PD-L1 IHC 22C3 pharmDx

SK006
50 tests for use with Autostainer Link 48

Intended use

For in vitro diagnostic use.

PD-L1 IHC 22C3 pharmDx is a qualitative immunohistochemical assay using Monoclonal Mouse Anti-PD-L1, Clone 22C3 intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) non-small cell lung cancer (NSCLC) tissue using EnVision FLEX visualization system on Autostainer Link 48. PD-L1 protein expression is determined by using Tumor Proportion Score (TPS), which is the percentage of viable tumor cells showing partial or complete membrane staining. The specimen should be considered PD-L1 positive if TPS ≥ 50% of the viable tumor cells exhibit membrane staining at any intensity.

PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying NSCLC patients for treatment with KEYTRUDA® (pembrolizumab).

Summary and explanation
Binding of the PD-1 ligands, PD-L1 and PD-L2, to the PD-1 receptor found on T cells, inhibits T cell proliferation and cytokine production. Up-regulation of PD-1 ligands occurs in some tumors and signaling through this pathway can contribute to inhibition of active T-cell immune surveillance of tumors. KEYTRUDA® (pembrolizumab) is a humanized monoclonal antibody that binds to the PD-1 receptor and blocks its interaction with PD-L1 and PD-L2, releasing PD-1 pathway-mediated inhibition of the immune response, including the anti-tumor immune response. In syngeneic mouse tumor models, blocking PD-1 activity resulted in decreased tumor growth.

Principle of procedure
PD-L1 IHC 22C3 pharmDx contains optimized reagents and protocol required to complete an IHC staining procedure of FFPE specimens using Autostainer Link 48. Following incubation with the primary monoclonal antibody to PD-L1 or the Negative Control Reagent (NCR), specimens are incubated with a Linker antibody specific to the host species of the primary antibody, and then 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 color of the chromogenic reaction is modified by a chromogen enhancement reagent. The specimen may then be counterstained and coverslipped. Results are interpreted using a light microscope.

Materials provided
PD-L1 IHC 22C3 pharmDx (Code SK006) is for automated staining using Autostainer Link 48.

Each kit includes 19.5 mL of PD-L1 primary antibody (approximately 3µg/mL protein concentration) and contains the reagents necessary to perform 50 tests in up to 15 individual runs. The materials listed below are sufficient for 50 tests (50 slides incubated with Primary Antibody to PD-L1 and 50 slides incubated with the corresponding Negative Control Reagent, 100 slides in total). The number of tests is based on the use of 2 x 150 µL per slide of each reagent except DAB+ and Target Retrieval Solution.

The kit provides materials sufficient for a maximum of 15 individual staining runs.

Quantity	*Description*
1 x 34.5 mL	**Peroxidase-Blocking Reagent** PEROXIDASE-BLOCKING REAGENT Buffered solution containing hydrogen peroxide, detergent and 0.015 mol/L sodium azide.
1 x 19.5 mL	**Primary Antibody: Monoclonal Mouse Anti-PD-L1, Clone 22C3** MONOCLONAL MOUSE ANTI-PD-L1 CLONE 22C3 Monoclonal mouse anti-PD-L1 in a buffered solution, containing stabilizing protein, and 0.015 mol/L sodium azide.
1 x 15 mL	**Negative Control Reagent** NEGATIVE CONTROL REAGENT Monoclonal mouse control IgG antibody in a buffered solution, containing stabilizing protein, and 0.015 mol/L sodium azide.
1 x 34.5 mL	**Mouse LINKER** LINKER, ANTI-MOUSE Rabbit secondary antibody against mouse immunoglobulins in a buffered solution containing stabilizing protein and 0.015 mol/L sodium azide.
1 x 34.5 mL	**Visualization Reagent-HRP**

VISUALIZATION REAGENT-HRP

Dextran coupled with peroxidase molecules and goat secondary antibody molecules against rabbit and mouse immunoglobulins in a buffered solution containing stabilizing protein and an antimicrobial agent.

15 x 7.2 mL **DAB+ Substrate Buffer**

DAB+ SUBSTRATE BUFFER

Buffered solution, containing hydrogen peroxide and an antimicrobial agent.

1 x 5 mL **DAB+ Chromogen**

DAB+ CHROMOGEN

3,3'-diaminobenzidine tetrahydrochloride in organic solvent.

1 x 34.5 mL **DAB Enhancer**

DAB ENHANCER

Cupric sulfate in water.

6 x 30 mL **EnVision FLEX Target Retrieval Solution, Low pH, 50x**

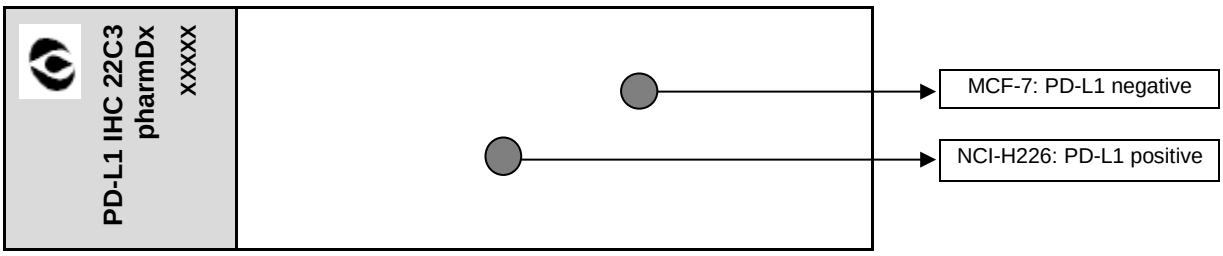
EnVision™ FLEX TARGET RETRIEVAL SOLUTION LOW pH (50X)

Buffered solution, pH 6.1, containing detergent and an antimicrobial agent.

15 slides **PD-L1 IHC22C3 pharmDx Control Slides**

CONTROL SLIDES

Each slide contains sections of two pelleted, formalin-fixed paraffin-embedded cell lines: NCI-H226 with moderate PD-L1 protein expression and MCF-7 with negative PD-L1 protein expression.



Note: All reagents included are formulated specifically for use with this kit. In order for the test to perform as specified, no substitutions, other than EnVision FLEX Target Retrieval Solution, Low pH, 50x (Code K8005) can be made. PD-L1IHC 22C3 pharmDx has been tailored for use with Autostainer Link 48. Please refer to the User Guides for your Autostainer Link 48 and PT Link for further information

Materials required, but not supplied

PT Link Pre-treatment Module (Code PT100)
Autostainer Link 48 (Code AS480)
EnVision FLEX Wash Buffer, 20x (Code K8007)
Hematoxylin (Code K8008)
Distilled or deionized water (reagent-quality water)
Timer
Positive and negative tissues to use as process controls (see Quality control section)
Microscope slides: Dako FLEX IHC Microscope Slides (Code K8020) or Fisherbrand Superfrost Plus charged slides
Coverslips
Permanent mounting medium and ancillary reagents required for mounting coverslips
Light microscope (4x–40x objective magnification)

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 (1).
4. Primary Antibody, Negative Control Reagent, Linker, and Visualization Reagent contain material of animal origin.
5. 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 (2).
6. Incubation times, temperatures, or methods other than those specified may give erroneous results.
7. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.
8. The Visualization Reagent, Liquid DAB+ chromogen and prepared DAB+ Substrate-Chromogen solution may be affected adversely if exposed to excessive light levels. Do not store system components or perform staining in strong light, such as direct sunlight.
9. Paraffin residuals may lead to false negative results.
10. Use of reagent volumes other than recommended may result in loss of visible PD-L1 immunoreactivity.

11. Results from a small study, showed a similar dynamic range of PD-L1 expression in primary and metastatic NSCLC specimen pairs. It is possible there may be differences in PD-L1 expression in primary tumors versus metastatic sites in the same patient.
12. Large tissue sections may require 3x150 µl of reagent.
13. 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.
14. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
15. Unused solution should be disposed of according to local, State and Federal regulations.
16. Safety Data Sheet available for professional users on request.

Danger

DAB+ Chromogen: 1–5% biphenyl-3,3',4,4'-tetrayltetraammonium tetrachloride

H350	May cause cancer.
H341	Suspected of causing genetic defects.
P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
P280	Wear protective gloves. Wear eye or face protection. Wear protective clothing.
P308 + P313	IF exposed or concerned: Get medical attention.
P405	Store locked up.
P501	Dispose of contents and container in accordance with all local, regional, national and international regulations.

Warning

EnVision FLEX Target Retrieval Solution, Low pH (50x): 1-5% Citric acid

H319	Causes serious eye irritation.
H411	Toxic to aquatic life with long lasting effects.
P280	Wear eye or face protection.
P273	Avoid release to the environment.
P264	Wash hands thoroughly after handling.
P305 + P351 + P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313	If eye irritation persists: Get medical attention.
P501	Dispose of contents and container in accordance with all local, regional, national and international regulations.

Storage
Store all components of PD-L1 IHC 22C3 pharmDx, including Control Slides, in the dark at 2-8 °C when not in use on Autostainer Link 48.

Do not use the kit after the expiration date printed on the outside of the kit box. If reagents are stored under any conditions other than those specified in this package insert, they must be validated by the user.

There are no obvious signs to indicate instability of this product, therefore, positive and negative controls should be run simultaneously with patient specimens.

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

Paraffin-embedded sections
Formalin-fixed, paraffin-embedded tissues are suitable for use. Alternative fixatives have not been validated and may give erroneous results. Fixation time for 12-72 hours in 10% neutral buffered formalin (NBF) is recommended, however, a study with limited samples showed fixation times of 4-168 hours in 10% NBF did not systematically alter PD-L1 detection. Fixation times of ≤3 hours may result in variable PD-L1 detection. 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. FFPE tissue blocks which are 5 years or older may result in a loss of PD-L1 immunoreactivity.

Tissue specimens should be cut into sections of 4-5 µm. After sectioning, tissues should be mounted on Fisherbrand Superfrost Plus slides, or Dako FLEX IHC microscope slides (Code K8020). To preserve antigenicity, tissue sections, once mounted on slides, should be held in the dark at 2-8 °C and stained within 6 months of sectioning.

The use of PD-L1 IHC 22C3 pharmDx, on decalcified tissues has not been validated and is not recommended.

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

EnVision FLEX Target Retrieval Solution, Low pH, 50x
Prepare a sufficient quantity of 1x Target Retrieval Solution, Low pH by diluting Target Retrieval Solution, Low pH, 50x 1:50 using distilled or deionized water (reagent-quality water); 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. One 30 mL bottle of Target Retrieval Solution, Low pH, 50x, diluted 1:50 will provide 1.5 L of 1x reagent, sufficient to fill one PT Link tank which will treat up to 24 slides per use. Discard 1x Target Retrieval Solution after three uses and do not use after 5 days following dilution.

Additional EnVision FLEX Target Retrieval Solution, Low pH, 50x, if required, is available as Code K8005.

EnVision FLEX Wash Buffer, 20x
Prepare a sufficient quantity of Wash Buffer by diluting Wash Buffer 20x 1:20 using distilled or deionized water (reagent-quality water) for the wash steps. Store unused 1x solution at 2-8 ºC for no more than one month. Discard buffer if cloudy in appearance. Refer to the User Guide for your Autostainer Link 48 for further information.
EnVision FLEX Wash Buffer, 20x is available as Code K8007.

DAB+ Substrate-Chromogen Solution
This solution should be mixed thoroughly prior to use. Any precipitate developing in the solution does not affect staining quality.

To prepare DAB+ Substrate-Chromogen Solution, add 1 drop of Liquid DAB+ Chromogen per mL of DAB+ Substrate Buffer and mix. Prepared Substrate-Chromogen is stable for 5 days if stored in the dark at 2-8 °C.

Important Notes:
- **If using an entire bottle of DAB+ Substrate Buffer, add 9 drops of DAB+ chromogen**. Although the label states 7.2 mL, this is the useable volume and does not account for the "dead volume" in the bottle.
- The color of the Liquid DAB+ Chromogen in the bottle may vary from clear to lavender-brown. This will not affect the performance of this product. Dilute per the guidelines above. Addition of excess Liquid DAB+ Chromogen to the DAB+ Substrate Buffer will result in deterioration of the positive signal.

Staining procedure on the Autostainer Link solution
Procedural Notes
The user should read these instructions carefully and become familiar with all components and instrumentation prior to use (see Precautions).

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

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

All of the required steps and incubation times for staining are preprogrammed in the Dako Link software. Please refer to the User Guides for Autostainer Link 48 and PT Link for further information on programming protocols and loading slides and reagents.

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

Staining Protocol
Please select the PD-L1 IHC 22C3 pharmDx staining protocol from the options in the Dako Link drop down menu.

All of the required steps and incubation times for staining are preprogrammed in the Autostainer Link 48. If the appropriate PD-L1 IHC 22C3 pharmDx protocols are not on your server please contact your local Technical Service Representative to obtain the protocols.

Step 1: Deparaffinization, Rehydration and Target Retrieval (3-in-1) Procedure

For details, please refer to the PT Link User Guide.

Set PT Link (Code PT100) Preheat and Cool to 65 °C. Set Heat to 97 °C for 20 minutes.

►Fill PT Link tanks with 1.5 L per tank of Target Retrieval Solution, Low pH, 1x working solution to cover the tissue sections.
►Preheat the Target Retrieval Solution to 65 °C.
►Immerse Autostainer racks containing mounted, FFPE tissue sections into the pre-heated Target Retrieval Solution, Low pH, (1x working solution) in PT Link tank. Incubate for 20 minutes at 97 °C.
►When target retrieval incubation has been completed and the temperature has cooled to 65 °C, remove each Autostainer slide rack with the slides from the PT Link tank and **immediately** place the Autostainer rack with slides into a tank (e.g., PT Link Rinse Station, Code PT109) containing diluted, room temperature Wash Buffer (Code K8007).
►Incubate slides in diluted, room temperature Wash Buffer for 5 minutes.

Step 2: Staining Procedure
After deparaffinization, rehydration and target retrieval (3-in-1) procedure, the Autostainer racks with slides are placed on Autostainer Link 48. The instrument will perform the staining process by applying the appropriate reagent, monitoring the incubation time and rinsing slides between reagents. The reagent times are preprogrammed in the Dako Link software.

Step 3: Counterstain
Slides should be counterstained for 5 minutes with Hematoxylin (Link) (Code K8008). The Hematoxylin incubation time is preprogrammed in the protocol.

Step 4: Mounting
Non-aqueous, permanent mounting media is required.

Note: 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 slides in the dark at room temperature (20-25 °C).

Quality Control
Reagents in PD-L1 IHC 22C3 pharmDx have been quality controlled by immunohistochemistry using the target retrieval and staining procedures outlined above.

Deviations in the recommended procedures for tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results. In-house controls should be included in each staining run.

Differences in tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls in addition to the Control Slides supplied in the kit (3). In the USA, consult the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry, see also CLSI Quality Assurance for Immunocytochemistry, Approved Guideline (4) for additional information.

Table 1: The Purpose of Daily Quality Control

Tissue	Reagents	Purpose
Positive Control: Tissue or cells containing target antigen to be detected. The ideal control is weakly positive staining tissue, which may be more sensitive in detecting reagent degradation.	Primary Antibody & Detection System	Controls all steps of the analysis. Validates reagents and procedures used for PD-L1 staining.
Negative Control: Tissues or cells expected to be negative (could be located in patient tissue or positive control tissue).	Primary Antibody & Detection System	Detection of unintended antibody cross-reactivity to cells/cellular components.
Control Slide supplied by Dako	Primary Antibody & Detection System	Controls staining procedure only
Patient tissue slide	*Negative Control Reagent & same Detection System as used with the Primary Antibody	Detection of non-specific background staining.

*From the same species as the primary antibody, but not directed against the same target antigen. To detect non-specific antibody binding, e.g. binding of Fc portion of antibody by the tissue.

Control Cell Line Slides (provided)
Each slide contains sections of two pelleted, formalin-fixed paraffin-embedded cell lines: NCI-H226 with moderate PD-L1 protein expression and MCF-7 with negative PD-L1 protein expression. One control slide should be stained with the Primary Antibody to PD-L1 in each staining run. The evaluation of the Control Slide cell lines supplied in the kit indicates the validity of the staining run. They should not be used as an aid in interpretation of patient results.

Positive Control Tissue
Controls should be fresh 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 sample(s). Positive tissue controls are indicative of correctly prepared tissues and proper staining techniques. One positive tissue control for each set of test conditions should be included in each staining run.

The tissues selected for use as the positive tissue controls should give weak to moderate positive staining so they can detect subtle changes in assay sensitivity. Specimens processed differently from the patient sample(s) validate reagent performance only and do not verify tissue preparation.

Known positive tissue controls should only be utilized for monitoring the correct performance of processed tissues and test reagents, NOT as an aid in formulating a specific diagnosis of patient samples. If the positive tissue controls fail to demonstrate appropriate positive staining, results with the test specimens should be considered invalid.

Negative Control Tissue
Use a negative control tissue (known to be PD-L1 negative) of the same tumor indication as the patient specimen, fixed processed and embedded in a manner similar to the patient sample(s) with each staining run to verify the specificity of the primary antibody and to provide an indication of non-specific background staining. The variety of different cell types present in most tissue sections offers internal negative control sites (this should be verified by the user).

If specific staining occurs in the negative control tissue, results with the patient specimens should be considered invalid.

Tonsil Control Tissue (optional)
Use human tonsil tissue fixed, processed and embedded in a manner similar to the patient sample(s) as an additional control material to verify sensitivity, specificity and nonspecific background staining of the assay.

Strong positive staining should be detected in portions of the crypt epithelium and weak to moderate staining of the follicular macrophages in the germinal centers. Negative staining should be observed in endothelium, fibroblasts as well as surface epithelium.

Negative Control Reagent
Use the supplied Negative Control Reagent in place of the primary antibody on a sequential section of each patient specimen to evaluate non-specific staining and allow better interpretation of specific staining at the antigen site. The incubation period for the Negative Control Reagent should be equivalent to that of the primary antibody.

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 in-house tissues with known IHC performance characteristics representing known positive and negative tissues. Refer to the quality control procedures previously outlined in this section of the product insert and, in the US, to the quality control requirements of the CAP Certification Program for Immunohistochemistry and/or CLSI Quality Assurance for Immunocytochemistry, Approved Guideline (4) These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters.

Scoring Interpretation - NSCLC

All viable tumor cells on the entire slide must be evaluated and included in the PD-L1 scoring assessment. A minimum of 100 viable tumor cells must be present for the specimen to be considered adequate for PD-L1 evaluation.

To successfully score PD-L1 IHC 22C3 pharmDx stained specimens, it is critical that the appropriate cells are evaluated, that the proper

cellular localization is identified and that the staining intensity is properly interpreted. Slide evaluation should be performed by a pathologist using a light microscope. For evaluation of the immunocytochemical staining and scoring, an objective of 10-40x magnification is appropriate. Any perceptible membrane staining of tumor cells should be included in the scoring.

Score partial or complete cell membrane staining (≥1+) that is perceived distinct from cytoplasmic staining. Cytoplasmic staining should be considered non-specific staining and is excluded in the assessment of staining intensity. Normal cells and tumor associated immune cells such as infiltrating lymphocytes or macrophages ***should not*** be included in the scoring for the determination of PD-L1 positivity. Tumor specimens stained with the NCR must have 0 specific staining and ≤1+ background staining.

Tumor Proportion Score (TPS) is the percentage of viable tumor cells showing partial or complete membrane staining (≥1+). The specimen should be considered PD-L1 positive if TPS ≥ 50% of the viable tumor cells exhibit membrane staining at any intensity (i.e. ≥1+).

For each staining run, slides should be examined in the order presented in Table 2 to determine the validity of the staining run and enable assessment of the staining of the sample tissue.

Refer to PD-L1 IHC 22C3 pharmDx Interpretation Manual for additional guidance.

Table 2. Recommended order of slide evaluation

Recommended order of slide interpretation	Rationale
1. Control Cell Line Slide containing the PD-L1-positive and PD-L1-negative cell lines	The Control Cell Line Slide stained with the PD-L1 primary antibody from PD-L1 IHC 22C3 pharmDx should be examined first to determine that all reagents are functioning properly. The presence of a brown (3,3'-diaminobenzidine, DAB) reaction product on the cell membrane is indicative of positive reactivity. *NCI-H226 (PD-L1-positive control cell line) acceptance criteria:* • Cell membrane staining of ≥70% of cells at ≥2+ average staining intensity. • Non-specific staining <1+ intensity. *MCF-7 (PD-L1-negative control cell line) acceptance criteria:* • No specific staining. • Non-specific staining <1+ intensity. ❖ *Note that staining of a few cells in the MCF-7 cell pellet may occasionally be observed. The following acceptance criteria are applicable: the presence of ≤10 total cells with distinct plasma membrane staining is acceptable.* ➢ If either of the Control Cell Lines does not meet these criteria, all results with the patient specimens should be considered invalid.
2. Positive Control Tissue Slide (NSCLC)	The Positive Control Tissue Slide should be examined next. This slide verifies that the fixation method and epitope retrieval process are effective. Use intact cells for interpretation of staining results because necrotic or degenerated cells often stain non-specifically. Presence of brown plasma membrane staining should be observed. Non-specific staining should be ≤1+.
3. Negative Control Tissue Slide (NSCLC)	The Negative Control Tissue Slide should be examined after the Positive Control Tissue to verify the specificity of the labeling of the target antigen by the primary antibody. The absence of specific staining in the Negative Control Tissue Slide confirms the lack of kit cross-reactivity to cells/cellular components. Alternatively, negative portions of the Positive Control Tissue may serve as the Negative Control Tissue, but this should be verified by the user. If unwanted specific cell membrane staining occurs in the Negative Control Tissue Slide, results with the patient specimen should be considered invalid.
4. Patient tissue slide stained using the Negative Control Reagent	Examine patient specimens stained with the Negative Control Reagent from PD-L1 IHC 22C3 pharmDx next. Absence of cell membrane staining verifies the specific labeling of the target antigen by the primary antibody. Staining occurring in the cytoplasm of the specimen treated with the Negative Control Reagent should be considered non-specific staining.
5. Patient tissue slide stained using the primary antibody	Examine the entire slide of the patient specimen stained with the PD-L1 primary antibody from PD-L1 IHC 22C3 pharmDx following review of all acceptable control slides. Positive staining intensity should be assessed within the context of any non-specific background staining observed in the Negative Control Reagent slide within the same run. As with any immunocytochemical test, a negative result means that the antigen was not detected, not necessarily that the antigen was absent in the cells/tissue assayed. Refer to Summary and Explanation, Limitations, and Performance Characteristics for specific information regarding PD-L1 IHC 22C3 pharmDx immunoreactivity. Complete circumferential and/or partial linear membrane staining of tumor cells indicates positive PD-L1 staining. Granular staining in the cytoplasm is not considered as positive staining.

Additional Recommendations for Interpretation of PD-L1 IHC 22C3 pharmDx Staining

1. A hematoxylin and eosin (H&E) stain of the tissue specimen is recommended for the first evaluation of the patient specimen. The PD-L1 IHC 22C3 pharmDx assay and H&E stain should be performed on serial sections from the same paraffin block of the specimen.
2. To verify cell membrane staining, use 10–40x objective magnification.
3. Various non-target elements, such as tumor associated immune cells (e.g. macrophages) and stroma may also stain positive for PD-L1, however should not be included in the scoring.

General Limitations

1. Immunohistochemistry is a multi-step diagnostic 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-negative 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. The clinical interpretation of any positive staining or its absence must be evaluated within the context of clinical presentation, morphology and other histopathological criteria. The clinical interpretation of any staining, or its absence, must be complemented by morphological studies and proper controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist, who is

familiar with the antibodies, reagents and methods used, to interpret the stained preparation. Staining must be performed in a certified, licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
5. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit non-specific staining with horseradish peroxidase (5).
6. Reagents may demonstrate unexpected reactions in previously untested tissue types. The possibility of unexpected reactions even in tested tissue types cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues. Contact Dako Technical Support with documented unexpected reactions.
7. 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) (5).
8. The reagents and instructions supplied in this system have been designed for optimal performance. Further dilution of the reagents or alteration of incubation times or temperatures may give erroneous or discordant results.

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 six months of mounting of tissues on slides when stored in the dark at 2-8°C.
2. For optimal and reproducible results, the PD-L1 protein requires target retrieval pre-treatment when tissues are routinely fixed (neutral buffered formalin) and paraffin embedded.
3. Do not substitute reagents from different lot numbers of this product, or from kits of other manufacturers. The only exception is the EnVision FLEX Target Retrieval Solution, Low pH 50x, which, if required, is available as Code K8005.
4. Stained control cell lines should be used only for validation of the staining run and should not be used to score the staining reaction in tissue sections.
5. Use of Dako PD-L1 IHC 22C3 pharmDx on tissues with fixatives other than formalin has not been validated.

Clinical performance evaluation
The clinical benefit of PD-L1 IHC 22C3 pharmDx was investigated in a multicenter, open-label, randomized clinical study conducted to assess the safety and efficacy of KEYTRUDA in patients with advanced NSCLC. Patients were PD-L1 positive by a research assay, and had progression of disease following treatment with platinum-containing chemotherapy. Patients with EGFR or ALK genomic tumor aberrations had disease progression on FDA-approved therapy for these aberrations prior to receiving KEYTRUDA. Assessment of tumor status was performed every 9 weeks. The major efficacy outcome measures were ORR (according to RECIST 1.1 as assessed by blinded independent central review) and duration of response (6).

To evaluate the clinical utility of PD-L1 IHC 22C3 pharmDx, archived clinical study samples were retrospectively tested at a U.S based reference laboratory with PD-L1 IHC 22C3 pharmDx. KEYTRUDA® (pembrolizumab) demonstrated a robust overall response rate with a clinically meaningful duration of response in NSCLC patients with positive PD-L1 expression as determined by PD-L1 IHC 22C3 pharmDx.

Based on the research assay, a total of 223 NSCLC patients were enrolled in the study. Out of the 223 patients, tumor tissue from 220 patients was retrospectively tested with the PD-L1 IHC 22C3 pharmDx test. Specimens from 61 patients were positive for PD-L1 expression (≥50% of viable tumor cells exhibiting membrane staining at any intensity) and samples from 104 patients were negative for PD-L1 expression (<50% of viable tumor cells exhibiting membrane staining at any intensity).

The baseline characteristics for this population of 61 patients with positive PD-L1 expression by PD-L1 IHC 22C3 pharmDx included: median age 60 years (34% age 65 or older); 61% male; 79% White; and 34% and 64% with an ECOG performance status 0 and 1, respectively. Disease characteristics were squamous and non-squamous (21% and 75% respectively); M1 (98%); brain metastases (11%); and one (26%), two (30%), or three or more (44%) prior therapies. The mutation status among patients was EGFR (10%), ALK (0%), or Kras (16%).

Efficacy results for NSCLC are summarized in Table 3.

Table 3: Response to KEYTRUDA® (pembrolizumab) in Previously Treated NSCLC Patients with PD-L1 tumor proportion score ≥50%

Endpoint	N=61
Overall Response Rate	
ORR %, (95% CI)	41% (29, 54)*
Complete Response	0%
Partial Response	41%
Response Duration	
Median in months (range)	Not reached (2.1+, 9.2+)
% ongoing	84%†
* In patients with a PD-L1 tumor proportion score <50% (n = 104), ORR was13% (8, 22)	
† Includes 11 patients with ongoing responses of 6 months or longer	

Among the 61 patients 25 patients (41%) experienced a partial response. Of these 25 patients, 21 (84%) had an ongoing response at the time of the data analysis cut-off date. At that time, the duration of response was between 2.1 and 9.2 months for all 25 responders. The patients with response durations of 2.1 and 9.2 months were both ongoing at the time of data cutoff, denoted by appending a + sign to those values in the table. Median DoR is calculated based on the number of events and their timing and had not been reached at the data cut-off date.

Non-clinical performance evaluation

Analytical Sensitivity
Analytical sensitivity of PD-L1 IHC 22C3 pharmDx was tested on 127 unique cases of non-small cell lung carcinoma (NSCLC) FFPE specimens staged I to IV using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a range of 0-100% positive tumor cells and 0-3 staining intensity.

Repeatability/External Reproducibility
The Repeatability/External Reproducibility of PD-L1 IHC 22C3 pharmDx was evaluated at Dako and three external testing sites. The performance data are provided in Table 4 and Table 5. For the repeatability studies performed at Dako, negative percent agreement (NPA), positive percent agreement (PPA) and overall agreement (OA) were determined as shown in Table 4. Since the repeatability studies resulted in 100% agreement, confidence intervals were calculated using the Wilson Score method, based on the number of independent pair-wise comparisons.

For external reproducibility studies, the average negative percent agreement (ANA), average positive percent agreement (APA) and overall percent agreement (OA) were determined as shown below in Table 5. Average agreements were calculated since no natural reference exists in reproducibility parameters such as site and observer. Confidence intervals for the average agreements were computed using a percentile bootstrap method.

(Note that the repeatability studies were not analyzed using bootstrap confidence intervals, since the aforementioned cannot be computed on data with zero discordance.)

Table 4: Repeatability of PD-L1 IHC 22C3 pharmDx tested at one site

Repeatability Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-instrument	≥50%	Each of 16 NSCLC specimens (10 PD-L1-negative and 6 PD-L1-positive) with a range of PD-L1 IHC expression was tested on each of six Autostainer Link 48 instruments.	≥50% cut-off: NPA 100% (92.9-100%) PPA 100% (88.6-100%) OA 100% (95.4-100%)
Inter-operator	≥50%	Each of 16 NSCLC specimens (10 PD-L1-negative and 6 PD-L1-positive) with a range of PD-L1 IHC expression was tested using six analysts on one Autostainer Link 48 instrument.	≥50% cut-off: NPA 100% (92.7-100%) PPA 100% (88.6-100%) OA 100% (95.4-100%)
Inter-day	≥50%	Each of 16 NSCLC specimens (10 PD-L1-negative and 6 PD-L1-positive) with a range of PD-L1 IHC expression was tested on six non-consecutive days on the Autostainer Link 48 instrument.	≥50% cut-off: NPA 100% (92.9-100%) PPA 100% (88.6-100%) OA 100% (95.4-100%)
Inter-lot	≥50%	Each of 16 NSCLC specimens (8 PD-L1-negative and 8 PD-L1-positive) with a range of PD-L1 IHC expression was tested with three replicates and each of three reagent lots on the Autostainer Link 48 instrument.	≥50% cut-off: NPA 100% (92.6-100%) PPA 100% (92.6-100%) OA 100% (96.2-100%)
Intra-run	≥50%	Each of 16 NSCLC specimens (10 PD-L1-negative and 6 PD-L1-positive) with a range of PD-L1 IHC expression was tested with six replicates within a run on the Autostainer Link 48 instrument.	≥50% cut-off: NPA 100% (92.9-100%) PPA 100% (88.6-100%) OA 100% (95.4-100%)
Intra-day	≥50%	Each of 16 NSCLC specimens (10 PD-L1-negative and 6 PD-L1-positive) with a range of PD-L1 IHC expression was tested on two runs within a day, repeated over three days, on the Autostainer Link 48 instrument.	≥50% cut-off: NPA 100% (88.3-100%) PPA 100% (82.4-100%) OA 100% (92.4-100%)

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

Table 5: Reproducibility of the PD-L1 IHC 22C3 pharmDx tested at three external sites

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	≥50%	Each of 36 NSCLC specimens (21 PD-L1-negative and 15 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 2700 pair-wise comparisons.	≥50%: ANA 90.3% (84.4-95.2%) APA 85.2% (75.6-92.9%) OA 88.3% (81.4-94.3%)
Intra-site	≥50%	Each of 36 NSCLC specimens (21 PD-L1-negative and 15 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days at each of three study sites. Intra-site analysis was performed for three sites on a total of 1080 pair-wise comparisons.	≥50%: ANA 91.9% (88.8-94.8%) APA 87.6% (82.5-92.2%) OA 90.2% (86.3-93.7%)
Inter-observer	≥50%	Scoring of 62 NSCLC specimens (30 PD-L1-negative and 32 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 1674 pair-wise comparisons.	≥50%: ANA 92.6% (87.8-96.7%) APA 92.8% (88.1-96.8%) OA 92.7% (88.1-96.8%)
Intra-observer	≥50%	Scoring of 62 NSCLC specimens (30 PD-L1-negative and 32 PD-L1-positive)with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 558 pair-wise comparisons.	≥50%: ANA 96.4% (94.0-98.5%) APA 96.5% (94.3-98.6%) OA 96.4% (94.3-98.6%)

ANA=Average Negative Agreement; APA=Average Positive Agreement; OA=Overall Agreement

Normal tissues: Plasma membrane staining was observed on immune cells and cells of epithelial origin. Cytoplasmic staining was noted in some cell types but was not recorded as positive staining. Table 6 summarizes Monoclonal Mouse Anti-PD-L1, Clone 22C3 immunoreactivity on the recommended panel of normal tissues. All tissues were formalin-fixed and paraffin-embedded and stained with PD-L1 IHC 22C3 pharmDx 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 PD-L1 IHC expression in normal tissues (7, 8).

Table 6: Summary of PD-L1 IHC 22C3 pharmDx normal tissue reactivity

Tissue Type (# tested)	Positive Plasma Membrane Staining: Tissue Elements	Positive Cytoplasmic Staining: Tissue Elements	Non-specific Staining
Adrenal (3)	0/3	1/3 Medullary cells	0/3
Bone marrow (3)	3/3 Megakaryocytes	3/3 Megakaryocytes	0/3
Breast (3)	0/3	0/3	0/3
Cerebellum (3)	0/3	0/3	0/3
Cerebrum (3)	0/3	0/3	0/3
Cervix (3)	1/3 Epithelium	0/3	0/3
Colon (3)	2/3 Macrophages	0/3	0/3
Esophagus (3)	0/3	0/3	0/3
Kidney (3)	1/3 Tubular epithelium	0/3	0/3
Liver (3)	1/3 Macrophages 1/3 Heptatocytes	0/3	0/3
Lung (3)	3/3 Alveolar macrophages	0/3	0/3
Mesothelial cells (2)	0/2	0/2	0/2
Muscle, cardiac (3)	0/3	0/3	0/3
Muscle, skeletal (3)	0/2	0/2	0/2
Nerve, peripheral (3)	0/3	1/3 Connective tissue/vessels	0/3
Ovary (3)	0/3	0/3	0/3
Pancreas (3)	0/3	0/3	0/3
Parathyroid (3)	1/3 Glandular epithelium	0/3	0/3
Pituitary (3)	1/3 Anterior hypophysis 1/3 Posterior hypophysis	1/3 Anterior hypophysis 1/3 Posterior hypophysis	0/3
Prostate (2)	2/2 Epithelium	0/2	0/2
Salivary gland (3)	0/3	0/3	0/3
Skin (3)	0/3	0/3	0/3
Small intestine (3)	0/3	0/3	0/3
Spleen (3)	2/3 Macrophages	0/3	0/3
Stomach (3)	2/3 Lymphocytes 1/3 Gastric glands	1/3 Gastric glands	0/3
Testis (3)	0/3	0/3	0/3
Thymus (3)	3/3 Medullary epithelium	0/3	0/3
Thyroid (3)	0/3	0/3	0/3
Tonsil (3)	3/3 Crypt epithelium 2/3 Germinal center (macrophages)	0/3	0/3
Uterus (3)	0/3	0/3	0/3

Neoplastic tissues: Plasma membrane staining was observed on immune cells and cells of epithelial origin. Cytoplasmic staining was noted in some cell types but was not recorded as positive staining. Table 7 summarizes Monoclonal Mouse Anti-PD-L1, Clone 22C3 immunoreactivity on a panel of neoplastic tissues. All tissues were formalin-fixed and paraffin-embedded and stained with PD-L1 IHC 22C3 pharmDx according to the instructions in this package insert. There were no unexpected results observed in the tumor specimens tested. The observed staining was consistent with the reported literature for PD-L1 IHC expression in neoplastic tissues (7-10).

Table 7: Summary of PD-L1 IHC 22C3 pharmDx neoplastic tissue reactivity

Tumor Type	Location	PD-L1 positive/total N=159
Adenocarcinoma	Appendix	0/1
	Breast, DCIS	0/2
	Breast, invasive ductal	0/7
	Breast, invasive ductal metastatic to lymph node	0/1
	Cervix, endocervical type	0/1
	Colon	0/5
	Colon, metastatic to liver	0/1
	Colon, mucinous	0/1
	Esophagus	0/1
	Gallbladder	1/5
	GI, metastatic to lung	0/1
	Head & neck, hard palate	0/1
	Lung	1/4
	Ovary	0/1
	Ovary, endometrioid	0/1
	Ovary, mucinous	0/1
	Ovary, serous	0/1
	Pancreas	0/2
	Pancreas, ductal	0/3
	Prostate	0/5
	Rectum	0/4
	Salivary/parotid gland	0/2
	Small intestine	0/2
	Stomach	0/6
	Stomach, mucinous	0/1
	Thyroid, follicular	0/1
	Thyroid, follicular-papillary	0/1
	Thyroid, papillary	0/3
	Uterus, clear cell	0/1
	Uterus, endometrium	0/3
Adrenocortical carcinoma	Adrenal	0/1
Astrocytoma	Cerebrum	0/3
Basal cell carcinoma	Skin	0/1
Carcinoma	Nasopharyngeal, NPC	0/1
Chondrosarcoma	Bone	0/1
Chordoma	Pelvic cavity	0/1
Embryonal carcinoma	Testis	0/1
Ependymoma	Brain	0/1
Glioblastoma	Brain	0/1
Hepatoblastoma	Liver	0/1
Hepatocellular carcinoma	Liver	0/5
Islet Cell tumor	Pancreas	0/1
Interstitialoma	Colon	0/1
	Rectum	0/1
	Small intestine	0/1
Leiomyosarcoma	Soft tissue, chest wall	0/1
	Bladder	0/1
Lymphoma		
Anaplastic Large Cell	Lymph node	0/1
Diffuse B-cell	Lymph node	0/4
Hodgkin	Lymph node	2/2
Non-Hodgkin	Lymph node	1/1
Medulloblastoma	Brain	0/1
Medullary carcinoma	Thyroid	0/1
Melanoma	Rectum	0/1
	Nasal cavity	0/1
Meningioma	Brain	0/2
Mesothelioma	Peritoneum	0/1
Neuroblastoma	Retroperitoneum	0/1
Neurofibroma	Soft tissue, lower back	0/1
Osteosarcoma	Bone	0/2
Pheochromocytoma	Adrenal	0/1
Primitive Neuroectodermal Tumor (PNET)	Retroperitoneum	0/1
Renal Cell carcinoma		
Papillary	Kidney	0/1

Tumor Type	Location	PD-L1 positive/total N=159
Clear Cell	Kidney	0/6
Rhabdomyosarcoma	Soft tissue, embryonal	0/1
	Prostate	0/1
	Retroperitoneum	0/1
Seminoma	Testis	0/2
Signet Ring Cell carcinoma	Metastatic colon signet ring cell carcinoma to ovary	0/1
	Colon	0/1
Small cell carcinoma	Lung	0/1
Spermatocytoma	Testis	0/2
Squamous Cell carcinoma	Metastatic esophageal squamous cell carcinoma to lymph node	0/1
	Cervix	2/5
	Esophagus	0/7
	Head & neck	0/2
	Lung	1/2
	Skin	0/2
	Uterus	0/1
Synovial Sarcoma	Pelvic cavity	0/1
Thymoma	Mediastinum	1/1
Transitional Cell carcinoma	Bladder	0/6
	Kidney	0/1

Troubleshooting

Table 8: Troubleshooting

Problem	Probable Cause	Suggested Action
1. No staining of slides	1a. Programming error.	1a. Verify that the PD-L1 IHC 22C3 pharmDx program was selected for programming of slides.
	1b. Lack of reaction with DAB+ Substrate-Chromogen Solution (DAB)	1b. Verify that DAB+ Substrate-Chromogen Solution was prepared properly.
	1c. Sodium azide in wash buffer.	1c. Use only Dako Wash Buffer (Code K8007).
	1d. Degradation of Control Slide	1d. Check kit expiration date and kit storage conditions on outside of package.
2a. Weak staining of specimen slides.	2a. Inappropriate fixation method used.	2a. Ensure that only approved fixatives and fixation methods are used.
2b. Weak staining of specimen slides or of the positive cell line on the Dako-provided Control Slide.	2b. Inadequate target retrieval.	2b. Verify that the 3-in-1 pre-treatment procedure was correctly performed.
3. Excessive background staining of slides.	3a. Paraffin incompletely removed.	3a. Verify that the 3-in-1 pre-treatment procedure was correctly performed.
	3b. Slides dried while loading onto Autostainer Link 48.	3b. Ensure slides remain wet with buffer while loading and prior to initiating run.
	3c. Nonspecific binding of reagents to tissue section.	3c. Check for proper fixation of the specimen and/or the presence of necrosis.
4. Tissue detached from slides.	4. Use of incorrect microscope slides.	4. Use Dako FLEX IHC Microscope Slides (Code K8020), or charged slides (such as Fisherbrand Superfrost Plus).
5. Excessively strong specific staining.	5a. Inappropriate fixation method used.	5a. Ensure that only approved fixatives and fixation methods are used.
	5b. Inappropriate wash buffer used.	5b. Use only Dako Wash Buffer (Code K8007).
6. Target Retrieval Solution is cloudy in appearance when heated.	6. When heated the Target Retrieval Solution turns cloudy in appearance.	6. This is normal and does not influence staining.

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 call Dako Technical Support for further assistance. Additional information on staining techniques and specimen preparation can be found in Dako Education Guide: Immunohistochemical Staining Methods (11) (available from Dako).

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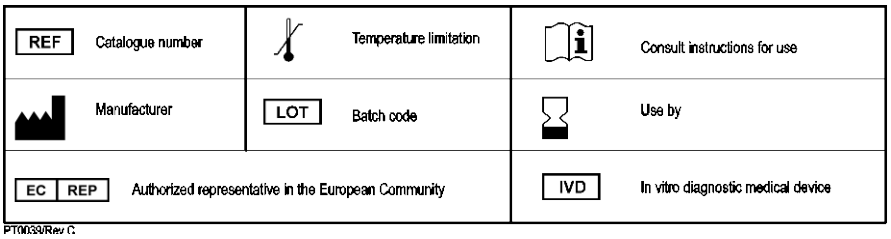

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