Ki-67 IHC MIB-1 pharmDx (Dako Omnis)

GE020

60 tests for use with Dako Omnis

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1. 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.

2. Summary and Explanation

The Ki-67 antigen is a nuclear protein, which is defined by its reactivity with monoclonal antibody from the Ki-67 clone (1). Two isoforms of 345 and 395 kDa have been identified (2). The Ki-67 antigen is preferentially expressed during all active phases of the cell cycle (G1, S, G2 and M-phases), but it is downregulated in resting cells (G0-phase) (1, 3). 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. The antigen is rapidly degraded as the cell enters the non-proliferative state (4), and there appears to be no expression of Ki-67 during DNA repair processes (5). Ki-67 expression has a prognostic role in common clinical subgroups of patients with human epidermal receptor 2-negative (HER2-), hormone receptor-positive (HR+) early breast cancer (6, 7).

3. Principle of Procedure

Ki-67 IHC MIB-1 pharmDx (Dako Omnis) contains optimized reagents and the protocol required to complete an IHC staining procedure of FFPE specimens using the Dako Omnis. Following incubation with the primary monoclonal antibody to Ki-67 or the Negative Control Reagent (NCR), 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. Ki-67 IHC MIB-1 pharmDx (Dako Omnis) (Code GE020) is applicable for automated staining using the Dako Omnis instrument. Please consult the Dako Omnis User Guide(s) for detailed instructions on loading and unloading of slides, reagents, bulk fluids, and waste.

4. Materials Provided

The materials listed below are sufficient for 60 tests: 60 slides incubated with primary antibody to Ki-67 protein and 60 slides incubated with the Negative Control Reagent (NCR). GE020 includes 12 mL of Primary Antibody (approximately 0.46 µg/mL protein concentration). GE020 has been optimized for use with the Dako Omnis instrument. Please refer to the Dako Omnis Basic User Guide for further information.

Quantity	Description
1 x 12 mL	**Primary Antibody: Monoclonal Mouse Anti-Human Ki-67, Clone MIB-1** **MONOCLONAL MOUSE ANTI-HUMAN Ki-67 CLONE MIB-1 (Dako Omnis)** Monoclonal mouse anti-human Ki-67 in a buffered solution, containing stabilizing protein, and 0.015 mol/L sodium azide.
1 x 12 mL	**Negative Control Reagent** **NEGATIVE CONTROL REAGENT (Dako Omnis)** Monoclonal mouse IgG antibody in a buffered solution, containing stabilizing protein, and 0.015 mol/L sodium azide.

NOTE: Primary antibody Ki-67 IHC MIB-1 pharmDx and Ki-67 NCR are formulated specifically for use with this test. For the test to perform as specified, no substitutions of Ki-67 primary antibody can be made. If including NCR with the test, no substitution of the NCR should be made.

5. Materials Required, but Not Supplied

Dako Omnis (Code GI100)
EnVision FLEX, High pH (Dako Omnis) (Code GV800) or EnVision FLEX Mini Kit, High pH (Dako Omnis) (Code GV823), containing:
- EnVision FLEX DAB+ Chromogen (Dako Omnis)
- EnVision FLEX Peroxidase-Blocking Reagent (Dako Omnis)
- EnVision FLEX Substrate Buffer (Dako Omnis)
- EnVision FLEX Target Retrieval Solution, High pH (50x) (Dako Omnis)*
- EnVision FLEX Visualization Reagent (Dako Omnis)

EnVision FLEX, Target Retrieval Solution Low pH (50x) (Dako Omnis), (Code GV805)
Hematoxylin (Dako Omnis) (Code GC808) or equivalent
Wash Buffer (20x) (Dako Omnis) (Code GC807)
Clearify™ clearing agent (GC810)
Dako Omnis Sulfuric Acid, 0.3 M (GC203)
Distilled or de-ionized water (reagent-grade water) **
Ethanol, absolute
Xylene, toluene, or xylene substitutes
Materials for permanent mounting
Timer
Microscope slides: Dako FLEX IHC Microscope Slides (Code K8020) or SuperFrost Plus slides
Bright field microscope (4-40x objective magnification)
Positive and negative tissue to use as process controls (see Quality Control section 11)

***NOTE: Use EnVision FLEX Target Retrieval Solution, Low pH (50x) (Dako Omnis), Code GV805, for heat-induced epitope retrieval (HIER) with Ki-67 IHC MIB-1 pharmDx (Dako Omnis). The color of the EnVision FLEX Target Retrieval Solution, Low pH (50x) (Dako Omnis), Code GV805 is red.**

****NOTE: Not all sources of distilled or de-ionized water may be of sufficient quality for IHC reagent preparation. Agilent recommends reagent-grade distilled or de-ionized water [corresponding to Clinical Laboratory Reagent Water (CLRW) standard as specified by CLSI (8), or water similar in quality to be used for reagent preparation.**

6. Precautions

1. For in vitro diagnostic use.
2. For professional users.
3. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, NaN_3 may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing (9).
4. Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection, and disposed of with proper precautions (10).
5. Incubation times, temperatures, or methods other than those specified may give erroneous results.
6. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.
7. Paraffin residuals may lead to false negative results.
8. Wear appropriate Personal Protective Equipment (PPE) to avoid contact with eyes and skin.
9. Unused solution should be disposed of according to local, State and Federal regulations.
10. As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper work procedures, the dangerous properties of the product and the necessary safety instructions. Please refer to Safety Data Sheet (SDS) for additional information.
11. Safety Data Sheets are available on www.agilent.com or on request.
12. Lack of adherence to the maintenance schedule for the Dako Omnis instrument may give erroneous results. Refer to Dako Omnis Basic and Advanced User Guides for additional information and for additional instrument-related precautions.

7. Storage

Store components of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) in the original container in the dark at 2-8 °C when not in use on Dako Omnis. During storage, the cap on each vial should be closed.

Do not use the reagent after the expiration date printed on the reagent vial label. If the reagents are stored under any conditions other than those specified in the instructions for use, they must be validated by the user.

Onboard reagent stability for Ki-67 IHC MIB-1 pharmDx (Dako Omnis) has been validated to 375 hours. After staining completion, the reagents should be removed from Dako Omnis, caps replaced securely on the vials, and stored in the dark at 2-8 °C. For onboard stability of all ancillary components including diluted working solutions of Wash Buffer and EnVision FLEX Target Retrieval Solution, refer to respective Instructions for Use. Onboard time of reagents is tracked by the Dako Omnis software; refer to Dako Omnis Basic and Advanced User Guides for details.

NOTE: There are no obvious visual signs to indicate incorrect product storage or handling of this product during the product's shelf life. Positive and negative controls should be run simultaneously with patient tissue, preferably on the same slide, to monitor product performance during the product's shelf life. If a problem is suspected with the antibody during the shelf life that cannot be explained by

incorrect product storage or handling, or other variations in laboratory procedures, contact Agilent Technical Support. Refer to Troubleshooting (section 16) and Quality Control (section11) for more information.

8. Specimen Preparation

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

8.1 Paraffin-Embedded Tissue

Formalin-fixed, paraffin-embedded (FFPE) tissues are suitable for use. Alternative fixatives have not been validated and may give erroneous results. Fixation time for 6-72 hours in 10% neutral buffered formalin (NBF) is recommended. Fixation time of < 6 hours may result in variable Ki-67 detection. Ischemia times should be kept at one hour or less. Specimens should be blocked into a thickness of 3 or 4 mm, fixed in formalin, and dehydrated and cleared in a series of alcohols and xylene, followed by infiltration with melted paraffin. The paraffin temperature should not exceed 60°C.

8.2 Tissue Sections

FFPE tissue specimens should be cut into sections of 4-5 µm. After sectioning, tissues should be mounted on Dako FLEX IHC Microscope Slides (Code K8020) or SuperFrost Plus microscope slides, and then placed in a 58 ± 2°C calibrated oven for 1 hour.

To preserve antigenicity, tissue sections mounted on slides should be stained within 2 months of sectioning when stored in the dark at 2-8 °C (preferred) or at room temperature up to 25 °C. Slide storage and handling conditions should not exceed 25 °C at any point post-mounting to ensure tissue integrity and antigenicity.

The tissue specimens must be mounted on the slide within the defined slide staining area. Please consult the Dako Omnis Basic User Guide for dimensions of slide staining area.

9. Reagent Preparation

The user should adhere to appropriate PPE requirements and become familiar with all components prior to use (see Precautions section 6).

EnVision FLEX Target Retrieval Solution, Low pH (50x) (GV805) and Wash Buffer (20x) (GC807) must be diluted to 1x concentration according to their Instructions for Use. The color of the FLEX Target Retrieval Solution, Low pH (50x) is red.

The pH of 1x Target Retrieval Solution must be 6.1 ± 0.2. 1x Target Retrieval Solution pH below 5.9 may give erroneous results. Do not adjust pH of 1x Target Retrieval Solution after preparation under any circumstance. If a problem is suspected with the Target Retrieval Solution pH, please refer to the Troubleshooting (section 16) for more information.

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.

10. Staining Procedure

10.1 Procedural Notes

The user should read these instructions carefully and become familiar with all the components and the instrumentation prior to use (see Precautions section 6).

The automated staining procedure for Ki-67 IHC MIB-1 pharmDx (Dako Omnis) on Dako Omnis includes deparaffinization of tissue sections, target retrieval, and staining. The slides are unloaded in the wet unloading station. All protocol steps are pre-programmed into the Dako Omnis software. The **"Ki-67 IHC pharmDx"** protocol is used with the Primary Antibody Ki-67 IHC MIB-1 pharmDx, and the **"Ki-67 IHC NCR pharmDx"** protocol is used with the isotype-matched Negative Control Reagent. Please refer to the Dako Omnis Basic User Guide for further information on loading slides and reagents and for instructions on how to display and print the parameters for the automated procedures.

The Ki-67 reagents and instructions have been designed for optimal performance. Further dilution of the antibody or alteration of incubation temperatures may give erroneous or discordant results. Differences in tissue processing and technical procedures in the user's laboratory may invalidate the assay results.

NOTE: Laboratories located at high elevations should determine the best method of maintaining the required temperature (95-99 °C) during heat-induced epitope retrieval. Any adjustments required to address elevation concerns must be validated by the user. See the Dako Omnis Advanced User Guide for further information on creating new protocols.

10.2 Pre-staining procedure

1. Choose the **Ki-67 IHC pharmDx** or **Ki-67 IHC NCR pharmDx** protocol to be applied for each slide from the Dako Link Omnis Workstation software.
2. Ensure the Dako Link Omnis Workstation software is configured to print slide labels with the protocol name displayed.
3. Print slide labels and attach them to the glass slides.
4. Place the slides in the Slide Rack. A Slide Rack can hold from one to five slides.
5. Ensure that the bulk bottles with fluids are onboard and registered by the Dako Omnis instrument. Bulk bottle fluids:
    a. Clearify clearing agent (Code GC810)

b. EnVision FLEX Target Retrieval Solution Low pH, (Code GV805), **diluted to 1x working concentration with distilled or de-ionized water**
c. Wash buffer (Code GC807) **diluted to 1x working concentration with distilled or de-ionized water**

6. Ensure that all flip top vial caps are open and locked in place before loading all required reagents in the Reagent Storage Module:
    a. Ki-67, Code GE020
    b. Negative Control Reagent, Code GE020
    c. EnVision FLEX Peroxidase-Blocking Reagent (Dako Omnis), Code GV800 or GV823
    d. EnVision FLEX Visualization Reagent (Dako Omnis), Code GV800 or GV823
    e. EnVision FLEX Substrate Buffer (Dako Omnis), Code GV800 or GV823
    f. EnVision FLEX DAB+ Chromogen (Dako Omnis), Code GV800 or GV823
    g. Optional: Hematoxylin (Dako Omnis) Code GC808 or equivalent
    h. Sulfuric Acid, 0.3 M, Code GC203
7. Load the Slide Rack onto Dako Omnis.
8. Follow the instructions on the Touch Screen and tap "Done" to initiate the staining procedure.
9. Ensure the slide unloading station is filled with distilled or de-ionized water to prevent slides from drying.

NOTE: When using the overnight staining feature (delayed start) slides must be removed from the Unloading drawer in the morning the staining has been completed.

NOTE: The Ki-67 IHC pharmDx and Ki-67 IHC NCR pharmDx protocols on the Dako Omnis instrument can be monitored on the Dako Link Omnis Workstation.

Table 1 provides an overview of the main steps in the locked protocol. The protocol cannot be edited with the exception of the hematoxylin counterstain step (see Counterstain section 10.3)

Table 1: Outline of "Ki-67 IHC pharmDx" and "Ki-67 IHC NCR pharmDx" staining protocols

Staining Protocol: Ki-67 IHC MIB-1 pharmDx (Dako Omnis)	
Protocol Step	Protocol Detail
Dewax: Clearify Clearing Agent	10 seconds top, 1 minute bottom
Target retrieval: EnV FLEX TRS, Low pH	30 minutes; 97±2 ˚C
Antibody (Primary) or NCR	20 minutes
Endogenous enzyme block	3 minutes
Labeled polymer	20 minutes
Substrate chromogen	5 minutes
Counterstain	3 minutes

10.3 Counterstain

Slides should be counterstained with Dako Hematoxylin (Code GC808). The Ki-67 IHC pharmDx and Ki-67 IHC NCR pharmDx protocols on Dako Omnis include a counterstaining step that is pre-programmed for 3 minutes with Hematoxylin (Dako Omnis) (Code GC808). Slides are ready for mounting when removed from the Dako Omnis unloading station. The counterstaining step is editable when a copy of the protocol is created. If counterstains other than the recommended Dako Hematoxylin (Code GC808) are preferred and adjustments are made to the protocol, they must be validated by the user. See the Dako Omnis Advanced User Guide for further information on creating new protocols.

10.4 Mounting

After staining onboard Dako Omnis, the sections must be dehydrated, cleared, and mounted using non-aqueous, permanent mounting methods.

10.5 Stained Slide Storage

Some fading of stained slides may occur, depending on several factors including, but not limited to, counterstaining, mounting materials and methods, and slide storage conditions. To minimize fading, store stained slides in the dark at room temperature (20-25 °C).

11. Quality Control

Ki-67 IHC MIB-1 pharmDx (Dako Omnis) has been quality-controlled by immunohistochemistry using the required reagents and staining procedures outlined in Sections 9 and 10. Deviations in the recommended procedures for tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results. In the USA, consult the quality control guidelines of the College of American Pathologists (CAP) Accreditation Program for Immunohistochemistry. Other countries should follow local guidelines as applicable. For additional information, see Dako Educational Guide, "Immunohistochemical Staining Methods", and CLSI Quality Assurance for Design Control and Implementation of Immunohistochemistry Assays; Approved Guideline (11, 8).

11.1 System-Level Controls

Positive and negative control tissues (lab-supplied) should be run for each staining procedure. These quality controls are intended to ensure the validity of the staining procedure, including reagents, tissue processing and instrument performance. It is recommended that control tissues be stained on the same slide as the patient tissue. Well-preserved normal tonsil or biopsy/surgical specimens of breast carcinoma

are suitable for use as control tissue. The positive control should be a tissue with positive biomarker expression. The negative control should be a tissue or tissue element with no biomarker expression. When using tonsil as a positive control tissue, negative control elements within the specimen may serve as the negative control tissue. The use of internal negative control elements in alternate positive control tissue types must be verified by the user. Control tissues should be fixed in the same way as the patient tissue. If controls are not fixed in the same way as the patient tissue, the control may only be used as a staining control for reagents and instrument performance. Refer to Table 5 for more information on quality controls including H&E stained patient tissue and lab-supplied positive and negative control tissues.

11.2 Assay Verification

Prior to initial use of a staining system in a diagnostic procedure, the user should verify the assay's performance by testing it on a series of lab-supplied tissues with known IHC performance characteristics representing known positive and negative tissues. Refer to the quality control procedures outlined in Quality Control (section 11) as well as to the quality control requirements of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry and/or CLSI Quality Assurance for Design Control and Implementation of Immunohistochemistry Assays; Approved Guideline (8) for additional information. Troubleshooting options for potential problems, their causes and suggested corrective actions are outlined in Table 11.

11.3 Negative Control Reagent (Discretionary)

Negative Control Reagent may be used in place of the primary antibody with a section of each patient tissue to evaluate non-specific staining and allow better interpretation of specific staining at the antigen site. If slides are stained with NCR, the NCR is applied in place of the primary antibody. Absence of cell nuclear staining verifies the specific labeling of the target antigen by the primary antibody. Use the "Ki-67 IHC NCR pharmDx" Dako Omnis protocol for slides stained with the Negative Control Reagent.

A study conducted by Agilent demonstrated that non-specific nuclear staining was absent in 8,012 out of 8,127 NCR stained specimens. This data is provided as an aid to the laboratory director to inform the decision to include or omit the use of NCR in their laboratory.

12. Staining and Scoring Interpretation

A hematoxylin and eosin (H&E) stained section adjacent to the IHC stained section is recommended for the evaluation of an acceptable sample. All viable invasive tumor areas on the entire slide must be evaluated and included in the Ki-67 scoring assessment. 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 to confirm:

1. The histological diagnosis of invasive breast cancer.
2. The specimen contains a minimum of 200 viable tumor cells in the invasive tumor component to determine the percentage of positive cells.
3. The specimen has been properly fixed and prepared for IHC analysis. Only well-preserved and well-stained areas of the specimen should be used to determine the percentage of positive tumor cells.
4. Tumor invasiveness. Only the invasive cancer component should be scored. Carcinoma in situ should not be scored.

Slide evaluation should be performed by a pathologist using a bright field microscope. For evaluation of the IHC staining and scoring, an objective of 10-40x magnification is appropriate. Convincing nuclear staining of tumor cells with 1+ intensity or higher should be included in the scoring. Cells that exhibit a "grey" coloring in the nucleus are considered as staining at an intensity <1+ and 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. Low intensity (1+) nuclear positivity should be evaluated using a high power (i.e., 40x) objective and positivity is defined by the following rules:

- Signal must be unequivocally brown
- The staining must correspond to a nucleus
- The staining must cover the whole chromatin distribution within the nucleus
- The staining must correspond to a non-apoptotic cell

Tumor areas and artifacts which should not be scored include:

- Necrotic areas
- In situ carcinoma areas
- Edge effects
- Fixation and processing artifacts

Ki-67 protein expression is determined by assessing the percentage of viable tumor cells showing convincing nuclear staining at intensities 1+ and higher.

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

Tables 2 and 3 provide detail about which tissues elements are included/excluded in the numerator and denominator, respectively, when determining the Ki-67 pharmDx Score.

Table 2. Ki-67 pharmDx Score numerator inclusion/exclusion criteria

Tissue Elements	Included in the Numerator	Excluded from the Numerator
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 3: Ki-67 pharmDx Score denominator inclusion/exclusion criteria

Tissue Elements	Included in the Denominator	Excluded from the Denominator
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

For each staining procedure, slides should be examined in the order presented in Table 5, to determine the validity of the staining procedure and enable assessment of patient tissue staining.
The specimen should be considered to have Ki-67 expression if Ki-67 pharmDx Score is ≥ 20%.
The specimen should be considered to have Ki-67 expression if ≥ 20% of the viable tumor cells in the invasive cancer component exhibit complete nuclear staining at any intensity 1+ or higher (Table 4).

Table 4. Ki-67 expression level based on Ki-67 pharmDx Score

	Ki-67 pharmDx Score	
Ki-67 Expression Level	Ki-67 pharmDx Score < 20%	Ki-67 pharmDx Score ≥ 20%

For additional guidance on scoring, please refer to the Ki-67 IHC MIB-1 pharmDx (Dako Omnis) Interpretation Manual.

13. Tissue Evaluation:

The following table provides the order of tissue evaluation for interpretation of Ki-67 IHC MIB-1 pharmDx (Dako Omnis)

Table 5: Recommended order of tissue evaluation

Specimens	Rationale	Requirements
1. Patient tissue stained with H&E	A hematoxylin and eosin (H&E) stain of the patient tissue is evaluated first to assess tissue histology and preservation quality.	The H&E and Ki-67 IHC MIB-1 pharmDx (Dako Omnis) antibody stain should be performed on serial sections from the same paraffin block of the specimen. Tissue specimens should be undamaged, well preserved, and should confirm tumor indication.
2. Positive control tissue stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) primary antibody	The positive control tissue stained with Ki-67 Primary Antibody should be examined next. Known positive control tissue should only be utilized for monitoring the correct performance of processed tissue and test reagents,	Controls should be normal tonsil or biopsy/surgical specimens of breast carcinoma, fixed, processed, and embedded as soon as possible in the same manner as the patient tissue(s).

Specimens	Rationale	Requirements
(Lab-supplied)	NOT as an aid in formulating a specific diagnosis of patient tissue.	Use well-preserved specimens for interpretation of staining results as necrotic or degenerated cells often stain non-specifically. The tissue selected for use as the positive tissue controls should include weak to moderate positive staining when stained with Ki-67 to aid in detection of subtle changes in assay sensitivity. Positive control tissue should be included in each staining procedure. On-slide tissue controls are recommended. Tissue sections stained with Ki-67 Primary Antibody: 1. Tonsil: 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. 2. Breast carcinoma: Presence of brown nuclear staining should be observed in invasive tumor cells. Cells labeled by the antibody display a nuclear staining pattern except in mitotic cells, where the chromosomes and the cytoplasm are labeled. Non-specific staining must be < 1+. If the positive control tissues fail to demonstrate appropriate positive staining, then results with the patient tissue should be considered invalid.
3.***Optional:*** Positive control tissue stained with Negative Control Reagent (NCR) (Lab-supplied)	Negative Control Reagent may be used to stain the positive control tissue specimen if needed for troubleshooting purposes.	**Tissue sections stained with Negative Control Reagent:** No nuclear staining. Non-specific staining should be < 1+.
4. Negative control tissue stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) primary antibody (Lab-supplied)	The negative control tissue stained with Ki-67 primary antibody should be examined next to verify the labeling specificity of the target antigen by the primary antibody. **Note:** When using tonsil as a positive control tissue, negative control elements within the specimen may serve as the negative control tissue. The use of internal negative control elements in alternate positive control tissue types must be verified by the user.	Controls should be normal tonsil or biopsy/surgical specimens of breast carcinoma, fixed, processed, and embedded as soon as possible in the same manner as the patient tissue(s). Use well preserved specimens for interpretation of staining results as necrotic or degenerated cells often stain non-specifically. Negative control tissue should be included in each staining procedure. On-slide tissue controls are recommended. Tissue sections stained with Ki-67 Primary Antibody: 1. Tonsil: The superficial layer and the majority of cells in the basal squamous epithelial layer should be negative. This internal negative control element eliminates the need for a separate negative control tissue. Non-specific staining must be < 1+. 2. Breast carcinoma: No nuclear staining should be observed in invasive tumor cells. Non-specific staining must be < 1+.

Specimens	Rationale	Requirements
		If the negative control tissues fail to demonstrate appropriate staining, results with the patient tissue should be considered invalid.
5. Optional: Negative control tissue stained with Negative Control Reagent (NCR) (Lab-supplied)	Negative Control Reagent may be used to stain the negative control tissue specimen if needed for troubleshooting purposes.	**Tissue sections stained with Negative Control Reagent**: No nuclear staining. Non-specific staining should be < 1+.
6.***Discretionary***: Patient tissue stained using the Negative Control Reagent (NCR)	Examine patient tissue stained with the Negative Control Reagent. Negative Control Reagent is used in place of the primary antibody and aids in interpretation of specific staining at the antigen site.	Tissue stained with NCR should exhibit no nuclear staining in tumor cells. Non-specific staining should be < 1+ average staining across the specimen. If weak staining is present in tumor nuclei, it should be used as a baseline to evaluate the Ki-67 antibody slide. Staining at the same intensity or lower that may occur in the Ki-67 antibody slide should be disregarded upon interpretation. If patient tissue stained with NCR fails to demonstrate appropriate staining, the corresponding patient tissue stained with primary antibody is considered non-evaluable and the patient tissue must be retested.
7. Patient tissue stained using the Ki-67 IHC MIB-1 pharmDx (Dako Omnis) primary antibody	Examine the patient tissue stained with the Ki-67antibody last to assess Ki-67 protein status. Refer to Summary and Explanation (section 2), Limitations (section 14), and Performance Evaluation (section 15) for specific information regarding Ki-67 IHC MIB-1 pharmDx (Dako Omnis) immunoreactivity.	All viable tumor cells in the invasive cancer component should be evaluated for Ki-67 and included in the Ki-67 scoring assessment. Positive staining intensity should be assessed within the context of any non-specific staining observed in the staining procedure. NCR is recommended for this assessment if non-specific staining is observed. As with any immunohistochemical test, a result showing no staining means that the antigen was not detected, not necessarily that the antigen was absent in the cells/tissue assayed. Refer to Interpretation Manual for details on patient tissue evaluation.

14. Limitations

14.1 General Limitations

1. Immunohistochemistry is a multi-step process that requires specialized training in the selection of the appropriate reagents; tissue selection, fixation, and processing; preparation of the immunohistochemistry slide; and interpretation of the staining results.
2. Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false results. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.
3. Excessive or incomplete counterstaining may compromise proper interpretation of results.
4. Staining artifacts caused by non-specific DAB chromogen particles require re-test of the stained slides if the artifacts impair the interpretation of Ki-67 staining. Background staining may be evaluated by comparing tissue stained with primary antibody to tissue stained with Negative Control Reagent.
5. The clinical interpretation of any positive staining or its absence must be evaluated within the context of clinical presentation, morphology, and other histopathological criteria. The clinical interpretation of any staining, or its absence, must be complemented by morphological studies and proper controls. It is the responsibility of a qualified pathologist, who is familiar with the antibodies, reagents and methods used, to interpret the stained preparation. Staining must be performed in a certified, licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
6. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit non-specific staining with horseradish peroxidase (12).
7. Reagents may demonstrate unexpected reactions in previously untested tissue types. The possibility of unexpected reactions even in tested tissue types cannot be eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues (13). Contact Agilent Technical Support with documented unexpected reactions.
8. False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (cytochrome C) (11).
9. The reagents and instructions supplied for this assay have been designed for optimal performance. Further dilution of the reagents or alteration of incubation times or temperatures may give erroneous or discordant results. Any protocol adjustments made must be validated by the user.

10. Slides flagged in the slide log on the Dako Omnis Workstation should be investigated by qualified personnel. See Dako Omnis Basic and Advanced User Guides.
11. Canceled slides indicate a significant issue occurred during staining and should not be used. The specimen will require re-staining. Refer to Dako Omnis Advanced User Guide for further details.

14.2 Product-Specific Limitations

1. 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. Refer to Storage (section 7) and Specimen Preparation (section 8).
2. 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.
3. Use of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) on fine needle aspirates has not been validated.
4. Membranous/cytoplasmic staining was reported in a study of 322 invasive ductal breast carcinomas, and membranous/cytoplasmic Ki-67 was found to associate with grade 3 tumors, ER negativity and HER2 amplification (14).

15. Performance Evaluation

15.1 Non-Clinical Performance Evaluation: Normal and Neoplastic Tissues

Normal tissues: Table 6 summarizes monoclonal mouse anti-Ki-67, Clone MIB-1, immunoreactivity on the recommended panel of normal tissues. Nuclear staining was observed in a subset of tissues. All tissues were formalin-fixed and paraffin-embedded and stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) according to the instructions in this package insert. There were no unexpected results observed in cell types or tissue types tested. The observed staining was consistent with the reported literature for Ki-67 IHC expression in normal tissues (15, 16).

Table 6: Summary of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) normal tissue reactivity

Tissue Type (# tested)	Positive Cell Staining: Tissue Elements*	Non-specific Staining*
Adrenal (3)	2/3 Scattered adrenal cortex cells	0/3
Bone marrow (3)	2/3 Marrow cells	0/3
Breast (3)	2/3 subset ductal epithelial cells 2/3 rare myoepithelial cells	0/3
Cerebellum (3)	0/3	0/3
Cerebrum (3)	1/3 Rare oligodendrocytes	0/3
Cervix (3)	2/3 Rare epithelial cells 1/3 parabasal squamous cells	0/3
Colon (3)	3/3 Crypt epithelial cells	0/3
Esophagus (3)	3/3 Parabasal squamous cells	0/3
Kidney (3)	3/3 Rare tubule cells	0/3
Liver (3)	3/3 Rare hepatocytes	0/3
Lung (3)	3/3 Rare type 1 alveolar cells 1/3 Pulmonary macrophages	0/3
Mesothelial cells (3)	1/3 Rare mesothelial cells	0/3
Muscle, cardiac (3)	1/3 Few cardiac myocytes	0/3
Muscle, skeletal (3)	0/3	0/3
Nerve, peripheral (3)	0/3	0/3
Ovary (3)	2/3 Follicle cyst lining cells 1/3 Rare ovarian stromal cells	0/3
Pancreas (3)	3/3 Rare acinar cells	0/3
Parathyroid (3)	3/3 Rare endocrine cells/Chief cells	0/3
Pituitary (3)	1/3 Lymphocytes 2/3 Pituicytes	0/3
Prostate (3)	3/3 Rare epithelial cells and stromal cells	0/3
Salivary gland (3)	1/3 Rare acinar epithelial cells	0/3
Skin (3)	3/3 Suprabasal squamous cells	0/3
Small intestine (3)	2/3 Crypt epithelium	0/3
Spleen (3)	3/3 Few red pulp cells 1/3 White pulp cells	0/3
Stomach (3)	3/3 Gastric epithelial cells	0/3
Testis (3)	3/3 Spermatogonia	0/3
Thymus (3)	3/3 Thymic cortex	0/3
Thyroid (3)	0/3	2/3 Scattered interspersed inflammatory cells
Tonsil (3)	3/3 Squamous parabasal cells 3/3 Germinal centers 3/3 interfollicular cells	0/3
Urinary bladder (3)	3/3 Urothelial cells	2/3 Interspersed inflammatory cells
Uterus (3)	3/3 Endometrial epithelium and stroma	0/3

*The numbers in each cell indicate the number of tissues showing staining out of the total number of tissues of that type tested.

Neoplastic tissues: Table 7 summarizes monoclonal mouse anti-Ki-67, Clone MIB-1, immunoreactivity on a panel of neoplastic tissues. Nuclear staining was observed in the majority of tumor types evaluated. All tissues were formalin-fixed and paraffin-embedded and stained with Ki-67 IHC MIB-1 pharmDx (Dako Omnis) according to the instructions in this package insert. There were no unexpected results observed in the tumor specimens tested.

Table 7: Ki-67 IHC MIB-1 pharmDx (Dako Omnis) neoplastic tissue reactivity grouped by tumor type and location and using the most common staining result for each tumor type (N=71).

Tumor Type by System	Number of Positive Cases with Any Staining (out of 3 replicates)*	Tumor Type by System	Number of Positive Cases with Any Staining (out of 3 replicates)*
Gastrointestinal tract		**Lung and mediastinum**	
Colon mucinous adenocarcinoma	1/3	Lung bronchioloalveolar carcinoma	3/3
Gastric adenocarcinoma	2/3	Lung squamous carcinoma	3/3
Gastric adenocarcinoma metastasis	2/3	Lung adenocarcinoma #1	1/3
Colon adenocarcinoma in liver	0/3	Lung adenocarcinoma #2	3/3
Gastrointestinal stromal tumor (GIST)	2/3	Lung carcinoma, NOS	3/3
Pancreatic adenocarcinoma	2/3	Fibrous tumor of pleura	0/3
Hepatoma (hepatocellular carcinoma)	3/3	Thymoma	3/3
Cholangiocarcinoma	3/3		
		Female reproductive tract	
Head and neck		Papillary serous carcinoma	3/3
Squamous carcinoma of the ear	3/3	Endometrial carcinoma	0/3
		Ovarian mucinous adenocarcinoma	0/3
Urinary tract		Well differentiated serous carcinoma	0/3
Kidney transitional cell carcinoma	3/3	Metastatic ovarian carcinoma, NOS	3/3
Bladder transitional cell carcinoma #1	3/3	Endometrial stromal sarcoma	3/3
Bladder transitional cell carcinoma #2	3/3	Ovary granulosa cell tumor	3/3
Papillary renal cell carcinoma	1/3	Squamous carcinoma, cervix	3/3
Renal cell carcinoma, NOS #1	0/3	Ovarian Dysgerminoma	3/3
Renal cell carcinoma, NOS #2	1/3		
Male reproductive tract		**Endocrine system (including neuroendocrine tumors)**	
Testicular yolk sac tumor	3/3	Islet cell tumor of Pancreas	2/3
Testicular embryonal carcinoma	3/3	Pancreatic glucagonoma	3/3
Prostate adenocarcinoma (metastatic)	3/3	Thymic carcinoid Tumor #1	3/3
Prostate adenocarcinoma	1/3	Thymic carcinoid Tumor #2	3/3
		Pheochromocytoma	3/3
Breast		Paraganglioma	0/3
Breast carcinoma, lobular #1	0/3	Thyroid medullary carcinoma	3/3
Breast carcinoma, lobular #2	1/3	Thyroid papillary carcinoma	1/3
Breast carcinoma**,** NOS #1	0/3	Thyroid follicular adenoma	3/3
Breast carcinoma**,** NOS #2	3/3		
Breast carcinoma**,** NOS #3	3/3	**Soft tissue and bone**	
Breast carcinoma**,** NOS #4	1/3	Pleomorphic rhabdomyosarcoma	3/3
Breast carcinoma, metastasis in lymph node	3/3	Spindle cell rhabdomyosarcoma	0/3
		Ewing sarcoma	0/3
Hematopoietic system		Primitive neuroendocrine tumor in scrotum	0/3
Splenic lymphoma	3/3	Leiomyosarcoma	3/3
Lymphoma of cecum	3/3	Round cell liposarcoma	3/3
		Synovial sarcoma	0/3
Skin		Malignant fibrous Histocytoma	3/3
Melanoma #1	3/3	Extraskeletal myxoid chondrosarcoma	0/3
Melanoma #2	3/3		
Melanoma #3	3/3		
Merkel cell tumor	3/3		
Basal cell carcinoma, skin	0/3		
Nervous system			
Glial tumor**,** NOS	3/3		
Meningioma	3/3		
Ganglioneuroma	0/3		
Schwannoma	3/3		

NOS = not otherwise specified

*The numbers in each cell indicate the number of tissues showing staining out of the total number of tissues of that type tested.

15.2 Non-Clinical Performance Evaluation: Breast Carcinoma

Analytical Sensitivity:
Analytical sensitivity of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) was tested on 148 unique specimens of FFPE breast carcinoma specimens using a manufactured production lot. Assessment of Ki-67 expression demonstrated staining across a range of 0-75% positive tumor cells and 0-3+ staining intensity.

Precision:
The precision of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) was evaluated at Agilent. Negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with two-sided 95% confidence intervals using the bootstrap method.

Table 8: Precision of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) tested at one site

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-day	≥ 20%	Each of 32 breast carcinoma specimens (16 Ki-67-negative and 16 Ki-67-positive) representing a range of Ki-67 expression were tested on a single Dako Omnis instrument over 5 non-consecutive days.	NPA 98.3% (96.2,100.0%) PPA 99.2% (97.5,100.0%) OA 98.8% (97.5, 99.8%)
Inter-instrument	≥ 20%	Each of 32 breast carcinoma specimens (16 Ki-67-negative and 16 Ki-67-positive) representing a range of Ki-67 expression were tested on each of three Dako Omnis instruments.	NPA 98.8% (96.7,100.0%) PPA 97.5% (94.6,100.0%) OA 98.1% (96.5, 99.6%)
Inter-lot (Ki-67 pharmDx IHC)	≥ 20%	Each of 40 breast carcinoma specimens (20 negative and 20 positive) representing a range of Ki-67 expression were tested using three unique lots of Ki-67 pharmDx reagents.	NPA 98.9% (97.2,100.0%) PPA 97.2% (92.8,100.0%) OA 98.1% (95.8, 99.7%)
Inter-lot (accessory reagents)	≥ 20%	Each of 32 breast carcinoma specimens (17 negative and 15 positive) representing a range of Ki-67 expression were tested using three unique lots of accessory reagents.	NPA 95.1% (88.2,100.0%) PPA 100.0% (95.9,100.0%) OA 97.4% (93.8,100.0%)
Intra-instrument/Intra-rack/Intra-day (Repeatability)	≥ 20%	Each of 32 breast carcinoma specimens (16 Ki-67-negative and 16 Ki-67-positive) representing a range of Ki-67 expression were tested within one rack on three different Dako Omnis instruments. Pairwise comparisons were only performed on slides that were stained within the same rack.	NPA 98.8% (96.7,100.0%) PPA 97.5% (94.6,100.0%) OA 98.1% (96.2, 99.6%)
Inter-observer	≥ 20%	One set of 60 stained specimens (32 Ki-67 negative and 28 Ki-67 positive) representing a range of Ki-67 expression was evaluated three times by three different trained and certified pathologists. Inter-observer analysis was performed between observers on a total of 537 comparisons.	NPA 98.9% (97.2,100.0%) PPA 95.2% (91.7, 98.0%) OA 97.2% (95.4, 98.7%)
Intra-observer	≥ 20%	One set of 60 stained specimens (32 Ki-67 negative and 28 Ki-67 positive) representing a range of Ki-67 expression was evaluated three times by the same trained and certified pathologist. Intra-observer analysis was performed on a total of 537 comparisons.	NPA 99.3% (98.3,100.0%) PPA 96.8% (94.4, 98.8%) OA 98.1% (96.8, 99.3%)

NPA= Negative Percent Agreement; PPA= Positive Percent Agreement; OA=Overall Percent Agreement

External Reproducibility:
The reproducibility of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) was evaluated at three external testing sites. Negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with two-sided 95% confidence intervals using the bootstrap method for the ≥ 20% cutoff.

Table 9: Reproducibility of Ki-67 IHC MIB-1 pharmDx (Dako Omnis) tested at three external sites

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	≥ 20%	Each of 30 breast carcinoma specimens (15 Ki-67 negative and 15 Ki-67 positive) representing a range of Ki-67 expression was tested on 5 non-consecutive days. Inter-site analysis was performed between three sites on a total of 450 comparisons.	NPA 94.7% (88.4,100.0%) PPA 100.0% (98.3,100.0%) OA 97.3% (94.2,100.0%)
Intra-site	≥ 20%	Each of 30 breast carcinoma specimens (15 Ki-67 negative and 15 Ki-67 positive) representing a range of Ki-67 expression was tested on 5 non-consecutive days at each of three study sites. Intra-site analysis was performed for three sites on a total of 450 comparisons.	NPA 100.0% (98.2,100.0%) PPA 98.8% (96.9,100.0%) OA 99.3% (98.2,100.0%)
Inter-observer	≥ 20%	One set of 60 stained specimens (29 Ki-67 negative and 31 Ki-67 positive) representing a range of Ki-67 expression was rotated across three sites and evaluated three times by the same pathologist at each site. Inter-observer analysis was performed between three sites on a total of 540 comparisons.	NPA 98.9% (97.7,100.0%) PPA 97.8% (95.3, 99.6%) OA 98.3% (96.9, 99.4%)
Intra-observer	≥ 20%	One set of 60 stained specimens (29 Ki-67 negative and 31 Ki-67 positive) representing a range of Ki-67 expression was rotated across three sites and evaluated three times by the same pathologist at each site. Intra-observer analysis was performed for three sites on a total of 540 comparisons.	NPA 98.5% (97.0, 99.6%) PPA 98.6% (97.1, 99.6%) OA 98.5% (97.4, 99.4%)

NPA= Negative Percent Agreement; PPA= Positive Percent Agreement; OA=Overall Percent Agreement

15.3 Clinical Performance Evaluation

Early Breast Cancer

Verzenio in Combination with Standard Endocrine Therapy (monarchE)

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 (17). 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% (18).

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 10 and Figure 1.

Table 10: Efficacy Results in monarchE in Cohort 1 Patients with Ki-67 Expression (Ki-67 pharmDx Score ≥ 20%)

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

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%).

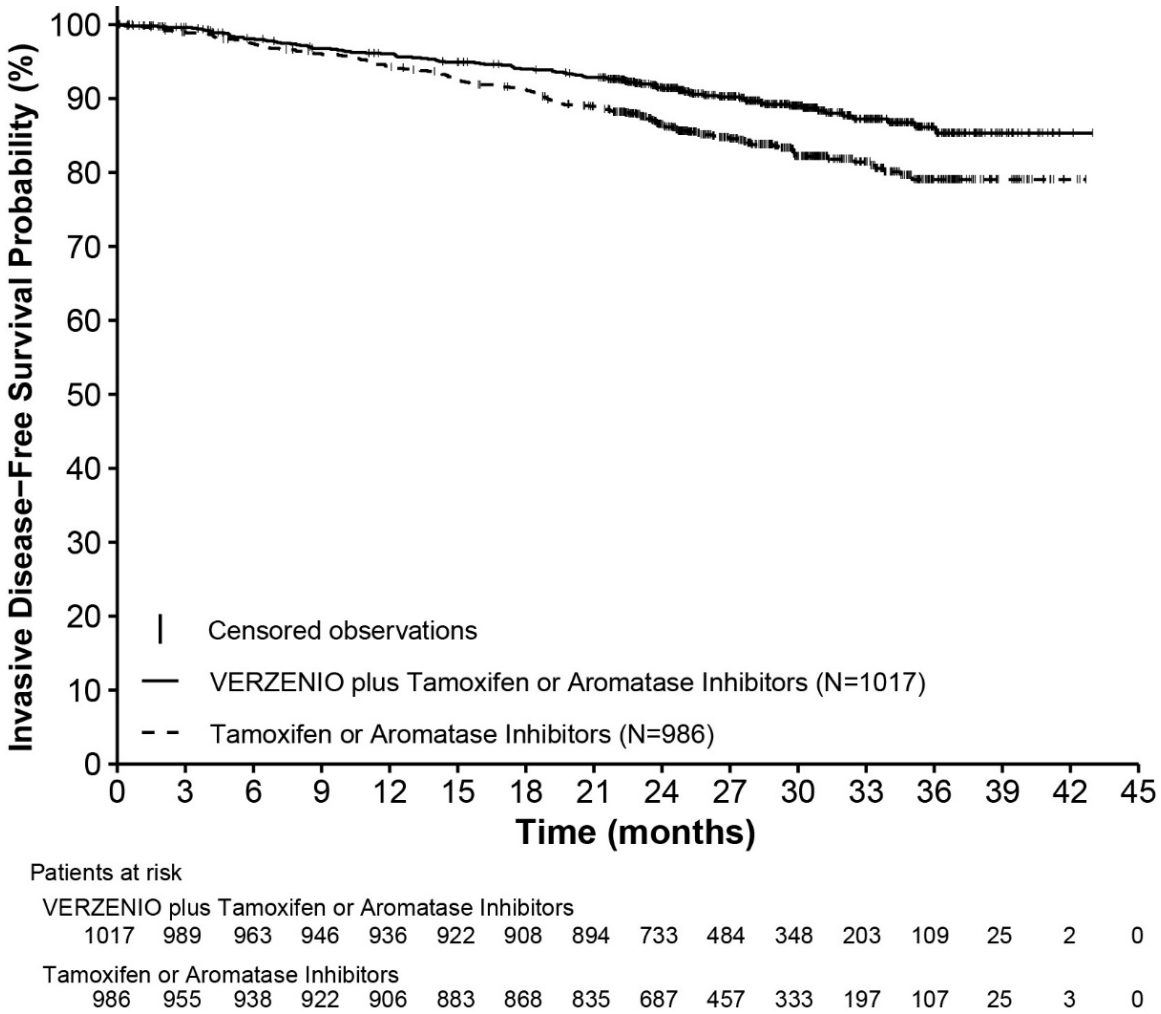

Figure 1: Kaplan-Meier Curves of Invasive Disease–Free Survival comparing Verzenio plus Tamoxifen or an Aromatase Inhibitor versus Tamoxifen or an Aromatase Inhibitor in Cohort 1 Patients with Ki-67 expression (Ki-67 pharmDx Score ≥ 20%) (monarchE)

16. Troubleshooting

Refer to the Troubleshooting section in the referenced Education Guide (11) for remedial action or contact Agilent Technical Support to report unusual staining.

Dako Omnis is an automated system designed to alert the user if anything in the run has been outside of specifications. Please refer to the Dako Omnis Basic and Advanced User Guides for details on what conditions are flagged and how. Below is a troubleshooting guide for results and conditions that are not easily identified through the Dako Omnis warning and alert system.

The user should always ensure adherence to the maintenance schedule for the Dako Omnis instrument.

Always use the appropriate controls as described in the Quality Control section.

Table 11: Troubleshooting

Problem	Probable Cause	Suggested Action
1. No or weak staining of slides	1a. Wrong storage conditions used for reagents.	1a. Check that reagents have been stored correctly according to listed storage conditions.
	1b. Reagent is used past its expiration date.	1b. Ensure reagent is not used past its expiration date.
	1c. Reagent is used past its onboard stability.	1c. Ensure reagent is not used past its onboard stability.
	1d. Inappropriate fixation method used.	1d. 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.
	1e. Excessive heating of mounted tissue sections prior to loading on Dako Omnis may lead to loss of immunoreactivity and morphology.	1e. Dry the tissue sections at *58* ± 2°C for a maximum of 1 hour, using a calibrated oven with uniform heat distribution (19).
	1f. Incorrect placement of dynamic gap lids in stainer modules.	1f. Check placement of dynamic gap lids.
	1g. Damaged dynamic gap lids.	1g. Check integrity of dynamic gap lids.
	1h. Distilled or de-ionized water is not used to dilute the Target Retrieval Solution concentrate.	1h. Ensure that distilled or de-ionized water is used to prepare 1x Target Retrieval Solution.
	1i. Incorrect Target Retrieval Solution is used.	1i. Ensure that correct Target Retrieval Solution specified in “Materials Required but not Supplied” and/or “Reagent Preparation” sections is used.
	1j. 1x Target Retrieval Solution does not meet pH specifications.	1j. Check pH of 1x Target Retrieval Solution. If pH is outside acceptable range (pH 6.1+/- 0.2), discard 1x Target Retrieval Solution. Do not adjust pH. Prepare new 1x Target Retrieval Solution. Check pH of new Target Retrieval Solution.
2. Excessively strong specific staining of slides	2a. Inappropriate fixation method used.	2a. Ensure that only approved fixatives and fixation methods are used.
	2b. Distilled or de-ionized water is not used to dilute the Target Retrieval Solution concentrate.	2b. Ensure that distilled or de-ionized water is used to prepare 1x Target Retrieval Solution.
	2c. Incorrect Target Retrieval Solution is used.	2c. Ensure that correct Target Retrieval Solution specified in “Materials Required but not Supplied” and/or “Reagent Preparation” sections is used.
	2d. 1x Target Retrieval Solution does not meet pH specifications.	2d. Check pH of 1x Target Retrieval Solution. If pH is outside acceptable range (pH 6.1+/- 0.2), discard 1x Target Retrieval Solution. Do not adjust pH. Prepare new 1x Target Retrieval Solution. Check pH of new Target Retrieval Solution.
3. Excessive non-specific staining of slides	3a. Starch additives used in mounting sections to slides.	3a. Avoid using starch additives for adhering sections to glass slides. Many additives are immunoreactive.
	3b. Sections dried after staining procedure.	3b. Verify that the unloading station is filled with sufficient water.
	3c. Sections dried prior to coverslipping.	3c. Avoid stained slides drying out between unloading from Dako Omnis and coverslipping.
	3d. Inappropriate fixation method used.	3d. Ensure that approved fixative was used. Alternative fixative may cause excessive background staining.
	3e. Paraffin incompletely removed.	3e. 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.

Problem	Probable Cause	Suggested Action
	3f. Non-specific binding of reagents to tissue.	3f. Ensure that correct fixation method of the specimen is used and avoid large areas of necrosis.
	3g. Re-use of mixing strip.	3g. Ensure that new mixing strips are used.
	3h. Distilled or de-ionized water is not used to dilute the Target Retrieval Solution concentrate.	3h. Ensure that distilled or de-ionized water is used to prepare 1x Target Retrieval Solution.
	3i. Incorrect Target Retrieval Solution is used.	3i. Ensure that correct Target Retrieval Solution specified in "Materials Required but not Supplied" and/or "Reagent Preparation" sections is used.
	3j. 1x Target Retrieval Solution does not meet pH specifications.	3j. Check pH of 1x Target Retrieval Solution. If pH is outside acceptable range (pH 6.1+/- 0.2), discard 1x Target Retrieval Solution. Do not adjust pH. Prepare new 1x Target Retrieval Solution. Check pH of new Target Retrieval Solution.
4. Tissue detaches from slides	4a. Use of incorrect slides.	4a. Use FLEX IHC Microscope Slides (Code K8020), or SuperFrost Plus slides.
5. Slide is flagged	5a. Reagent is used beyond its expiration date.	5. Flagged slides should be evaluated by qualified personnel. Contact an Agilent Technologies representative if further action is needed.
	5b. Reagent is stored onboard Dako Omnis beyond its validated onboard stability.	
	5c. Maintenance overdue or other factors.	
6. 1x Target Retrieval Solution does not meet pH specifications	6a. pH meter is not calibrated correctly.	6a. Ensure pH meter is calibrated per manufacturer's recommendations. After re-calibration, re-test pH of 1x Target Retrieval Solution. Do not modify the pH of 1x Target Retrieval Solution TRS. If pH is outside acceptable range (pH 6.1+/- 0.2), discard 1x Target Retrieval Solution. Prepare new 1x Target Retrieval Solution. Check pH of new 1x Target Retrieval Solution.
	6b. Target Retrieval Solution pH is measured at incorrect temperature.	6b. Ensure that 1x Target Retrieval Solution pH is measured at ambient temperature.
	6c. Distilled or de-ionized water is not used to dilute the Target Retrieval Solution concentrate.	6c. Ensure that distilled or de-ionized water is used to prepare 1x Target Retrieval Solution
	6d. Incorrect Target Retrieval Solution is used.	6d. Ensure that the correct Target Retrieval Solution specified in Section 5 "Materials Required but not Supplied" and/or Section 9 "Reagent Preparation" sections is used.

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 previously referenced Education Guide*: Immunohistochemical Staining Methods* (11) (available from www.agilent.com), Atlas of Immunohistology (20), and Immunoperoxidase Techniques. A Practical Approach to Tumor Diagnosis (21).

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Explanation of symbols

REF	Catalogue number		Temperature limitation	IVD	In vitro diagnostic medical device
	Manufacturer	LOT	Batch code	Σ	Contains sufficient for <n> tests
	Use by		Consult instructions for use	EC REP	Authorized representative in the European Community

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