For In Vitro Diagnostic Use. FDA-approved as an aid in identifying colorectal cancer patients eligible for treatment with Erbitux® (cetuximab) and Vectibix® (panitumumab).
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Introduction

Welcome to the EGFR pharmDx™ Interpretation Manual

This guide for pathologists includes key technical histological staining and interpretation tips when using the EGFR pharmDx™ kit. Utilization of the suggestions that follow will ensure that your laboratory achieves the quality results expected from EGFR pharmDx™.

The EGFR pharmDx™ Interpretation Manual objectives are simple:

- Provide an understanding of Epidermal Growth Factor Receptor biology.
- Give procedure recommendations to ensure the EGFR pharmDx™ assay is performed consistently for optimal results.
- Present a standard approach to staining and interpretation to ensure reproducible results.
- Supply pathologists with guidelines for consistent interpretation of EGFR pharmDx™ to aid in assessing colorectal cancer patients for Erbitux® (cetuximab), or Vectibix™ (panitumumab) for EGFR-expressing metastatic colorectal cancer.
- To troubleshoot the EGFR pharmDx™ kit if problems occur.

We hope this EGFR pharmDx™ Interpretation Manual is useful, and we encourage you to provide feedback on how we can improve this tool. Contact your local Dako representative with feedback (see back panel for contact information).

EGFR pharmDx™ is an FDA-approved assay indicated as an aid in identifying patients eligible for treatment with Erbitux® (cetuximab), or Vectibix™ (panitumumab) for EGFR-expressing metastatic colorectal cancer.
EGFR Overview

Epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane receptor encoded by the human HER1 gene. The EGFR protein contains an extracellular ligand binding domain, a transmembrane region and an intracellular domain with intrinsic protein-tyrosine kinase activity (see Figure 1). Ligand binding of the EGFR receptor activates the EGFR tyrosine kinase resulting in cell growth and differentiation (1).

EGFR Signaling Networks (2)

Figure 1

EGFR and the HER Family

EGFR is a member of the EGF/erbB receptor family of related growth factor receptors that includes HER2/erbB2 or neu, HER3/erbB3 and HER4/erbB4 (1). Mouse monoclonal anti-EGFR clone 2-18C9 was selected for its high specificity for EGFR. The specificity of clone 2-18C9 for EGFR (HER1) and lack of cross-reactivity with the related HER family receptors were demonstrated by immunocytochemistry and Western blotting using CHO cells transiently transfected with vectors expressing HER2, HER3 and HER4. Further specificity testing by flow cytometry and Western blotting showed that clone 2-18C9 recognizes both the wild type and the EGFRvIII mutant form of the receptor. The epitope bound by clone 2-18C9 was found to be a structural epitope in the extracellular cysteine-rich region of the molecule spanning sub-domain S2 and proximal to the transmembrane region (3).
EGFR Expression in Normal Tissue

The EGFR protein is expressed on a variety of normal cells including many epithelial cell types (4-10). Non-epithelial cell types that express EGFR include smooth muscle cells, fibroblasts and perineurium (11).

Some examples of normal tissue stained with EGFR pharmDx™ are summarized in the table below. All tissues were formalin-fixed and paraffin-embedded.

**Table 1. Evaluation of normal tissue staining by Dako EGFR pharmDx™**

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Positive Tissue Element Staining and Staining Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal</td>
<td>Cortical cells (2+): Cytoplasmal</td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>None</td>
</tr>
<tr>
<td>Breast</td>
<td>Lobular epithelial cells (2+): Membrane and cytoplasmic</td>
</tr>
<tr>
<td>Brain (Cerebellum)</td>
<td>Molecular layer (1+): Extracellular</td>
</tr>
<tr>
<td>Brain/Cerebrum</td>
<td>None</td>
</tr>
<tr>
<td>Cervix</td>
<td>Basalar squamous epithelial cells (2+): Membrane</td>
</tr>
<tr>
<td>Colon**</td>
<td>None</td>
</tr>
<tr>
<td>Esophagus</td>
<td>Basalar squamous epithelial cells (2+): Membrane</td>
</tr>
<tr>
<td>Heart</td>
<td>None</td>
</tr>
<tr>
<td>Kidney</td>
<td>Tubules (1+): Cytoplasmic staining (granular)</td>
</tr>
<tr>
<td>Liver</td>
<td>Hepatocytes (sinusoids) (3+): Membrane</td>
</tr>
<tr>
<td></td>
<td>Bile ducts (3+): Membrane and cytoplasmic</td>
</tr>
<tr>
<td>Lung†</td>
<td>Alveolar lining cells/basalar bronchial cells (myoepithelial cells) (2+): Membrane and cytoplasmic</td>
</tr>
<tr>
<td>Mesothelial Cells</td>
<td>Mesothelial cells (2+): Membrane and cytoplasmic</td>
</tr>
<tr>
<td>Ovary</td>
<td>None</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Ducts (2+): Membrane</td>
</tr>
<tr>
<td>Parathyroid</td>
<td>None</td>
</tr>
<tr>
<td>Peripheral Nerve</td>
<td>Nerve cell processes (1+): Fibrous</td>
</tr>
<tr>
<td>Pituitary</td>
<td>None</td>
</tr>
<tr>
<td>Prostate+</td>
<td>Glandular epithelial cells (2+): Membrane</td>
</tr>
<tr>
<td>Salivary Gland</td>
<td>Ductal elements (1+): Cytoplasmic</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>None</td>
</tr>
<tr>
<td>Skin†</td>
<td>Basalar squamous cells, adnexal structures (2+): Membrane and cytoplasmic</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>None</td>
</tr>
<tr>
<td>Spleen</td>
<td>None</td>
</tr>
<tr>
<td>Stomach</td>
<td>None</td>
</tr>
<tr>
<td>Testis</td>
<td>None</td>
</tr>
<tr>
<td>Thymus</td>
<td>None</td>
</tr>
<tr>
<td>Thyroid</td>
<td>None</td>
</tr>
<tr>
<td>Tonsil</td>
<td>Basalar squamous epithelium (3+): Membrane and cytoplasmic</td>
</tr>
<tr>
<td>Uterus</td>
<td>Endometrial gland epithelium (2+): Membrane and cytoplasmic</td>
</tr>
<tr>
<td></td>
<td>Endometrial stromal cells (2+): Membrane and cytoplasmic</td>
</tr>
<tr>
<td></td>
<td>Myometrium: None</td>
</tr>
</tbody>
</table>

† Recommended for positive control tissue (See page 9 for photomicrograph of normal positive tissue staining of cervix)

* Recommended for negative control tissue

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

** Colon tissue may exhibit positive staining of enterocytes, smooth muscle cells, endothelial cells and perineurium.
The EGFR pharmDx™ Kit

The EGFR pharmDx™ assay is a qualitative immunohistochemical (IHC) kit system to identify epidermal growth factor receptor (EGFR) expression in normal and neoplastic tissues routinely fixed for histological evaluation. EGFR pharmDx™ specifically detects the EGFR (HER1) protein in EGFR-expressing cells.

EGFR pharmDx™ is FDA-approved as an aid in identifying colorectal cancer patients eligible for treatment with Erbitux® (cetuximab) or Vectibix™ (panitumumab).

Following incubation with the primary monoclonal antibody to human EGFR protein, this kit employs a ready-to-use visualization reagent based on dextran technology. This reagent consists of both secondary goat anti-mouse 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 human cell lines with staining intensity scores of 2+ and 0 are provided for validation of the kit reagent performance.

Two EGFR pharmDx™ Kit Configurations are Available

<table>
<thead>
<tr>
<th>Kit Code</th>
<th>Kit Name</th>
<th>Test Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1492</td>
<td>EGFR pharmDx™ Kit for Manual Use</td>
<td>35 Test</td>
</tr>
<tr>
<td>K1494</td>
<td>EGFR pharmDx™ Kit for the Dako Autostainer/Autostainer Plus</td>
<td>50 Test</td>
</tr>
</tbody>
</table>

The EGFR pharmDx™ Kit Includes:

- Proteinase K
- Peroxidase Block
- Dako EGFR pharmDx™ Monoclonal Mouse Antibody
- Mouse IgG1 Negative Control Reagent
- Labeled Polymer, HRP
- DAB+ Substrate Buffer
- Liquid DAB+ Chromogen
- Dako Wash Solution 10x
- Dako EGFR pharmDx™ Control Slides
Expression Rates and Recommended Data Tracking for EGFR pharmDx™ Immunostaining

Clinical trials of EGFR-targeted therapies (cetuximab and panitumumab) have been performed using Dako EGFR pharmDx™-positive results as one of the criteria for study eligibility.

Summary of EGFR-Expressing Percent in Colon Cancer Patients

<table>
<thead>
<tr>
<th>Therapeutic</th>
<th>Study ID</th>
<th>EGFR-Expressing Ratio (# expressing/# tested)</th>
<th>% EGFR-Expressing</th>
<th>95% Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erbitux™/cetuximab</td>
<td>Pivotal Trial EMR 62202-007</td>
<td>474 / 577</td>
<td>82.1%</td>
<td>78.1 - 86.1%</td>
</tr>
<tr>
<td></td>
<td>Supportive Study IMCL CP02-0141</td>
<td>105 / 140</td>
<td>75.0%</td>
<td>66.9 - 83.1%</td>
</tr>
<tr>
<td></td>
<td>Prototype EGFR Study IMCL CP02-9923</td>
<td>292 / 401</td>
<td>72.8%</td>
<td>68.0 - 77.6%</td>
</tr>
<tr>
<td>Vectibix™/panitumumab</td>
<td>Pivotal Trial 20020408</td>
<td>735 / 1004</td>
<td>73.2%</td>
<td>70.4 - 75.9%</td>
</tr>
</tbody>
</table>

Recommended Data Tracking

**EGFR pharmDx™ Testing**

Use EGFR pharmDx™ data to determine an average number of percent positive colorectal adenocarcinomas.

- **73–82 percent positives**
  - If the average percent of positives falls within 73-82 percent, report results. Continue to use EGFR pharmDx™ by following protocol. Continue to monitor results.

- **<73% or >82% positives**
  - Review Technical Procedure
    - See pages 7–9
  - Review Interpretation Procedure
    - See pages 10–22

**Review Patient Demographics**

If results are <73% or >82%, review EGFR pharmDx™ procedures.
Technical Tips for Optimal EGFR pharmDx™ Performance

For accurate and consistent results one must adhere to the EGFR pharmDx™ protocol. 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 EGFR pharmDx™ assay itself. Technical issues relating to the performance of the assay are generally related to procedural deviations from the EGFR pharmDx™ protocol and can be alleviated.

Tissue Fixation and Variables

Procedural deviations that are related to sample handling and processing can affect the EGFR pharmDx™ results. Some of the variables that may affect results are as follows:

- Non-representative tissue samples
- Specimens drying prior to fixation
- Fixation with PreFer fixative (suitable fixatives include 10% (v/v) neutral buffered formalin, 10% (v/v) unbuffered formalin, 25% (v/v) unbuffered formalin, AFA, Pen-Fix, and Bouin’s)\(^1\)
- Age, pH and storage conditions of fixative
- Length of fixation
- Length of storage of unstained tissue sections

EGFR pharmDx™ Protocol Recommendations

- If staining must be interrupted, slides may be kept in wash buffer following incubation of the primary antibody for up to one hour at room temperature (20–25 °C).
- Specimens preserved in generally used fixatives (10% v/v neutral buffered formalin, 10% v/v unbuffered formalin, 25% v/v unbuffered formalin, AFA, Pen-Fix, and Bouin’s) are suitable for testing with EGFR pharmDx™. Use of EGFR pharmDx™ on PreFer fixed tissues may result in unsatisfactory preservation of morphology.\(^1\)
- Automated Staining: Dako recommends the use of EGFR pharmDx™ on a Dako Autostainer or Autostainer Plus. Use of EGFR pharmDx™ on alternative automated platforms has not been validated and may give erroneous results.
- Wash Buffer: Dilute the provided wash buffer 1:10 using distilled or deionized water. Store unused solution at 2–8 °C no more than seven days. Discard diluted solution if cloudy in appearance. Only use wash buffer supplied in the EGFR pharmDx™ kit or TBS Wash Buffer, code S3006.
- Storage of Reagents: Reagent 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.
- False-negative immunostaining can be caused by degradation of the antigen in the tissue over time. Specimens should be stained within two months of mounting of tissues on slides when stored at room temperature (20–25 °C).
- 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.
- For high-quality results, review the EGFR pharmDx™ Training Checklist (Table 2) prior to beginning your staining run.
# Table 2. EGFR pharmDx™ Training Checklist

Customer Name/Institution

Person Trained/Title

- Manual Staining Run
- Dako Autostainer Software Version
- Dako Autostainer Serial Number

Trainer

Date

EGFR pharmDx™ is a complete assay system requiring controls to ensure reproducible results.

### Manual or Dako Autostainer Procedure

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Antibody applied for 30 minutes and specimen fully covered?</td>
<td></td>
</tr>
<tr>
<td>Labeled Polymer applied for 30 minutes and specimen fully covered?</td>
<td></td>
</tr>
<tr>
<td>DAB+ Substrate-Chromogen prepared properly?</td>
<td></td>
</tr>
<tr>
<td>One drop DAB+ Chromogen to 1 mL DAB+ Substrate Buffer.</td>
<td></td>
</tr>
<tr>
<td>Substrate-Chromogen solution applied for 10 minutes and specimen fully covered?</td>
<td></td>
</tr>
</tbody>
</table>

### Dako Autostainer Procedure

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slides placed in buffer five minutes (± 1) before loading onto the Dako Autostainer?</td>
<td></td>
</tr>
<tr>
<td>Appropriate protocol template used?</td>
<td></td>
</tr>
<tr>
<td>Was the Dako Autostainer programming reviewed for accuracy?</td>
<td></td>
</tr>
<tr>
<td>Slides rinsed with buffer between steps and double rinsed after the labeled polymer step with an additional five-minute rinse hold?</td>
<td></td>
</tr>
<tr>
<td>Substrate-Chromogen prepared properly?</td>
<td></td>
</tr>
<tr>
<td>Add 11 drops of Liquid DAB+ Chromogen to one vial of DAB+ Substrate Buffer and mix.</td>
<td></td>
</tr>
<tr>
<td>Substrate-Chromogen solution applied for two five-minute applications?</td>
<td></td>
</tr>
</tbody>
</table>

### Instrumentation / Equipment

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is regular preventative maintenance performed on the Dako Autostainer?</td>
<td></td>
</tr>
<tr>
<td>Do you have all the necessary equipment to perform the EGFR pharmDx™ assay according to protocol?</td>
<td></td>
</tr>
<tr>
<td>If not, specify what is missing in comments below.</td>
<td></td>
</tr>
<tr>
<td>If you answered “No” to any of the above, you have deviated from protocol and should consult with your local Dako Technical Support Representative for assistance.</td>
<td></td>
</tr>
</tbody>
</table>

Additional observations or comments:
Quality Control

The first quality control step for interpretation is the evaluation of the control cell lines. Positive and negative cell lines are included in each EGFR pharmDx™ kit (Figures 3 and 4) to validate staining runs every time the assay is performed. Appropriate staining of the control cell lines provides evidence that the EGFR pharmDx™ assay is functioning properly. No membrane staining of the CAMA-1 control cell line (0) and moderate brown complete or incomplete membrane staining in the HT-29 control cell line (2+) indicate that the staining run is valid. If either of the control cell lines have staining results outside of these criteria, test slides stained simultaneously within the same run should be considered invalid and repeated.

The second quality control step is the positive control tissue (Figure 5). Use a positive control tissue (known to be EGFR protein 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 to provide an indication of specific background staining. This validation is performed to ascertain proper tissue preparation and staining techniques. The presence of a brown reaction product at the cell membrane is indicative of positive reactivity. One positive tissue control for each set of 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 only 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.

The third quality control step is the negative control tissue (Figure 6). Use a negative control tissue (known to be EGFR protein 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 to provide an indication of specific background staining. The variety of different cell types present in most tissue sections offers internal negative control. If specific staining occurs in the negative control tissue, results with the patient specimens should be considered invalid.

EGFR positive tissue elements such as nerve and fibroblasts are present in most normal and neoplastic tissues. These positive elements can serve as intrinsic internal controls in both patient specimens and positive and negative control tissue.
EGFR pharmDx™ Evaluation and Reporting

Slide Evaluation

Slide evaluation should be performed by a pathologist using a light microscope. EGFR pharmDx™ stains a variety of normal and neoplastic tissues. Observed EGFR staining patterns are heterogeneous or homogeneous depending on the tissue and/or tumor type. Heterogeneity includes various intensity areas within a single neoplasm. The staining can show a range of 0–3+ staining intensity. Cell staining patterns can also be heterogeneous including membrane staining, cytoplasmic staining or both. When only cytoplasmic staining is present, in the absence of membrane staining, the result is negative.

The performance characteristics of EGFR pharmDx™ make the visualization of four staining intensity levels possible from 0 to 3+. EGFR staining at the cellular level has been observed on both the membrane and in the cytoplasm at all intensity levels.

Dako recommends the following interpretive approach in the assessment of EGFR Immunostaining.

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

Steps to EGFR pharmDx™ Evaluation

1. Evaluate the control cell lines to validate the assay staining run. Cell lines should not be used as an interpretive aid.

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 EGFR pharmDx™ stained slide.

4. Evaluate the EGFR-stained section for identification of neoplasm at low power, 4x magnification. 3+ intensity staining of 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 obvious at 10x magnification.

6. If the staining pattern is an artifact, it should be disregarded for evaluation. Find another representative area(s). Refer to page 17 for “Interpreting Artifacts.”

7. If membrane staining at 10x is not obvious, use 20x or 40x magnification to assess staining pattern (membranous versus cytoplasmic). Confirmation of EGFR pharmDx™ staining at 20x magnification may be useful in those neoplasms with abundant cytoplasmic staining. Some tumors have several populations of cells with different intensities of EGFR membrane staining.

8. If there is no tumor staining and normal elements are not staining, review control slides of that staining run to confirm that appropriate levels of EGFR expression are observed. If positive elements in the control slides are negative, repeat the staining run.
Table 3. EGFR pharmDx™ Pathology Report Form

<table>
<thead>
<tr>
<th>Description</th>
<th>Clinical Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deparaffinized tissue and appropriate control tissue sections are stained using the FDA-approved Dako EGFR pharmDx™ immunohistochemistry kit.</td>
<td>Several clinical trials of EGFR-targeted therapies (cetuximab and panitumumab) have been performed. Patients whose tumors had EGFR expression as demonstrated using the Dako EGFR pharmDx™ assay were eligible for study enrollment. The response rate for EGFR-negative patients and patients with EGFR-positive staining in less than one percent of tumor cells is unknown as no such patients were present in the clinical drug trials. Tumors with EGFR-positive staining in ≥1% of their cells are considered EGFR expressing with regard to the current EGFR-targeted therapy indications for use.</td>
</tr>
<tr>
<td>Tumors should be reported as EGFR positive or EGFR negative using membrane staining as the evaluable structure. A tumor cell is EGFR positive if it exhibits any membrane staining above background, whether or not it is completely circumferential. A tumor with no membrane staining above background in any tumor cell is reported as an EGFR-negative tumor.</td>
<td></td>
</tr>
</tbody>
</table>

Patient Result

EGFR protein

- Positive
- Negative

EGFR pharmDx™ is indicated as an aid in identifying colorectal cancer patients eligible for treatment with Erbitux® (cetuximab) or Vectibix™ (panitumumab).

EGFR pharmDx™ Staining Results

<table>
<thead>
<tr>
<th>Report to Treating Physician</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR-Negative Tumor</td>
<td>Absence of membrane staining above background in all tumor cells.</td>
</tr>
<tr>
<td>EGFR-Positive Tumor</td>
<td>EGFR-positive staining is defined as any IHC staining of tumor cell membranes above background level; whether it is complete or incomplete circumferential staining.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Staining Intensity</th>
<th>Percent of Tumor Cells Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+, 2+, or 3+</td>
<td>&gt;0%</td>
</tr>
</tbody>
</table>

These definitions of positive and negative results are in accord with published literature, but may require modification in specific contexts.
EGFR pharmDx™ Interpretation Guidelines

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

Examples of EGFR pharmDx™ Staining Intensity

**Figure 7**

Colorectal adenocarcinoma, no staining, 0 staining intensity; 10x magnification.

Colorectal adenocarcinoma, no staining, 0 staining intensity; 20x magnification.

Colorectal adenocarcinoma, membrane staining, 1+ staining intensity; 20x magnification.

Colorectal adenocarcinoma, membrane staining, 1+ staining intensity; 40x magnification.

Colorectal adenocarcinoma, membrane staining, 2+ staining intensity; 20x magnification.

Colorectal adenocarcinoma, membrane staining, 2+ staining intensity; 40x magnification.

Colorectal adenocarcinoma, membrane staining, 3+ staining intensity; 20x magnification.

Colorectal adenocarcinoma, membrane staining, 3+ staining intensity; 40x magnification.
Staining Patterns

The Dako EGFR pharmDx™ stains a variety of normal and neoplastic tissues. Observed EGFR staining patterns are heterogeneous or homogeneous depending on the tissue and/or tumor type.

Heterogeneous Staining

Heterogeneity includes various staining intensities within a single neoplasm. The staining can show a range of 0-3+ staining intensity. Cell staining patterns can also be heterogeneous, including membrane staining, cytoplasmic staining or both.

Homogeneous Staining

Cancers with EGFR protein expression can also exhibit homogeneous staining patterns.

Figure 8
Colorectal adenocarcinoma, example of heterogeneous positive staining; 10x magnification.

Figure 9
Colorectal adenocarcinoma, example of leading edge heterogeneous positive staining; 10x magnification.

Figure 10
Colorectal adenocarcinoma, example of homogeneous positive staining; 20x magnification.
EGFR Staining of Normal and Benign Tissues

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

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

- esophageal squamous epithelial cells
- hepatocytes
- mesothelial cells
- fibroblasts
- tonsillar and cervical squamous epithelial cells
- ductal and myoepithelial cells of the breast
- pulmonary alveolar epithelial and bronchial basal cells
- prostatic luminal cells
- ductal elements of the salivary gland
- endometrial gland and stromal cells
- normal colonic surface epithelial cells may or may not display weak positive staining of EGFR
- smooth muscle cells

Figure 11
Colon, example of perineural fibroblast staining; 40x magnification. Perineural fibroblasts are found in most tissues and serve as a good internal positive control.
Hepatocyte Staining in the Liver

Figure 12
Colorectal adenocarcinoma infiltrating the liver. Example of positively stained normal hepatocytes and negative tumor cells; 10x magnification. The sample is negative.

Figure 13
Colorectal adenocarcinoma infiltrating the liver. Example of positively stained normal hepatocytes and negative tumor cells; 20x magnification. The sample is negative.

Figure 14
Colorectal adenocarcinoma infiltrating the liver. Example of positively stained normal hepatocytes and negative tumor cells; 40x magnification. The sample is negative.

Needle Biopsy from Liver

Figure 15
Needle biopsy from liver exhibiting strong tumor staining; 10x magnification. The sample is positive.
Factors to Consider in Evaluation of EGFR pharmDx™ Stains

Non-specific Background
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.

Possible Causes of Non-specific Background

Biologic Activity
- Pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (granulocytes) may result in non-specific background staining due to decomposition of H₂O₂ within the substrate.

Technological Processes
- Use of fixatives other than those recommended (see page 7)
- Incomplete deparaffinization of tissue sections prior to staining
- Use of alternative buffers (use kit-supplied buffer or equivalent 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)

Staining of Granulocytes

Figure 16
Colorectal adenocarcinoma, example of endogeneous peroxidase staining in granulocytes; 20x magnification. The sample is negative.
Interpreting Artifacts

Overdigestion

EGFR pharmDx™ includes pretreatment by means of a proteolytic enzyme digestion step. Tissue sections may occasionally be overdigested, causing disruption of cell membranes and overall tissue architecture. Run the assay with careful attention to the duration of the proteolytic digestion step.

**Note**

If overdigestion is a persistent problem with tissues fixed in 10% (v/v) neutral buffered formalin, tissue sections may be post-fixed in 10% (v/v) neutral buffered formalin for 10 minutes after deparaffinization. See procedure right:

**Post-Fixation Procedure**

*(Validated for tissues originally fixed in 10% (v/v) NBF)*

1. Deparaffinize sections and immerse in reagent quality water.
2. Immerse slides in a 10 percent neutral buffered formalin for 10 minutes.
3. Rinse slides twice in deionized or distilled water.
4. Continue with the EGFR pharmDx™ staining procedure.

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.

Figure 17
Colorectal adenocarcinoma, example of overdigestion; 20x magnification.

Figure 18
Colorectal adenocarcinoma, example of lymphoid nodule overdigested; 10x magnification.

Figure 19
Colorectal adenocarcinoma, example of lymphoid nodule overdigested; 20x magnification.
Edge Artifact

Frequently, increased staining is observed around the periphery of the tissue specimen, 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 Crushing 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 peripheral positive reaction is only at the edge of the tissue section, evaluation of EGFR staining should exclude tumor cells within the region’s edge artifact.

Retraction Artifact

Stromal retraction around tumor cell glands can create clefts where pooled antibody can non-specifically stain. Thorough washing after the primary antibody incubation step may prevent this reaction. Retraction space staining manifests itself as a hemicircumferential reaction around the periphery of the gland.

Thermal Artifact

Thermal electrocautery may alter nuclei and cell membranes. Dako recommends that the evaluation of EGFR pharmDx™ staining be performed on tissue with no or minimal thermal electrocautery artifacts.

Non-Evaluable Areas of Tissue

Areas of 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.).
Examples of Cancer Stained with EGFR pharmDx™

Colorectal Cancer

Figure 21
Colorectal adenocarcinoma, example of poorly differentiated carcinoma; 2+ staining intensity; 20x magnification.

Figure 22
Colorectal adenocarcinoma, example of well-differentiated carcinoma; 3+ staining intensity; 10x magnification.

Examples of Negative EGFR pharmDx™ Immunostaining

Figure 23
Colorectal adenocarcinoma, example of H&E; 20x magnification.

Figure 24
Colorectal adenocarcinoma, example of negative staining; 20x magnification.

Figure 25
Colorectal adenocarcinoma, example of H&E; 40x magnification.

Figure 26
Colorectal adenocarcinoma, example of negative staining; 40x magnification.
ColoRECTal Cancer

Figure 27
Colorectal adenocarcinoma, example of negative staining; 20x magnification.

Figure 28
Colorectal adenocarcinoma, example of negative staining; 40x magnification.

Figure 29
Colorectal adenocarcinoma, example of cytoplasmic staining with no membrane staining; 20x magnification. EGFR positive cells can be observed to exhibit cytoplasmic and membrane immunostaining. When only cytoplasmic staining is present the immunostaining is interpreted as negative.

Figure 30
Colorectal adenocarcinoma, example of cytoplasmic staining with no membrane staining; 20x magnification. EGFR positive cells can be observed to exhibit cytoplasmic and membrane immunostaining. When only cytoplasmic staining is present the immunostaining is scored as negative.

Examples of Weak to Moderate EGFR Immunostaining

Figure 31
Colorectal adenocarcinoma, example of H&E; 20x magnification.

Figure 32
Colorectal adenocarcinoma, example of weak membrane staining, 1+ staining intensity; 20x magnification.
Figure 33
Colorectal adenocarcinoma, example of H&E, 40x magnification.

Figure 34
Colorectal adenocarcinoma, example of weak positive staining, 1+ staining intensity, 40x magnification.

Figure 35
Colorectal adenocarcinoma, example of weak to moderate positive membrane staining, 2+ staining intensity, 20x magnification.

Figure 36
Colorectal adenocarcinoma, example of weak to moderate positive membrane staining, 2+ staining intensity, 40x magnification.

Figure 37
Colorectal adenocarcinoma, example of weak to moderate positive membrane staining, 2+ staining intensity, 20x magnification.

Figure 38
Colorectal adenocarcinoma, example of moderate positive membrane staining, 2+ staining intensity, 40x magnification.
Colorectal Cancer

Examples of Moderate to Strong EGFR Immunostaining

Figure 39
Colorectal adenocarcinoma, example of strong positive membrane staining, 3+ staining intensity; 10x magnification.

Figure 40
Colorectal adenocarcinoma, example of strong positive membrane staining, 3+ staining intensity; 20x magnification.

Figure 41
Colorectal adenocarcinoma, example of strong positive membrane staining, 3+ staining intensity; 40x magnification.

Figure 42
Colorectal adenocarcinoma, example of strong positive membrane staining, 3+ staining intensity; 10x magnification.

Figure 43
Colorectal adenocarcinoma, example of strong positive membrane staining, 3+ staining intensity; 20x magnification.

Figure 44
Colorectal adenocarcinoma, example of strong positive membrane staining, 3+ staining intensity; 40x magnification.
EGFR pharmDx™ Immunostaining in a Variety of Solid Tumors

EGFR is expressed in a number of solid tumors. The EGFR pharmDx™ assay is a qualitative immunohistochemical (IHC) kit system useful in identifying epidermal growth factor receptor expression in normal and neoplastic tissues routinely fixed for histological evaluation.

Results from tissue specimens stained using EGFR pharmDx™ for purposes other than Erbitux® or Vectibix™ assessment have no known clinical utility.
Additional EGFR Resources

- Hong WK, Ulrich A. The role of EGFR in solid tumors and implications for therapy. ONCOLOGY BIOTHERAPEUTICS, Volume 1, Number 1, 2000.

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