Ki-67 IHC MIB-1 pharmDx (Dako Omnis) Interpretation Manual – Breast Carcinoma

FDA approved for in vitro diagnostic use
For countries outside of the United States, see the local Verzenio product label for approved prescription information to guide therapy.
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Intended Use

For In Vitro Diagnostic Use.
Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is a qualitative immunohistochemical (IHC) assay using monoclonal mouse anti-Ki-67, Clone MIB-1, intended for use in the detection of Ki-67 protein in formalin-fixed, paraffin-embedded (FFPE) breast carcinoma tissue using the EnVision FLEX visualization system on Dako Omnis. Ki-67 protein expression in breast carcinoma is determined by using the Ki-67 pharmDx Score, which is the overall percentage of viable tumor cells in the invasive cancer component showing Ki-67 nuclear staining. The specimen should be considered to have Ki-67 expression if Ki-67 pharmDx Score is ≥ 20%.
Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is indicated as an aid in identifying patients with early breast cancer at high risk of disease recurrence for whom adjuvant treatment with Verzenio® (abemaciclib) in combination with endocrine therapy is being considered.
Note: The front page image is breast carcinoma (FFPE) stained with Ki-67 IHC MIB-1 pharmDx on Dako Omnis (10× magnification).
Ki-67 IHC MIB-1 pharmDx (Dako Omnis), in combination with high-risk clinical and pathological features, is the first companion diagnostic that is FDA-approved as an aid in identifying patients with early breast cancer at high risk of disease recurrence for whom Verzenio (abemaciclib) treatment in combination with standard adjuvant endocrine therapy is being considered. This Interpretation Manual is provided as a tool to help guide pathologists and laboratory personnel in achieving accurate and reproducible results in assessing Ki-67 expression in FFPE breast carcinoma specimens. Ki-67 expression evaluation may be used to identify patients with hormone receptor-positive (HR+)/human epidermal growth factor receptor 2-negative (HER2−) early breast cancer at high risk of recurrence who may be eligible for treatment with Verzenio in combination with endocrine therapy.

The manual provides detailed scoring guidelines and technical information from the Ki-67 IHC MIB-1 pharmDx (Dako Omnis) Instructions for Use (IFU) to ensure high-quality staining and reliable diagnostic assessment. To help familiarize you with the requirements for scoring breast carcinoma stains with Ki-67 IHC MIB-1 pharmDx (Dako Omnis), example cases of various Ki-67 expression levels are provided as reference. These example cases and in-depth recommendations for interpretation of breast carcinoma specimens stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) can help individual laboratories achieve reproducible and reliable results.

Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is considered a qualitative immunohistochemical assay. Ki-67 expression in breast carcinoma is determined by using the Ki-67 pharmDx Score, which is the number of Ki-67 staining viable tumor cells in the invasive cancer component divided by the total number of viable tumor cells in the invasive cancer component, multiplied by 100.

Breast carcinoma tissue specimens that are tested for Ki-67 expression are scored and divided into Ki-67 expression levels based on the Ki-67 pharmDx Score for the whole tissue:
- Ki-67 pharmDx Score < 20%
- Ki-67 pharmDx Score ≥ 20%

For more details on staining and interpretation, please refer to the current version of the IFU provided with Ki-67 IHC MIB-1 pharmDx (Dako Omnis), Code GE020 or visit www.agilent.com.
Assay interpretation

The clinical interpretation of staining, or the absence of staining, must be complemented by the evaluation of proper controls. Evaluation must be made by a qualified pathologist within the context of the patient’s clinical history and other diagnostic tests. This product is intended for in vitro diagnostic (IVD) use.

Reporting results

To help understand what information should be reported to the treating physician, please refer to the Reporting Results section of this manual on page 30.

Images

The included images from digital scans are of breast carcinoma or normal tonsil tissue as specified in the image descriptions.

Note: Image magnification levels may appear different than indicated in respective annotations due to adjustment of image size.

Tissue samples supplied by BioIVT Asterand®

Tissue samples were provided by the Cooperative Human Tissue Network which is funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects.
Ki-67 Overview

Ki-67 is a marker of cell proliferation

Ki-67 antigen (also known as antigen identified by monoclonal antibody Ki-67) is a nuclear protein expressed during all active phases of the mammalian cell cycle (G1, S, G2, and M-phases) and downregulated in resting cells (G0-phase).\(^3\)\(^4\) During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes.\(^5\) Localization of Ki-67 antigen correlates with distinct functions. During interphase, Ki-67 is required for normal cellular distribution and nucleolar association of heterochromatin. During mitosis, Ki-67 plays a role in the formation of the perichromosomal layer and prevents aggregation of mitotic chromosomes.\(^4\)

The antigen is rapidly degraded as the cell enters the non-proliferative state and there appears to be no expression of Ki-67, as detected by IHC, during DNA repair processes.\(^5\)\(^6\)

In cancer cells, the CDK4 and 6-Rb pathway contributes to enhanced cell proliferation

The cyclin-dependent kinase 4 and 6/cyclin D1 complex is responsible for the phosphorylation and inhibition of the retinoblastoma protein (Rb). When Rb is phosphorylated, the cell proceeds from G1 to S-Phase of the cell cycle, replicating DNA and preparing for mitosis (Figure 1).\(^7\)

Verzenio is a CDK4 and 6 inhibitor that induces cell cycle arrest

Verzenio (abemaciclib) is an ATP-competitive inhibitor of cyclin-dependent kinases 4 and 6 (CDK4 and 6). CDK4 and 6 promote cell growth by facilitating the progression of cells from the G1 to the S-phase of the cell cycle. This promotion of cell growth occurs primarily by counteracting the effects of the Rb protein, whereby the reversal of Rb-mediated suppression is achieved by the phosphorylation of this protein by CDK4 and/or 6. The CDK4 and 6-Rb pathway is commonly altered in cancer cells, whereby the activation of this pathway contributes to enhanced growth. Accordingly, in cancer cells, Verzenio inhibits CDK4 and 6-dependent phosphorylation of Rb, which subsequently blocks proliferation by inhibiting the progression of these cells from the G1-phase into the S and G2/M-phases of the cell cycle (Figure 2).\(^8\)

Ki-67 IHC MIB-1 pharmDx (Dako Omnis) detects Ki-67 in breast carcinoma specimens

Ki-67 expression has been reported as an independent prognostic factor in early breast cancer. In HR+ breast cancer, patients with high levels of Ki-67 following surgery have been shown to have higher recurrence rates while receiving adjuvant endocrine therapy.\(^9\)\(^10\) High-risk patient selection into the monarchE clinical trial was based on clinicopathological features or Ki-67 expression (Ki-67 pharmDx Score ≥ 20% and 1–3 positive axillary nodes).
The CDK4 and 6-Rb pathway

Figure 1: D-type cyclins activate CDK4 and 6, which phosphorylate Rb, thereby partially derepressing E2F, which is critical for DNA synthesis. CDK 4 and 6 = cyclin-dependent kinase 4 and 6, Rb = retinoblastoma protein, PO₄ = phosphate.

Figure 2: Continuous inhibition of CDK4 and 6 prevents the phosphorylation of Rb, ultimately leading to prolonged cell cycle arrest in G1 and the initiation of apoptosis.
Ki-67 IHC MIB-1 pharmDx (Dako Omnis) Overview

What is Ki-67 IHC MIB-1 pharmDx (Dako Omnis)?
Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is FDA-approved as an aid in identifying patients with early breast carcinoma at high risk of disease recurrence for treatment with Verzenio (abemaciclib). Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is a qualitative immunohistochemical (IHC) assay intended for use in the detection of Ki-67 protein in FFPE breast carcinoma tissue samples using EnVision FLEX visualization system on the Dako Omnis automated staining instrument.

Components of Ki-67 IHC MIB-1 pharmDx (Dako Omnis)
Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is a modular assay configured for the Dako Omnis workflow and contains optimized, purified primary antibody and Negative Control Reagent (NCR)* ready to use (RTU). Accessory system reagents required to complete an IHC staining procedure on FFPE breast carcinoma specimens are available in individual packaging from Agilent. All reagents (supplied and accessory) are required for proper use of the assay. Deparaffinization, rehydration, and target retrieval are performed onboard the Dako Omnis automated staining instrument using a two-step incubation of Clearify™, followed by Low pH Target Retrieval Solution (TRS). The specimens are then incubated with monoclonal mouse primary antibody to Ki-67 or the NCR prior to Peroxidase Block. Following incubation with the primary antibody/NCR and Peroxidase Block, specimens are incubated with a ready-to-use visualization reagent consisting of secondary antibody molecules and horseradish peroxidase molecules coupled to a dextran polymer backbone. The enzymatic conversion of the subsequently added chromogen results in precipitation of a visible reaction product at the site of antigen. The specimen may then be counterstained and coverslipped. Results are interpreted using a bright field microscope. Please consult the Dako Omnis User Guide(s) for detailed instructions on loading and unloading of slides, reagents, bulk fluids and waste.

* Use of Negative Control Reagent is discretionary for Ki-67 IHC MIB-1 pharmDx (Dako Omnis), Code GE020
Ki-67 IHC MIB-1 pharmDx (Dako Omnis) (Code GE020) is a modular assay configured for the Dako Omnis workflow (Figure 3). Primary antibody and NCR reagents are supplied to perform 60 tests in multiple individual runs (Figure 3):

1. Primary Antibody (purified): Monoclonal Mouse Anti-Human Ki-67, Clone MIB-1 Ready-to-Use (RTU)
2. Negative Control Reagent*

The following Dako Omnis bulk and visualization reagents are also required:

- EnVision FLEX, High pH (Dako Omnis) (Code GV800)* or EnVision Mini Kit, High pH (Dako Omnis) (Code GV823)*:
  - EnVision FLEX DAB+ Chromogen (Dako Omnis)
  - EnVision FLEX Peroxidase-Blocking Reagent (Dako Omnis)
  - EnVision FLEX Substrate Buffer (Dako Omnis)
  - EnVision FLEX Visualization Reagent (Dako Omnis)
- EnVision FLEX Target Retrieval Solution, Low pH (50×) (Dako Omnis) Code GV805
- Hematoxylin (Dako Omnis) Code GC808
- Wash Buffer (20×) (Dako Omnis) Code GC807
- Clearify™ Clearing Agent (Dako Omnis) Code GC810
- Dako Omnis Sulfuric Acid, 0.3 M Code GC203

* Use of Negative Control Reagent is discretionary for Ki-67 IHC MIB-1 pharmDx (Dako Omnis), Code GE020

† Note: GV800 and GV823 include an additional reagent, EnVision FLEX Target Retrieval Solution, High pH (50×), that is not required for the assay. The required target retrieval solution for the assay is EnVision FLEX Target Retrieval Solution, Low pH (50×) (Dako Omnis), Code GV805. The color of the EnVision FLEX Target Retrieval Solution, Low pH (50×) (Dako Omnis), Code GV805 is red.

** Figure 4: Ki-67 IHC MIB-1 pharmDx (Dako Omnis) staining procedure.
Technical Considerations

Deviations from the recommended procedures including tissue fixation, processing, and embedding may produce significant variability in results. Quality controls are described below and include lab-supplied positive and negative control tissues.

Specimen preparation

Specimens must be handled to preserve the tissue for immunohistochemical staining. Determine intact tumor morphology and the presence of sufficient tumor cells for evaluation. Use standard methods of tissue processing for all specimens.

In-house control tissue

Differences in processing and embedding may produce significant variability in results. It is recommended that control tissues be stained on the same slide as the patient tissue.

Include positive and negative in-house control tissue in each staining run. Controls should be normal tonsil or biopsy/surgical specimens of the same tumor indication as the patient specimen, fixed, processed, and embedded as soon as possible in the same manner as the patient tissue(s). When using tonsil as a positive control tissue, negative control elements within the tonsil specimen may serve as the negative control tissue.

Control tissues processed differently from the patient specimen validate reagent performance only and do not verify proper patient tissue preparation.

The tissue selected for use as the positive tissue controls should include weak to moderate positive staining when stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) to aid in detection of subtle changes in assay sensitivity.

Tonsil stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) should demonstrate moderate to strong brown nuclear expression in the majority of germinal center B cells. The parabasal layer of squamous epithelium should show a strong nuclear pattern. Cells in the intermediate layer of squamous epithelium should demonstrate a low to moderate nuclear expression. The superficial layer and the majority of cells in the basal squamous epithelial layer should be negative. See the Slide Evaluation Section (Figure 5 and Figure 6) for representative images of tonsil staining.

When using biopsy/surgical specimens as positive control tissue that are the same tumor indication as the patient's specimen, the presence of brown nuclear staining should be observed in tumor cells. The ideal positive control tissue provides a complete dynamic representation of weak to moderate staining of tumor cells. The ideal negative control tissue should demonstrate no staining on tumor cells.
**Tissue processing**

FFPE tissues have been validated for use. Other tissue preparations (e.g., cytology specimens, fine-needle aspirates or bone decalcifications) have not been validated. Block specimens into a thickness of 3 mm or 4 mm, fix in formalin and dehydrate and clear in a series of alcohols and xylene, followed by infiltration with melted paraffin. The paraffin temperature should not exceed 60 °C. Feasibility studies on breast carcinoma tissue samples were performed with fixation in 10% neutral buffered formalin for 6–72 hours. Tissues processed with other fixatives and other fixation times have not been validated and are not recommended.

Cut tissue specimens into sections of 4–5 µm. After sectioning, tissues should be mounted on Dako FLEX IHC Microscope Slides (Code K8020) or Superfrost Plus slides, and then placed in an oven and dried at 58 ± 2 °C for 1 hour. All tissues, specimens and controls must be mounted within the validated area of the slide. See Dako Omnis User Guide for further details. To preserve antigenicity, store tissue sections in the dark at 2–8 °C (preferred) or at room temperature up to 25 °C and stain within 2 months of sectioning.
Ki-67 IHC MIB-1 pharmDx (Dako Omnis) Staining Procedure

The Ki-67 IHC MIB-1 pharmDx (Dako Omnis) reagents and instructions have been designed for optimal performance. All required steps and incubation times for staining are pre-programmed in the Dako Omnis software.

**Reagent storage**
Store all components according to the IFU when not in use.

**Reagent preparation**
Reagents do not need to be equilibrated to room temperature before loading into the instrument. However, they should be loaded into the instrument before starting the staining procedure, which allows sufficient time for equilibration. Do not use after the expiration date printed on the outside of the package.

**EnVision FLEX Target Retrieval Solution, Low pH and Wash Buffer**
EnVision FLEX Target Retrieval Solution, Low pH (50×) (GV805) and Wash Buffer (20×) (GC807) must be diluted to 1× concentration according to their Instructions for Use. Please refer to their product specific limitations in their individual Instructions for Use.
Further dilution of the reagents, substitution of other MIB-1 antibody preparations, alteration of incubation times, temperatures, or materials may give erroneous results.
Deparaffinization, rehydration, target retrieval, staining, and counterstaining

- Select the Ki-67 IHC MIB-1 pharmDx protocol or Ki-67 IHC NCR pharmDx protocol for the slides to be stained
- Place the Dako Omnis staining rack with slides on the Dako Omnis instrument
- Load all required reagents onboard the Dako Omnis as prompted by the instrument. Ensure that the appropriate quality and amount of water is added to the unloading rack to prevent specimen dehydration
- The instrument performs the pretreatment, staining, and counterstaining procedures by applying the appropriate reagent, monitoring the incubation time, and rinsing slides between reagents

Mounting

Use non-aqueous permanent mounting media. To minimize fading, store slides in the dark at room temperature (20–25 °C).

Product-specific limitations

False-negative results could be caused by degradation of the antigen in the tissues over time. Specimens should be stained within the cut section storage recommendations listed in the Ki-67 IHC MIB-1 pharmDx (Dako Omnis) IFU.

The use of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) on specimens fixed in fixatives other than 10% neutral buffered formalin and for fixation times other than 6–72 hours is not recommended.

Membranous/cytoplasmic staining has been reported in invasive ductal breast carcinoma. In the absence of convincing nuclear immunoreactivity, cytoplasmic and/or membrane staining should be excluded from the Ki-67 pharmDx Score.

Use of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) on fine needle aspirates has not been validated.
Technical Checklist

Use the checklist below to ensure correct usage of Ki-67 IHC MIB-1 pharmDx (Dako Omnis):

Customer Name/Institution ________________________________

Name and Title __________________________________________

Instrument Serial Number _________________________________  Software Version ____________________

Regular preventive maintenance is performed on the Dako Omnis as indicated by instrument?  

Yes  No  

Ki-67 IHC MIB-1 pharmDx (Dako Omnis) and ancillary reagents are used before the expiration date printed on the outside of the box?  

Yes  No  

All Ki-67 IHC MIB-1 pharmDx (Dako Omnis) and ancillary reagents are stored in the dark at 2–8 °C?  

Yes  No  

Appropriate positive and negative control tissues from breast carcinoma or tonsil are identified?  

Yes  No  

Tissues are fixed in 10% neutral buffered formalin?  

Yes  No  

Tissues are infiltrated with melted paraffin, at or below 60 °C?  

Yes  No  

Tissue sections of 4–5 µm are mounted on Dako FLEX IHC Microscope Slides or Superfrost Plus slides?  

Yes  No  

Specimens are oven-dried at 58 ± 2 °C for 1 hour?  

Yes  No  

Specimens and control tissues are mounted within the validated area for Dako Omnis.  

Yes  No  

Specimens are stained within 2 months of sectioning when stored in the dark at 2–8 °C (preferred) or in the dark at room temperature up to 25 °C?  

Yes  No  

EnVision FLEX Target Retrieval Solution, Low pH is prepared properly? pH of 1× Target Retrieval Solution must be 6.1 ± 0.2.  

Yes  No  

EnVision FLEX Wash Buffer is prepared properly?  

Yes  No  

Slides are counterstained with EnVision FLEX Hematoxylin?  

Yes  No  

The Ki-67 IHC MIB-1 pharmDx (or NCR) protocol is selected on the Dako Omnis instrument for the appropriate slides?  

Yes  No  

Do you have all the necessary equipment to perform the Ki-67 IHC MIB-1 pharmDx (Dako Omnis) according to protocol? If not, specify what is missing in comments below.  

Yes  No  

Additional observations or comments: ____________________________________________________________

___________________________________________________________

___________________________________________________________
Slide Evaluation

General considerations

Ki-67 IHC MIB-1 pharmDx (Dako Omnis) evaluation should be performed by a qualified pathologist using a light microscope. Details of the Ki-67 IHC MIB-1 pharmDx (Dako Omnis) interpretation guidelines are reviewed on page 28. Before examining the patient specimen for Ki-67 staining, it is important to examine the controls to assess staining quality.

Ki-67 interpretation is best assessed by requesting two—three serial tissue sections (hematoxylin and eosin [H&E], Ki-67 stain, and NCR stain [discretionary]). If the H&E is first assessed and is acceptable, the remaining serial sections are likely to be acceptable for use in IHC staining.

Specimen adequacy

**Confirm the presence of at least 200 viable invasive tumor cells**

An H&E stain of the tissue specimen is evaluated first to assess tissue histology and preservation quality. Ki-67 IHC MIB-1 pharmDx (Dako Omnis) and the H&E staining should be performed on serial sections from the same paraffin block of the specimen. Tissue specimens should be intact, well preserved, and should confirm tumor indication of invasive breast carcinoma.

A minimum of 200 viable invasive tumor cells must be present in the Ki-67 stained slide for the specimen to be considered adequate for Ki-67 evaluation.

**Instructions for patient specimens with less than 200 viable tumor cells**

Tissue from a deeper level of the block, or potentially another block, could have a sufficient number of viable tumor cells for Ki-67 IHC MIB-1 pharmDx (Dako Omnis) testing.
Positive and negative in-house control tissue

Examine the positive in-house control tissue (tonsil or breast carcinoma) to determine that the tissues are correctly prepared and the reagents are functioning properly. It is recommended that control tissues be stained on the same slide as the patient tissue. The ideal positive control tissue provides a complete dynamic representation of weak to moderate staining of cells (Figure 7). If staining of positive in-house control tissue is not satisfactory, all results with the patient specimen should be considered invalid.

When using tonsil as a positive control tissue, negative control elements within the specimen may serve as the negative control tissue

Tonsil stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) should demonstrate moderate to strong nuclear expression in the majority of germinal center B cells (Figure 5). The parabasal layer of squamous epithelium can show a strong nuclear pattern. Cells in the intermediate layer of squamous epithelium can demonstrate low to moderate nuclear expression. The superficial layer and the majority of cells in the basal squamous epithelial layer should be negative (Figure 6). Cells labeled by the antibody display a nuclear staining pattern except in mitotic cells, where both the mitotic nuclei and the cytoplasm are labeled.

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**Figure 5:** Tonsil stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting strong nuclear staining in the germinal center B cells as well as scattered interfollicular lymphocytes and parabasal keratinocytes in the squamous epithelium (10× magnification).

**Figure 6:** Tonsil stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting strong nuclear staining in the parabasal keratinocytes in the squamous epithelium (A). Cells in the intermediate layer of the squamous epithelium demonstrate low to moderate staining (B). The superficial layer (C) and the majority of cells in the basal squamous epithelial layer should be negative (D) (20× magnification).
Using breast carcinoma as in-house control tissue

The ideal positive control tissue provides a complete dynamic representation of weak to moderate staining of cells (Figure 7). It is recommended that control tissues be stained on the same slide as the patient tissue. When using on-slide control tissue, the positive control tissue should be run on the same slide as patient specimen. The negative control may be run on a separate slide. If the staining of positive in-house control tissue is not satisfactory, all results with the patient specimen should be considered invalid.

Figure 7: Positive in-house control tissue stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) showing different intensities of nuclear Ki-67 expression by invasive breast carcinoma cells (20× magnification).

The ideal negative control tissue should demonstrate no staining of tumor cells (Figure 8) while retaining positive signal in relevant non-tumor cells. Examine the negative in-house control tissue to determine the expected staining. The variety of different cell types present in most breast carcinoma tissue sections offers internal negative control sites; this should be verified by the user.

If inappropriate staining occurs in the in-house control tissues, results with the patient specimen should be considered invalid.

Figure 8: Negative in-house control tissue stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) demonstrating lack of nuclear Ki-67 expression by invasive breast carcinoma cells (20× magnification).

Do not use in-house control tissue as an aid in interpretation of patient results.
Negative Control Reagent (Discretionary)

Negative Control Reagent (NCR) may be used in place of the primary antibody on a section of the patient specimen to evaluate non-specific staining and allow better interpretation of specific staining at the antigen site. Examine the slides stained with the NCR to identify non-specific background staining that may interfere with Ki-67 staining interpretation, making the specimen non-evaluable. Satisfactory performance is indicated by the absence of specific staining (Figure 9).

Examine the patient specimens stained with the NCR to determine if there is any non-specific staining that may interfere with interpreting the Ki-67 stained slide. For examples of non-specific staining, see Figures 34 and 35.

Figure 9: Absence of staining in an invasive breast carcinoma tissue specimen stained with NCR (Code GE020) (20× magnification).

NCR-stained slides indicate non-specific background staining, if present, which aids correct interpretation of patient specimens stained with the primary antibody.
Figure 10: Recommended order of slide evaluation.

- **Tissue block**
  - Serial sections are cut/prepared
  
  One section is stained with H&E (H&E Patient Specimen)
  - **Is H&E slide adequate?**
    - (intact, well-preserved, breast carcinoma)
    - **Yes**
      - Positive control tissue adequate?
      - **No**
        - Repeat staining run
      - **Yes**
        - Negative control tissue adequate?
        - **No**
          - Repeat staining run
        - **Yes**
          - Patient specimen stained with Negative Control Reagent acceptable?
          - **No**
            - Repeat staining run
          - **Yes**
            - Patient specimen stained with primary antibody exhibiting ≥ 200 viable tumor cells?
            - **No**
              - Repeat staining run with a deeper cut in the block or a new patient specimen
            - **Yes**
              - Scored by Pathologist

- **Sections of 4–5 µm thickness are mounted on glass microscope slides**

Exclude from scoring:
- Membrane and cytoplasmic staining
- Carcinoma in situ
- Normal cells
- Necrotic cells

*Use of Negative Control Reagent is discretionary for Ki-67 IHC MIB-1 pharmDx (Dako Omnis), Code GE020
Ki-67 pharmDx Score

Definition of Ki-67 pharmDx Score

Ki-67 expression in breast carcinoma stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is determined by using Ki-67 pharmDx Score. To determine the Ki-67 pharmDx Score, the entire viable tumor area must be evaluated. Ki-67 pharmDx Score is the number of Ki-67 staining viable tumor cells in the invasive cancer component divided by the total number of viable tumor cells in the invasive cancer component, multiplied by 100.

\[
\text{Ki-67 pharmDx Score} = \frac{\# \text{ Ki-67 staining viable tumor cells in the invasive cancer component}}{\text{Total # of staining and non-staining viable tumor cells in the invasive cancer component}} \times 100
\]

Any convincing nuclear staining (≥ 1+) of viable invasive tumor cells is considered Ki-67 staining and should be included in the scoring. See Figure 15 on page 32 for an example of 1+ staining intensity.

Nuclear staining (≥ 1+) of any other cell type, including carcinoma in situ, non-neoplastic breast epithelium or other non-neoplastic cells should be excluded from the Ki-67 pharmDx Score determination.

See Tables 1 and 2 on page 24 for additional Ki-67 pharmDx Score inclusion/exclusion criteria.
Determining Ki-67 pharmDx Score

- At lower magnifications, examine all well-preserved tumor areas in the entire slide. Evaluate overall areas of Ki-67 staining and non-staining tumor cells, keeping in mind that 1+ nuclear staining may be difficult to see at low magnifications.
- A minimum of 200 viable invasive tumor cells must be present in the Ki-67 stained slide for the specimen to be considered adequate for evaluation.
- For specimens with less than 200 viable tumor cells, sections from a deeper level of the block or potentially another block could have a sufficient number of tumor cells for evaluation of Ki-67 expression.
- At higher magnification, evaluate Ki-67 expression and determine Ki-67 pharmDx Score:
  - Determine the total number of viable invasive tumor cells, both Ki-67 staining and non-staining (Ki-67 pharmDx Score denominator).
  - Determine the number of Ki-67 staining viable invasive tumor cells (Ki-67 pharmDx Score numerator; see Tables 1 and 2 on page 24 for additional Ki-67 pharmDx Score inclusion/exclusion criteria).
  - Determine Ki-67 pharmDx Score.
Table 1: Ki-67 pharmDx Score numerator inclusion/exclusion criteria

<table>
<thead>
<tr>
<th>Tissue Elements</th>
<th>Included in the Numerator</th>
<th>Excluded from the Numerator</th>
</tr>
</thead>
</table>
| Tumor cells     | Viable tumor cells in the invasive cancer component with convincing and complete nuclear staining (at any intensity 1+ or higher) | - Non-staining tumor cells  
- Tumor cells with only cytoplasmic or membrane staining

- Non-invasive neoplasia (including carcinoma in situ)
- Non-viable/necrotic tumor cells
- Apoptotic nuclei/nuclear debris
- Tumor cells in areas with obscuring artifacts (i.e., poorly preserved areas and processing artifacts)

| Other cells     | Not included | - Benign epithelial cells  
- Other non-neoplastic cells |

Table 2: Ki-67 pharmDx Score denominator inclusion/exclusion criteria

<table>
<thead>
<tr>
<th>Tissue Elements</th>
<th>Included in the Denominator</th>
<th>Excluded from the Denominator</th>
</tr>
</thead>
</table>
| Tumor cells     | All viable tumor cells in the invasive cancer component | - Non-invasive neoplasia (including carcinoma in situ)
- Non-viable/necrotic tumor cells
- Apoptotic nuclei/nuclear debris
- Tumor cells in areas with obscuring artifacts (i.e., poorly preserved areas and processing artifacts)

| Other cells     | Not included | - Benign epithelial cells  
- Other non-neoplastic cells |
Suggested methods

Agilent recommends that scoring be performed within the context of the pathologist's past experience and best judgment in interpreting IHC stains. We offer three different examples of techniques that may be used when determining the respective Ki-67 pharmDx Scores of various staining patterns.

The entire IHC slide should be reviewed to determine which of the following example techniques may be used.

Example 1: Determination of Ki-67 pharmDx Score based on a small Ki-67 staining area

First: Evaluate the tumor area for convincing staining as described in "Determining Ki-67 pharmDx Score" on page 23.

Assessment: 10% of area shows staining, 90% of area shows no staining

Second: Evaluate the area of staining to determine the number of Ki-67 staining invasive tumor cells.

Assessment: There are approximately 100 viable tumor cells in the area of staining and about 80 Ki-67 staining cells (per the Ki-67 pharmDx Score numerator)

Calculate the Ki-67 pharmDx Score of the entire tumor area:

Assessment:

\[
\text{Ki-67 pharmDx Score of area with staining:} \\
\frac{\text{# Ki-67 staining viable tumor cells in the invasive cancer component}}{\text{Total # of staining and non-staining viable tumor cells in the invasive cancer component}} \times 100 = \frac{80 \text{ Ki-67 staining tumor cells}}{100 \text{ tumor cells}} \times 100 = 80%
\]

Ki-67 pharmDx Score of entire tumor area: 10% × 80% = 8%

Clinical interpretation: Ki-67 pharmDx Score < 20%

Figure 11: Example of tumor with small Ki-67 staining area.
Example 2: Determination of Ki-67 pharmDx Score based on a heterogeneous Ki-67 staining area

First: Visually divide the tumor area into regions with equal numbers of tumor cells.

Second: Observe each region and determine the total number of viable tumor cells and Ki-67 staining tumor cells. Determine the Ki-67 pharmDx Score for each region.

**Assessment:** The four sections have 60, 30, 20, and 10 Ki-67 staining tumor cells. Each section has a total of 100 tumor cells (including Ki-67 staining cells). The Ki-67 pharmDx Score for each section: Ki-67 pharmDx Score 60%, Ki-67 pharmDx Score 30%, Ki-67 pharmDx Score 20%, and Ki-67 pharmDx Score 10%.

Calculate the Ki-67 pharmDx Score of the entire tumor area:

**Assessment:**

\[
\text{Ki-67 pharmDx Score} = \frac{(60\% + 30\% + 20\% + 10\%)}{4} = 30\%
\]

Clinical interpretation: Ki-67 pharmDx Score ≥ 20%

Figure 12: Example with heterogeneous Ki-67 staining area.
Example 3: Determination of Ki-67 pharmDx Score for a near cutoff specimen

First: Evaluate the specimen for convincing staining as described in "Determining Ki-67 pharmDx Score" on page 23.

Second: Examine the specimen at a higher objective (20×) to confirm that there is no weak (1+) staining in areas that appeared devoid of staining at lower objectives. Evaluate all staining areas and determine the total number of Ki-67 staining tumor cells.

Then re-evaluate the entire specimen (staining and non-staining areas) and determine the total number of viable invasive tumor cells (Ki-67 staining and non-staining tumor cells). Determine the Ki-67 pharmDx Score.

**Assessment:** Tumor specimen has perceptible and convincing staining. 30 Ki-67 staining invasive tumor cells. There are approximately 200 viable invasive tumor cells present in the entire specimen.

Calculate the Ki-67 pharmDx Score of the entire tumor area:

**Assessment:**

\[
\text{Ki-67 pharmDx Score (\%) = } \frac{\text{# Ki-67 staining viable tumor cells in the invasive cancer component}}{\text{Total # of staining and non-staining viable tumor cells in the invasive cancer component}} \times 100 = \frac{30 \text{ Ki-67 staining tumor cells}}{200 \text{ tumor cells}} \times 100 = 15\%
\]

**Clinical interpretation:** Ki-67 pharmDx Score < 20%

*Figure 13:* Example of near cutoff specimen.
Interpretation of Ki-67 pharmDx Score

The Ki-67 pharmDx Score determines the percentage of viable invasive tumor cells with nuclear Ki-67 expression. See the table below for scoring interpretation examples.

**Table 3: Ki-67 pharmDx Score and corresponding Ki-67 staining characteristics**

<table>
<thead>
<tr>
<th>Ki-67 pharmDx Score</th>
<th>Staining Pattern</th>
<th>Image (20× magnification)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 20%</td>
<td>Convincing nuclear staining (≥ 1+) in &lt; 20% of viable tumor cells in the invasive cancer components</td>
<td></td>
</tr>
<tr>
<td>≥ 20%</td>
<td>Convincing nuclear staining (≥ 1+) in ≥ 20% of viable tumor cells in the invasive cancer component</td>
<td></td>
</tr>
</tbody>
</table>
Clinical features have been used to assess whether an early breast cancer has a high risk of disease recurrence, but an additional patient population can be identified through the biological parameter of tumor cell proliferation. Ki-67 expression is an indirect indicator of tumor cell proliferation. Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is FDA-approved as an aid in identifying patients with early breast cancer at high risk of disease recurrence for whom Verzenio (abemaciclib) treatment is being considered in combination with standard adjuvant endocrine therapy.

**Clinical validation of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) in patients with invasive early breast carcinoma**

The clinical validity of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) in identifying Ki-67 expression (Ki-67 pharmDx Score ≥ 20%) in patients with breast cancer is based on the monarchE study, sponsored by Eli Lilly and Company. Specimens from patients with invasive early breast cancer were evaluated for Ki-67 expression using Ki-67 IHC MIB-1 pharmDx (Dako Omnis). Efficacy of Verzenio treatment in patients who had Ki-67 expression of Ki-67 pharmDx Score ≥ 20% by Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is presented in the Clinical Performance Evaluation section on pages 71–72.

Use the following flowchart to help you understand which patients are indicated for treatment with Verzenio based on their Ki-67 pharmDx Score. See Verzenio prescribing information for additional selection criteria.

**Figure 14:** Testing scheme for Ki-67 IHC MIB-1 pharmDx (Dako Omnis).
Reporting Results

Suggested information to include when reporting results with Ki-67 IHC MIB-1 pharmDx (Dako Omnis).

**Ki-67 IHC MIB-1 pharmDx (Dako Omnis) summary of sample tested**

- Date of run: 
- Ki-67 IHC MIB-1 pharmDx (Dako Omnis) lot: 
- Staining run log ID: 
- Specimen ID: 
- Patient identifiers: 
- Type of service: IHC stain with manual interpretation 
- Other: 

**Ki-67 testing results**

- Positive control tissue results: Pass: ☐ Fail: ☐ 
- Negative control tissue results: Pass: ☐ Fail: ☐ 
- Patient Specimen, Negative Control Reagent (discretionary): Pass: ☐ Fail: ☐ Not included: ☐ 
- Adequate tumor cells present (≥ 200 cells): Yes: ☐ No: ☐

**Ki-67 IHC MIB-1 pharmDx (Dako Omnis) result to treating physician**

- Ki-67 pharmDx Score (%): * 
- Ki-67 pharmDx Score < 20%: ☐ Ki-67 pharmDx Score ≥ 20%: ☐

**Note:**
- Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is indicated as an aid in identifying patients with early breast cancer at high risk of disease recurrence for whom adjuvant treatment with Verzenio (abemaciclib) in combination with endocrine therapy is being considered. The specimen should be considered to have Ki-67 expression if Ki-67 pharmDx Score is ≥ 20%. See the Verzenio prescribing information for specific clinical circumstances guiding Ki-67 testing.
- * FDA has reviewed and approved this assay as a qualitative assay at a Ki-67 pharmDx Score ≥ 20% cutoff. Other correlations between the raw Ki-67 pharmDx Score and clinical outcome have not been established and have not been reviewed or approved by FDA.
By definition, Ki-67 staining areas in invasive breast carcinoma are:
- Viable tumor cells with convincing nuclear staining (at intensity 1+ or higher) that corresponds to the chromatin distribution within the nucleus

Ki-67 expression status in breast carcinoma is determined by Ki-67 pharmDx Score, which is the number of Ki-67 staining tumor cells in the invasive cancer component divided by the total number of viable tumor cells in the invasive cancer component, multiplied by 100.

This section will define and illustrate scoring inclusions and exclusions for accurate determination of Ki-67 pharmDx Score.
Ki-67 staining cells included in Ki-67 pharmDx Score

Tumor cells exhibiting appropriate Ki-67 expression are defined as Ki-67 staining areas showing convincing nuclear staining $\geq 1+$ intensity. All Ki-67 staining viable invasive tumor areas are included in the Ki-67 pharmDx Score numerator for determination of the Ki-67 pharmDx Score (see Tables 1 and 2 on page 24 for additional Ki-67 pharmDx Score inclusion/exclusion criteria). All viable tumor cells in the invasive cancer component should be included in the denominator. Below are common staining characteristics of Ki-67 staining cells that must be included in the Ki-67 pharmDx Score numerator. All images are of invasive breast carcinoma.

Tumor cells

Nuclear staining

Nuclear staining of tumor cells at intensities 1+–3+ should be included. Tumor cells exhibiting convincing nuclear staining are considered Ki-67 staining cells. Convincing nuclear staining is determined by the following parameters:

1. The signal must be unequivocally brown
2. The staining must correspond to a nucleus
3. The staining must cover the whole chromatin distribution within the nucleus
4. The staining must correspond to viable (non-apoptotic, non-necrotic) cells

Nuclear staining: 1+ intensity

Figure 15: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting 1+ nuclear staining of tumor cells (arrows) (20× magnification).
Nuclear staining: 2+ intensity

Figure 16: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting 2+ nuclear staining of tumor cells (arrows) (20× magnification).

Nuclear staining: 3+ intensity

Figure 17: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting 3+ nuclear staining of tumor cells (arrows) (20× magnification).

Key Point

Convincing nuclear staining of tumor cells at intensities 1+ or higher should be included in the Ki-67 pharmDx Score numerator.
Convincing staining of tumor cells is often heterogeneous, with various staining intensities present in the same sample.

Figure 18: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting 1–3+ nuclear staining of tumor cells. Red arrows indicate 3+ staining intensities, yellow indicate 2+ staining intensities, and green indicate 1+ staining intensities (20× magnification).

Negative vs. weakly positive cells

Cells that exhibit a "grey" coloring in the nucleus are excluded from Ki-67 scoring. If the nucleus is not unequivocally brown in color, then the cell is considered to not be exhibiting convincing nuclear staining.

Figure 19: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting both negative and weakly positive staining. Negative cells show grey nuclear staining and are indicated with black arrows, and weak 1+ staining is indicated with green arrows (20× magnification).

Key Point

Convincing nuclear staining of viable invasive tumor cells at ≥ 1+ intensity should be included in the Ki-67 pharmDx Score numerator.
Membrane and cytoplasmic staining

Tumor cells with membrane and/or cytoplasmic staining at any objective should not be included in the Ki-67 pharmDx Score numerator unless the nucleus is also clearly staining as defined in the previous sections.

Figure 20: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting cytoplasmic staining with distinct nuclear staining (arrows) (20× magnification).

Figure 21: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting cytoplasmic staining with no distinct nuclear staining (arrows) (40× magnification).
Figure 22: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting membrane staining with no distinct nuclear staining (arrows) (20× magnification).

**Key Point**

Tumor cells exhibiting perceptible membrane and/or cytoplasmic staining without nuclear staining are not included in the Ki-67 pharmDx Score numerator. Tumor cells that do show distinct nuclear staining in addition to membrane and/or cytoplasmic staining should be included in the Ki-67 pharmDx Score numerator.
Uneven chromatin distribution

Occasionally tumor cells can exhibit an incomplete nuclear staining pattern due to uneven chromatin distribution or nuclear pseudo-inclusions, resulting in a “nuclear clearing” aspect. These nuclear features should also be observed on the corresponding H&E stain. Any convincing nuclear staining of tumor cells that covers the entirety of the chromatin distribution within the nucleus (≥ 1+ intensity) should be included in the Ki-67 pharmDx Score numerator, even if incomplete in appearance. If distinct nucleolar staining is observed in an otherwise negative nucleus, the tumor cell should be scored as negative and included in the denominator.

Figure 23: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) and corresponding H&E stained slide exhibiting incomplete nuclear staining pattern due to uneven chromatin distribution that results in a "nuclear clearing" aspect (arrows) (20× magnification).
Figure 24: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting distinct nucleolar staining in an otherwise negative tumor cell nucleus (arrow) (40× magnification).

**Key Point**

Tumor cells must exhibit a convincing nuclear staining pattern corresponding to the chromatin distribution within the nucleus to be included in the Ki-67 pharmDx Score numerator.
Cells excluded from Ki-67 pharmDx Score

Only viable invasive tumor cells exhibiting convincing Ki-67 nuclear staining should be included in the Ki-67 pharmDx Score numerator. Below are cells that can exhibit staining but should be excluded from the Ki-67 pharmDx Score determination (Ki-67 pharmDx Score numerator and/or denominator).

Carcinoma in situ

Carcinoma in situ cells exhibiting Ki-67 nuclear staining should be excluded from the Ki-67 pharmDx Score.

Figure 25: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting staining of ductal carcinoma in situ (DCIS) and corresponding H&E (10× magnification).

Key Point

Any tumor cells associated with carcinoma in situ should be excluded from scoring
Normal epithelium

Benign epithelial cells can show Ki-67 nuclear staining. These cells should be excluded from the Ki-67 pharmDx Score.

![Image of normal epithelium](image.png)

Figure 26: Ki-67 IHC MIB-1 pharmDx (Dako Omnis) staining in normal epidermal cells adjacent to invasive area of breast carcinoma specimen (20× magnification).

Non-neoplastic breast epithelium

Non-neoplastic breast epithelium such as normal ducts and lobules adjacent to breast carcinoma may show Ki-67 nuclear staining. These cells should not be included in the Ki-67 pharmDx Score.

![Image of breast carcinoma specimen](image.png)

Figure 27: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis). Ki-67 staining in normal epithelial cells within ducts and lobules can be seen (arrows). The left half of the image above shows tumor cells that should be included in the Ki-67 pharmDx Score. The right side of the image shows normal breast epithelium that should be excluded from the Ki-67 pharmDx Score estimation (10× magnification).

**Key Point**

Staining in non-neoplastic cells should be excluded from scoring.
Non-viable, necrotic, and apoptotic cells

Only viable tumor cells should be included in the Ki-67 pharmDx Score. Non-viable, necrotic and apoptotic cells that may or may not be exhibiting Ki-67 staining should be excluded from both the numerator and denominator.

Figure 28: Necrotic area of breast carcinoma specimen stained with Ki-67 primary antibody and corresponding H&E stained slide (10× magnification).

**Key Point**

Only viable tumor cells should be counted in the Ki-67 pharmDx Score. Non-viable, necrotic and apoptotic cells should be excluded.
Lymphocytes

Lymphocytes often exhibit nuclear staining and should not be included in the Ki-67 pharmDx Score scoring algorithm.

Nuclear staining of lymphocytes is often heterogeneous, with various staining intensities present.

**Figure 29:** Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting staining of scattered positive lymphocytes within a lymphoid aggregate (arrows) (20× magnification).

**Figure 30:** Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting staining of lymphocytes (arrows) admixed with positive tumor cells (20× magnification).

**Key Point**

Lymphocytes with nuclear staining should not be included in the Ki-67 pharmDx Score numerator
Stromal cells

Stromal cells exhibiting Ki-67 nuclear staining should be excluded from the Ki-67 pharmDx Score.

Figure 31: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting stromal cell nuclear staining (arrows), as well as staining of tumor cells (20× magnification).

Key Point

Stromal cells exhibiting nuclear staining should not be included in the Ki-67 pharmDx Score
Artifacts

The following pages provide examples of artifacts you may see when staining with Ki-67 IHC MIB-1 pharmDx (Dako Omnis).

Non-specific background staining

Background staining is defined as diffuse, non-specific staining of a specimen. It is caused by several factors. These factors include, but are not limited to:

- Pre-analytic fixation and processing of the specimen
- Incomplete removal of paraffin from the section
- Incomplete rinsing of slides during staining
- Improper deparaffinization procedure
- Incomplete rinsing of reagents from slides

The non-specific background staining of the NCR-stained test specimen is useful in determining the level of background staining in the Ki-67 stained test specimen. All specimens must have < 1+ non-specific background staining. The use of fixatives other than 10% NBF may be a source of background staining and is not recommended. Background staining with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) is rare.
Pre-analytical artifacts

Areas of the examined section exhibiting distorted morphology secondary to pre-analytical artifacts such as poor fixation, crush and/or cautery artifact should be excluded from scoring.

Figure 32: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting crush artifact; these areas should be excluded from the score (20× magnification).

Figure 33: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting cautery artifact; these areas should be excluded from the score (20× magnification).

**Key Point**

Scoring of areas with processing artifacts such as crush and/or cautery artifact should be avoided
Poorly-adhering sections may trap DAB between the tissue and the slide, leading to diffuse non-specific background. Areas of the examined section exhibiting non-specific background staining should be excluded from scoring.

**Figure 34:** Breast carcinoma NCR exhibiting weak (≤ 1+) cytoplasmic staining which is considered acceptable non-specific staining, excluded from the scoring (arrows) (20× magnification).

**Figure 35:** Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting cytoplasmic blush (arrows). These represent non-specific staining that should be excluded from the score. Weak nuclear staining is also present and should be included (20× magnification).

**Key Point**

All specimens must have < 1+ non-specific background staining
Edge artifact

Commonly, edge artifact is linked to the following pre-analytic factors:
- Thick tissue sections
- Tissue cautery
- Drying of tissue prior to fixation or during staining procedure

These factors can lead to accentuation of staining at the periphery of the section. In this case, Ki-67 staining at the edge of the tissue section is excluded from scoring.

**Note:** Although edge artifact can be present, it is not common.

![Figure 36: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis). Edge staining artifact should be excluded from the score (5× magnification).](image)

**Key Point**

Scoring of the edge of a specimen should be avoided if staining due to edge artifact is inconsistent with the rest of the specimen.
Case 1: Ki-67 pharmDx Score < 20%

Figure 37a: 10× magnification.

Figure 37b: 20× magnification.
Figures 37a–c: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 0% (10–40× magnification).
Case 2: Ki-67 pharmDx Score < 20%

Figure 38a: 10× magnification.

Figure 38b: 20× magnification.
Figures 38a–c: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 5% (10–40× magnification).
Case 3: Ki-67 pharmDx Score < 20%

Figure 39a: 10× magnification.

Figure 39b: 20× magnification.
Figures 39a–c: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 7% (10–40× magnification).
Case 4: Ki-67 pharmDx Score < 20%

Figure 40a: 10× magnification.

Figure 40b: 20× magnification.
Figures 40a–c: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 11% (10–40x magnification).
Ki-67 pharmDx Score ≥ 20%
case examples

Case 1: Ki-67 pharmDx Score ≥ 20%

Figure 41a: 10× magnification.

Figure 41b: 20× magnification.

Figure 41c: 40× magnification.

Figures 41a–c: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 43% (10–40× magnification).
Case 2: Ki-67 pharmDx Score ≥ 20%

Figures 42a–c: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 52% (10–40× magnification).
Case 3: Ki-67 pharmDx Score ≥ 20%

Figure 43a: 10× magnification.

Figure 43b: 20× magnification.
Figure 43c: 40× magnification.

**Figures 43a–c:** Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 82% (10–40× magnification).
Near cutoff case examples
(Ki-67 pharmDx Score range of ≥ 10% but < 20%)

Near cutoff case 1: Ki-67 pharmDx Score range of ≥ 10% but < 20%

Figure 44a: 10× magnification.

Figure 44b: 20× magnification.
Figures 44a–c: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 15% (10–40× magnification).
Near cutoff case 2: Ki-67 pharmDx Score range of $\geq 10\%$ but $< 20\%$

Figure 45a: 10× magnification.

Figure 45b: 20× magnification.
Figures 45a–c: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 19% (10–40× magnification).
Near cutoff case examples: (Ki-67 pharmDx Score range of ≥ 20% but ≤ 30%)

Near cutoff case 3: Ki-67 pharmDx Score range of ≥ 20% but ≤ 30%

Figure 46a: 10× magnification.

Figure 46b: 20× magnification.
Figures 46a–c: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 21% (10–40× magnification).
Near cutoff case 4: Ki-67 pharmDx Score range of ≥ 20% but ≤ 30%

Figure 47a: 10× magnification.

Figure 47b: 20× magnification.
Figures 47a–c: Breast carcinoma specimen stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) exhibiting a Ki-67 pharmDx Score of 28% (10–40× magnification).
For further troubleshooting help, contact your local Agilent representative.

Table 4: Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Probable Cause</th>
<th>Suggested Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wrong storage conditions used for reagents</td>
<td>Check that reagents have been stored correctly according to listed storage conditions</td>
<td></td>
</tr>
<tr>
<td>Reagent is used past its expiration date</td>
<td>Ensure reagent is not used past its expiration date</td>
<td></td>
</tr>
<tr>
<td>Reagent is used past its onboard stability</td>
<td>Ensure reagent is not used past its onboard stability</td>
<td></td>
</tr>
<tr>
<td>Inappropriate fixation method used</td>
<td>Ensure that patient tissue is not fixed for too short or too long a time period, that ischemia time has been minimized, and that the correct fixative (10% NBF) was used</td>
<td></td>
</tr>
<tr>
<td>Excessive heating of mounted tissue sections prior to loading on Dako Omnis may lead to loss of immunoreactivity and morphology</td>
<td>Dry the tissue sections at 58 ± 2 °C for a maximum of 1 hour, using a calibrated oven with uniform heat distribution</td>
<td></td>
</tr>
<tr>
<td>Incorrect placement of dynamic gap lids in stainer modules</td>
<td>Check placement of dynamic gap lids</td>
<td></td>
</tr>
<tr>
<td>Damaged dynamic gap lids</td>
<td>Check integrity of dynamic gap lids</td>
<td></td>
</tr>
<tr>
<td>Distilled or de-ionized water is not used to dilute the Target Retrieval Solution concentrate</td>
<td>Ensure that distilled or de-ionized water is used to prepare 1× Target Retrieval Solution</td>
<td></td>
</tr>
<tr>
<td>Incorrect Target Retrieval Solution is used</td>
<td>Ensure that correct Target Retrieval Solution specified in &quot;Materials Required but not Supplied&quot; and/or &quot;Reagent Preparation&quot; sections is used</td>
<td></td>
</tr>
<tr>
<td>1× Target Retrieval Solution does not meet pH specifications</td>
<td>Check pH of 1× Target Retrieval Solution. If pH is outside acceptable range (pH 6.1± 0.2), discard 1× Target Retrieval Solution. Do not adjust pH. Prepare new 1× Target Retrieval Solution. Check pH of new Target Retrieval Solution</td>
<td></td>
</tr>
<tr>
<td>Problem</td>
<td>Probable Cause</td>
<td>Suggested Action</td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
<td>-----------------</td>
</tr>
<tr>
<td><strong>Excessively strong specific staining of slides</strong></td>
<td>Inappropriate fixation method used</td>
<td>Ensure that only approved fixatives and fixation methods are used</td>
</tr>
<tr>
<td></td>
<td>Distilled or de-ionized water is not used to dilute the Target Retrieval Solution concentrate</td>
<td>Ensure that distilled or de-ionized water is used to prepare 1× Target Retrieval Solution</td>
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</tr>
<tr>
<td><strong>Excessive non-specific staining of slides</strong></td>
<td>Starch additives used in mounting sections to slides</td>
<td>Avoid using starch additives for adhering sections to glass slides. Many additives are immunoreactive</td>
</tr>
<tr>
<td></td>
<td>Sections dried after staining procedure</td>
<td>Verify that the unloading station is filled with sufficient water</td>
</tr>
<tr>
<td></td>
<td>Sections dried prior to coverslipping</td>
<td>Avoid stained slides drying out between unloading from Dako Omnis and coverslipping</td>
</tr>
<tr>
<td></td>
<td>Inappropriate fixation method used</td>
<td>Ensure that approved fixative was used. Alternative fixative may cause excessive background staining</td>
</tr>
<tr>
<td></td>
<td>Paraffin incompletely removed</td>
<td>Check appearance of solvent couplings. Gently scrub the couplings to remove impurities. Check the integrity of the couplings on the backside of the bulk bottles after cleaning. Refer to Dako Omnis Basic User Guide for additional details</td>
</tr>
<tr>
<td></td>
<td>Non-specific binding of reagents to tissue</td>
<td>Ensure that correct fixation method of the specimen is used and avoid large areas of necrosis</td>
</tr>
<tr>
<td></td>
<td>Re-use of mixing strip</td>
<td>Ensure that new mixing strips are used</td>
</tr>
<tr>
<td></td>
<td>Distilled or de-ionized water is not used to dilute the Target Retrieval Solution concentrate</td>
<td>Ensure that distilled or de-ionized water is used to prepare 1× Target Retrieval Solution</td>
</tr>
<tr>
<td></td>
<td>Incorrect Target Retrieval Solution is used</td>
<td>Ensure that correct Target Retrieval Solution specified in &quot;Materials Required but not Supplied&quot; and/or &quot;Reagent Preparation&quot; sections is used</td>
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<tr>
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<td>1× Target Retrieval Solution does not meet pH specifications</td>
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</table>

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<th>Suggested Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue detaches from slides</td>
<td>Use of incorrect slides</td>
<td>Use FLEX IHC Microscope Slides (Code K8020), or SuperFrost Plus slides</td>
</tr>
<tr>
<td>Slide is flagged</td>
<td>Reagent is used beyond its expiration date</td>
<td>Flagged slides should be evaluated by qualified personnel. Contact an Agilent Technologies representative if further action is needed</td>
</tr>
<tr>
<td></td>
<td>Reagent is stored onboard Dako Omnis beyond its validated onboard stability</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maintenance overdue or other factors</td>
<td></td>
</tr>
<tr>
<td>1× Target Retrieval Solution does not meet pH specifications</td>
<td>pH meter is not calibrated correctly</td>
<td>Ensure pH meter is calibrated per manufacturer’s recommendations. After re-calibration, re-test pH of 1× Target Retrieval Solution. Do not modify the pH of 1× Target Retrieval Solution TRS. If pH is outside acceptable range (pH 6.1± 0.2), discard 1× Target Retrieval Solution. Prepare new 1× Target Retrieval Solution. Check pH of new 1× Target Retrieval Solution</td>
</tr>
<tr>
<td></td>
<td>Target Retrieval Solution pH is measured at incorrect temperature</td>
<td>Ensure that 1× Target Retrieval Solution pH is measured at ambient temperature</td>
</tr>
<tr>
<td></td>
<td>Distilled or de-ionized water is not used to dilute the Target Retrieval Solution concentrate</td>
<td>Ensure that distilled or de-ionized water is used to prepare 1× Target Retrieval Solution</td>
</tr>
<tr>
<td></td>
<td>Incorrect Target Retrieval Solution is used</td>
<td>Ensure that the correct Target Retrieval Solution specified in “Materials Required but not Supplied” and/or “Reagent Preparation” sections is used</td>
</tr>
</tbody>
</table>

**Note:** If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please contact Agilent Technical Support for further assistance. Additional information on staining techniques and specimen preparation can be found in the Education Guide: *Immunohistochemical Staining Methods*¹¹ (available from www.agilent.com), *Atlas of Immunohistology*,¹² and *Immunoperoxidase Techniques. A Practical Approach to Tumor Diagnosis*.¹³
monarchE (NCT03155997) was a randomized (1:1), open-label, two cohort, multicenter study in adult women and men with HR-positive, HER2-negative, node-positive, resected, early breast cancer with clinical and pathological features consistent with a high risk of disease recurrence. To be enrolled in cohort 1, patients had to have HR-positive, HER2-negative, early breast cancer with tumor involvement in at least 1 axillary lymph nodes (pALN), and either:
- ≥ 4 pALN or
- 1–3 pALN and at least one of:
  - tumor grade 3
  - tumor size ≥ 50 mm

Patients with available untreated breast tissue samples were tested retrospectively using Ki-67 IHC MIB-1 pharmDx (Dako Omnis) at central testing sites. The assay was used to determine if Ki-67 pharmDx Score was ≥ 20% or < 20%.

Patients were randomized to receive 2 years of Verzenio plus physician's choice of standard endocrine therapy or standard endocrine therapy alone. Randomization to treatment was stratified by prior treatment (neoadjuvant chemotherapy versus adjuvant chemotherapy versus no chemotherapy); menopausal status (premenopausal versus postmenopausal), and region (North America/Europe versus Asia versus other). Men were stratified as postmenopausal. After the end of the study treatment period, standard adjuvant endocrine therapy was continued for a duration of at least 5 years if deemed medically appropriate.

Among the 2,003 patients with Ki-67 expression (Ki-67 pharmDx Score ≥ 20%) in cohort 1, patient median age was 51 years (range, 24–88 years), 99% were women, 68% were White, and 25% were Asian, 2.1% were Black or African American, 1.5% were American Indian or Alaska Native, and 0.2% were Native Hawaiian or Other Pacific Islander. Forty-six percent of patients were premenopausal. Most patients received prior chemotherapy (37% neoadjuvant, 60% adjuvant) and prior radiotherapy (95%). Fifty-seven percent of the patients had 4 or more positive lymph nodes with 20% having ≥ 10 positive lymph nodes, 58% had Grade 3 tumor, and 19% had pathological tumor size ≥ 50 mm. Nearly all patients (99%) were estrogen receptor positive and most patients were progesterone receptor positive (84%). Initial endocrine therapy received by patients included letrozole (39%), tamoxifen (33%), anastrozole (19%), or exemestane (8%).
The major efficacy outcome measure was invasive disease-free survival (IDFS). IDFS was defined as the time from randomization to the first occurrence of: ipsilateral invasive breast tumor recurrence, regional invasive breast cancer recurrence, distant recurrence, contralateral invasive breast cancer, second primary non-breast invasive cancer, or death attributable to any cause. Overall survival (OS) was an additional outcome measure.

Efficacy results are summarized in Table 5 and Figure 48.

**Table 5: Efficacy Results in monarchE in Cohort 1 Patients with Ki-67 Expression (Ki-67 pharmDx Score ≥ 20%)**

<table>
<thead>
<tr>
<th></th>
<th>Verzenio Plus Tamoxifen or an Aromatase Inhibitor N=1017</th>
<th>Tamoxifen or an Aromatase Inhibitor N=986</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive Disease-Free Survival (IDFS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients with an event (n, %)</td>
<td>104 (10.2)</td>
<td>158 (16.0)</td>
</tr>
<tr>
<td>Hazard ratio (95% CI)</td>
<td>0.626 (0.488, 0.803)</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.0042[a]</td>
<td></td>
</tr>
<tr>
<td>IDFS at 36 months (% 95% CI)</td>
<td>86.1 (82.8, 88.8)</td>
<td>79.0 (75.3, 82.3)</td>
</tr>
</tbody>
</table>

*Abbreviation: CI = confidence interval

[a] This p-value is from the pre-specified final IDFS analysis for cohort 1 patients with Ki-67 expression (Ki-67 pharmDx Score ≥ 20%)

**Figure 48:** Kaplan-Meier Curves of Invasive Disease-Free Survival comparing Verzenio plus Tamoxifen or an Aromatase Inhibitor versus Tamoxifen or an Aromatase Inhibitor in Cohort 1 Patients with Ki-67 expression (Ki-67 pharmDx Score ≥ 20%) (monarchE).
References


