the hood in the closed position and that it is not exposed to excessive heat or drafts.
	3e. Slides dried while loading the Autostainer.	3e. Ensure slides remain wet with buffer while loading and prior to initiating run.
	3f. Nonspecific binding of reagents to tissue section.	3f. Check for proper fixation of the specimen and/or the presence of necrosis.
4. Tissue detaches from slides.	4a. Use of incorrect slides.	4a. Use charged (Fisher's SuperFrost Plus) or silanized slides such as DakoCytomation's Silanized Slides (code S3003).
5. Excessively strong specific staining.	5a. Inappropriate fixation method used.	5a. Ensure that only approved fixatives and fixation methods are used.
	5b. Reagent incubation times too long.	5b. Review Staining procedure instructions.
	5c. Inappropriate wash buffer used.	5c. Use only the wash buffer that is recommended for use with the kit.
6. Weak staining of the 2+ control slide cell line.	6a. Inadequate target retrieval.	6a. Verify that the target retrieval procedure was performed properly.
	6b. Inadequate reagent incubation times.	6b. Verify that the incubation times are accurate in programming run.
	6c. Degradation of Control Slide.	6c. Check expiration date and kit storage conditions printed on package label.

Note: Refer to the Troubleshooting section in the DakoCytomation Handbook: Immunohistochemical Staining Methods, 3rd Edition¹⁸, the Atlas of Immunohistochemistry²³, or Immunoperoxidase Techniques. A Practical Approach to Tumor Diagnosis²⁰. Contact DakoCytomation Technical Support to report unusual staining.

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 Manufacturer	 LOT Batch Code	 Contains Sufficient for <math>\leq n> Tests
 Use By	 Consult <i>Instructions for Use</i>	 EC REP Authorized Representative in the European Community



DakoCytomation, Inc.
6392 Via Real
Carpinteria, California 93013 USA

Tel 805 566 6655
Fax 805 566 6688
Technical Support 800 424 0021
Customer Service 800 235 5763



DakoCytomation Denmark A/S
Produktionsvej 42
DK-2600 Glostrup Denmark

Tel +45 4485 9500
Fax +45 4485 9595
www.dakocytomation.com