

PD-L1 IHC 28-8 pharmDx

SK005

50 tests for use with Autostainer Link 48

Intended Use

For in vitro diagnostic use.

PD-L1 IHC 28-8 pharmDx is a qualitative immunohistochemical assay using Monoclonal Rabbit Anti-PD-L1, Clone 28-8 intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) non-squamous non-small cell lung cancer (NSCLC) tissue using EnVision FLEX visualization system on Autostainer Link 48. PD-L1 protein expression is defined as the percentage of tumor cells exhibiting positive membrane staining at any intensity.

PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in non-squamous NSCLC may be associated with enhanced survival from OPDIVO® (nivolumab).

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 (1). OPDIVO® (nivolumab) is a human immunoglobulin G4 (IgG4) 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 (2). In syngeneic mouse tumor models, blocking PD-1 activity resulted in decreased tumor growth (3).

Detection of PD-L1 expressing tumor cells in a non-squamous non-small cell lung cancer patient specimen may indicate an enhanced survival benefit to OPDIVO® (nivolumab) treatment for the patient. Specimens from patients in OPDIVO® clinical studies sponsored by Bristol-Myers Squibb were tested using PD-L1 IHC 28-8 pharmDx. The studies investigated the clinical validity of PD-L1 IHC 28-8 pharmDx for the assessment of PD-L1 status in patients treated with OPDIVO®.

OPDIVO® is a trademark owned by Bristol-Myers Squibb.

Principle of Procedure

PD-L1 IHC 28-8 pharmDx contains optimized reagents and protocol required to complete an IHC staining procedure of FFPE specimens using Autostainer Link 48 and Dako PT Link Pre-treatment Module (4). 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 the 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. Control Slides containing two formalin-fixed, paraffin-embedded human cell lines are provided to validate staining runs.

Materials Provided

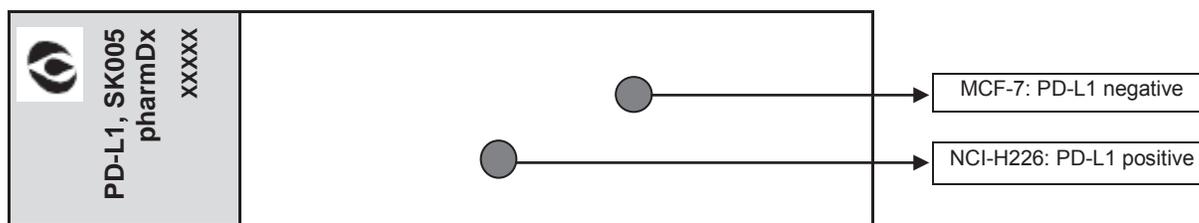
PD-L1 IHC 28-8 pharmDx (Code SK005) is for automated staining using Autostainer Link 48.

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 test 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 <div style="border: 1px solid black; padding: 2px; display: inline-block;">PEROXIDASE-BLOCKING REAGENT</div> Buffered solution containing hydrogen peroxide, detergent and 0.015 mol/L sodium azide.
1 x 19.5 mL	Primary Antibody: Monoclonal Rabbit Anti-PD-L1, Clone 28-8 <div style="border: 1px solid black; padding: 2px; display: inline-block;">MONOCLONAL RABBIT ANTI-PD-L1 CLONE 28-8</div> Monoclonal rabbit anti-PD-L1 in a buffered solution, containing stabilizing protein, and 0.015 mol/L sodium azide.
1 x 15 mL	Negative Control Reagent <div style="border: 1px solid black; padding: 2px; display: inline-block;">NEGATIVE CONTROL REAGENT</div> Monoclonal rabbit control IgG antibody in a buffered solution, containing stabilizing protein, and 0.015 mol/L sodium azide.

- 1 x 34.5 mL **LINKER, Anti-Rabbit**
LINKER, ANTI-RABBIT
 Mouse secondary antibody against rabbit 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 an 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 IHC 28-8 pharmDx Control Slides**
CONTROL SLIDES
 Each slide contains sections of two pelleted, formalin-fixed paraffin-embedded cell lines: NCI-H226 with positive 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-L1 IHC 28-8 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 (NaN₃), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, NaN₃ 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 (5).

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 (6).
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. Large tissue sections may require 3x150µl of reagent.
12. 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.
13. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
14. Unused solution should be disposed of according to local, State and Federal regulations.
15. 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 28-8 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. Recommended handling and processing conditions are: <30 minutes ischemia time prior to immersion in fixative, and 24- 48 hours fixation time in neutral buffered formalin. Alternative fixatives have not been validated and may give erroneous results. 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.

Tissue specimens should be cut into sections of 4-5µm. After sectioning, tissues should be mounted on Fisherbrand Superfrost Plus charged slides or Dako FLEX IHC microscope slides (Code K8020) and then placed in a 58 ± 2 °C oven for 1 hour. To preserve antigenicity, tissue sections, once mounted on slides, should be stored in the dark at 2-8 °C and stained within 4 months of sectioning.

The use of PD-L1 IHC 28-8 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 should be 6.1 ± 0.2 . 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 °C 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 28-8 pharmDx staining protocol from the options in the DakoLink drop down menu.

All of the required steps and incubation times for staining are preprogrammed in the DakoLink. If the appropriate PD-L1 IHC 28-8 pharmDx protocols are not on your server please contact your local Technical Service Representative.

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 are counterstained for 7 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 28-8 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 (refer to Table 1).

Differences in tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results, necessitating regular performance of lab-supplied controls in addition to the Control Slides supplied by Dako (7). 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 (8) for additional information.

Table 1: The Purpose of Daily Quality Control

Specimen	Reagents	Purpose
Lab-supplied Positive Control: Tissue 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.
Lab-supplied Negative Control: Tissues 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.
Lab-supplied Patient tissue stained with Negative Control Reagent	Negative Control Reagent & same Detection System as used with the Primary Antibody	Detection of non-specific background staining.

Lab-supplied Positive Control Tissue

Controls should be 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.

Lab-supplied 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.

Control Slide (provided)

Each slide contains sections of two pelleted, formalin-fixed paraffin-embedded cell lines: NCI-H226 with positive 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 by Dako indicates the validity of the staining run. They should not be used as an aid in interpretation of patient results.

Negative Control Reagent

Use the supplied Negative Control Reagent in place of the primary antibody on a sequential section of each patient specimen, positive control tissue, and negative control tissue 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 (3). These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters.

Staining Interpretation – Non-Squamous NSCLC

A hematoxylin and eosin (H&E) stain of the tissue specimen is evaluated first. An H&E stain and PD-L1 IHC 28-8 pharmDx should be performed on serial sections from the same paraffin block of the specimen. The H&E stained slide is used to assist with assessment of histology and preservation quality.

All viable tumor cells on the entire PD-L1 stained patient slide must be evaluated and included in the PD-L1 scoring assessment. A minimum of 100 viable tumor cells should be present in the PD-L1 stained patient slide to determine the percentage of stained cells.

Slide evaluation must be performed by a pathologist using a light microscope. For evaluation of the PD-L1 immunohistochemical staining and scoring, 4x objective magnification can be used for initial assessment of the entire specimen, followed by the 10-40x objectives for scoring.

PD-L1-positive staining is defined as complete circumferential and/or partial linear plasma membrane staining of tumor cells at any intensity. Cytoplasmic staining, if present, is not considered positive for scoring purposes. The diagnostic status of the patient tissue slide is derived by determination of the percentage of positive tumor cells at any staining intensity present in the entire specimen. Non-malignant cells and immune cells (e.g., such as infiltrating lymphocytes or macrophages) may also stain with PD-L1; however, these should not be included in the scoring for the determination of PD-L1 positivity.

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 28-8 pharmDx Interpretation Manual Non-Squamous Non-Small Cell Lung Cancer for additional guidance.

Table 2: Recommended Order of Slide Evaluation

Slide Order	Rationale
1. H&E	A hematoxylin and eosin (H&E) stain of the tissue specimen is evaluated first. The PD-L1 IHC 28-8 pharmDx and H&E stain should be performed on serial sections from the same paraffin block of the specimen. The H&E stained slide is used to assist with assessment of histology and preservation quality.
2. Control Slide containing the PD-L1 positive and PD-L1 negative cell lines	The Control Slide stained with the PD-L1 primary antibody from PD-L1 IHC 28-8 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 plasma membrane is indicative of positive reactivity. <i>NCI-H226 (PD-L1-positive control cell line) acceptance criteria:</i> <ul style="list-style-type: none">• Plasma membrane staining of $\geq 80\%$ of cells at $\geq 2+$ average staining intensity.• Non-specific staining $< 1+$ intensity. <i>MCF-7 (PD-L1-negative control cell line) acceptance criteria:</i> <ul style="list-style-type: none">• No specific staining.• Non-specific staining $< 1+$ intensity. <ul style="list-style-type: none">❖ <i>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, or cytoplasmic staining with $\geq 1+$ intensity within the boundaries of the MCF-7 cell pellet are acceptable.</i>➤ If either of the Control Cell Lines does not meet these criteria, all results with the patient specimens should be considered invalid.
3. Positive Control Tissue Slides	The Positive Control Tissue Slides stained with both PD-L1 primary antibody and NCR should be examined next. These slides verify 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 $\leq 1+$.
4. Negative Control Tissue Slides	The Negative Control Tissue Slides stained with both PD-L1 primary antibody and NCR should be examined after the Positive Control Tissue to verify the specificity of the labeling of the target antigen by the primary antibody. Alternatively, negative portions of the Positive Control Tissue may serve as the Negative Control Tissue, but this should be verified by the user. If specific plasma membrane staining occurs in the Negative Control Tissue Slides, results with the patient specimen should be considered invalid. Non-specific staining should be $\leq 1+$.
5. Patient tissue slide stained using the Negative Control Reagent	Examine patient specimens stained with the Negative Control Reagent from PD-L1 IHC 28-8 pharmDx. Absence of plasma 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. Non-specific staining should be $\leq 1+$.
6. 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 28-8 pharmDx last. Positive staining intensity should be assessed within the context of any non-specific background staining observed in the Negative Control Reagent slide in 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 Evaluation for specific information regarding PD-L1 IHC 28-8 pharmDx immunoreactivity. Complete circumferential and/or partial linear plasma membrane staining of tumor cells indicates positive PD-L1 staining. Cytoplasmic staining is not considered positive for scoring purposes.

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 (9).
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) (7).
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 four 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 other 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 PD-L1 IHC 28-8 pharmDx on tissues with fixatives other than formalin has not been validated.

Non-clinical Performance Evaluation

Analytical Specificity

The primary antibody for PD-L1 IHC 28-8 pharmDx is a rabbit monoclonal anti-human PD-L1, clone 28-8. The immunogen used for the antibody generation is a purified recombinant human PD-L1 containing the extracellular domain (Phe19-Thr239) of human PD-L1. IHC staining with the PD-L1 primary antibody showed no cross-reactivity for PD-L2 exogenously expressed in Chinese hamster ovary (CHO) cells.

PD-L1 IHC 28-8 pharmDx specifically detects PD-L1 membrane protein expressed in tumor cells in FFPE blocks, which can be completely abolished by the addition of PD-L1 antigen to the protein-rich antibody diluents. PD-L1 IHC 28-8 pharmDx detects no PD-L1 positive staining in PD-L1 knock-out tumor cells when PD-L1 gene is genetically deleted. A survey of PD-L1 detection by PD-L1 IHC 28-8 pharmDx in 30 FFPE human specimens is provided in Table 5.

Analytical Sensitivity

Analytical sensitivity of PD-L1 IHC 28-8 pharmDx was tested on 112 unique cases of non-squamous 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 and External Reproducibility of PD-L1 IHC 28-8 pharmDx was evaluated at Dako and three external testing sites respectively. The performance data are provided in Table 3 and Table 4. Negative percent agreement (NPA), positive percent agreement (PPA) and overall percent agreement (OA) of independent pair-wise comparison of the tests were determined for each PD-L1 expression level evaluated. The most frequently occurring observation was applied as a reference to calculate the NPA, PPA, OA and the corresponding 95% Wilson Score confidence intervals, therefore one of the results with this observation was excluded from the pair-wise comparisons.

Table 3: Repeatability of PD-L1 IHC 28-8 pharmDx: Non-squamous NSCLC

Repeatability	Method	% Agreement (95% CI)		
		≥1% Expression Level	≥5% Expression Level	≥10% Expression Level
Inter-instrument	Each of 10 non-squamous NSCLC specimens with a range of PD-L1 IHC expression was tested with three replicates on each of three Autostainer Link 48 instruments. A total of 60 independent pair-wise comparisons were performed.	NPA 100 (82.4, 100) PPA 100 (91.6, 100) OA 100 (94.0, 100)	NPA 100 (86.2, 100) PPA 100 (90.4, 100) OA 100 (94.0, 100)	NPA 100 (91.6, 100) PPA 100 (82.4, 100) OA 100 (94.0, 100)
Inter-analyst	Each of 12 non-squamous NSCLC specimens with a range of PD-L1 IHC expression was tested with three replicates by three analysts on one Autostainer Link 48 instrument. A total of 72 independent pair-wise comparisons were performed.	NPA 100 (86.2, 100) PPA 100 (92.6, 100) OA 100 (94.9, 100)	NPA 100 (91.6, 100) PPA 100 (88.6, 100) OA 100 (94.9, 100)	NPA 100 (93.4, 100) PPA 100 (82.4, 100) OA 100 (94.9, 100)
Inter-day	Each of 10 non-squamous NSCLC specimens with a range of PD-L1 IHC expression was tested with three replicates over five non-consecutive days on the Autostainer Link 48 instrument. A total of 80 independent pair-wise comparisons were performed.	NPA 100 (86.2, 100) PPA 100 (93.6, 100) OA 100 (95.4, 100)	NPA 100 (89.3, 100) PPA 100 (92.6, 100) OA 100 (95.4, 100)	NPA 98.2 (90.6, 99.7) PPA 100 (86.2, 100) OA 98.8 (93.3, 99.8)
Inter-lot	Each of 20 non-squamous NSCLC specimens with a range of PD-L1 IHC expression was tested with two replicates with each of five reagent lots on the Autostainer Link 48 instrument. A total of 160 independent pair-wise comparisons were performed.	NPA 100 (94.3, 100) PPA 100 (96.2, 100) OA 100 (97.7, 100)	NPA 100 (95.4, 100) PPA 100 (95.4, 100) OA 100 (97.7, 100)	NPA 100 (96.4, 100) PPA 100 (93.6, 100) OA 100 (97.7, 100)
Intra-run	Each of 10 non-squamous NSCLC specimens with a range of PD-L1 IHC expression was tested with eight replicates within a run on the Autostainer Link 48 instrument. A total of 70 independent pair-wise comparisons were performed.	NPA 100 (84.5, 100) PPA 100 (92.7, 100) OA 100 (94.8, 100)	NPA 100 (87.9, 100) PPA 100 (91.6, 100) OA 100 (94.8, 100)	NPA 100 (92.7, 100) PPA 100 (84.5, 100) OA 100 (94.8, 100)

Table 4: Reproducibility of the PD-L1 IHC 28-8 pharmDx tested at three external sites

Reproducibility	Method	% Agreement (95% CI)	
		≥1% Expression Level	≥5% Expression Level
Inter-site assay (three sites)	Each of 10 non-squamous NSCLC 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 140 independent pair-wise comparisons.	NPA 100 (93.6, 100) PPA 98.8 (93.6, 99.8) OA 99.3 (96.1, 99.9)	NPA 91.4 (82.5, 96.0) PPA 97.1 (90.2, 99.2) OA 94.3 (89.1, 97.1)
Intra-site assay	Each of 10 non-squamous NSCLC 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 120 independent pair-wise comparisons.	NPA 100 (92.6, 100) PPA 98.6 (92.5, 99.8) OA 99.2 (95.4, 99.9)	NPA 96.4 (87.9, 99.0) PPA 95.3 (87.1, 98.4) OA 95.8 (90.6, 98.2)
Inter-observer (one observer at each of three sites)	Scoring of 15 non-squamous NSCLC specimens with a range of PD-L1 IHC expression, stained with PD-L1 IHC 28-8 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 120 independent pair-wise comparisons.	NPA 96.9 (89.3, 99.1) PPA 100 (93.6, 100) OA 98.3 (94.1, 99.5)	NPA 100 (94.3, 100) PPA 89.3 (78.5, 95.0) OA 95.0 (89.5, 97.7)
Intra-observer (one observer at each of three sites)	Scoring of 15 non-squamous NSCLC specimens with a range of PD-L1 IHC expression, stained with PD-L1 IHC 28-8 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 90 independent pair-wise comparisons.	NPA 95.8 (86.0, 98.8) PPA 100 (91.6, 100) OA 97.8 (92.3, 99.4)	NPA 100 (93.1, 100) PPA 100 (90.8, 100) OA 100 (95.9, 100)

Normal and Neoplastic tissues

Table 5 summarizes Monoclonal Rabbit Anti-Human PD-L1 antibody immunoreactivity on the recommended panel of normal tissues. Table 6 summarizes Monoclonal Rabbit Anti-Human PD-L1 antibody immunoreactivity on neoplastic tissues in multi-tumor tissue microarrays. All tissues were formalin-fixed and paraffin-embedded and stained with PD-L1 IHC 28-8 pharmDx according to the instructions in the package insert. PD-L1 IHC 28-8 pharmDx detected PD-L1 protein localized in the plasma membrane of cell types known to express the PD-L1 antigen such as immune cells and cells of epithelial origin mainly tumor cells.

Table 5: Summary of PD-L1 IHC 28-8 pharmDx Normal Tissue Reactivity

Tissue Type (# tested)	Positive Plasma Membrane Staining: Tissue Elements	Positive Cytoplasmic Staining: Tissue Elements
Adrenal (3)	3/3 Medullary cells	3/3 Medullary cells
Bone marrow (3)	3/3 Megakaryocytes	3/3 Megakaryocytes
Breast (3)	0/3	0/3
Cerebellum (3)	0/3	0/3
Cerebrum (3)	0/3	0/3
Cervix (3)	1/3 Epithelium	1/3 Epithelium
Colon (3)	2/3 Macrophages	0/3
Esophagus (3)	0/3	0/3
Kidney (3)	3/3 Tubular epithelium	3/3 Tubular epithelium
Liver (3)	2/3 Immune cells	2/3 Immune cells
Lung (3)	3/3 Alveolar macrophages	0/3
Mesothelial cells (3)	0/3	0/3
Muscle, cardiac (3)	0/2	0/2
Muscle, skeletal (3)	0/2	0/2
Nerve, peripheral (3)	0/3	0/3
Ovary (3)	0/3	0/3
Pancreas (3)	3/3 Epithelium (mainly islet cells)	3/3 Epithelium (mainly islet cells)
Parathyroid (3)	3/3 Epithelium	0/3
Pituitary (3)	1/3 Anterior adenohypophysis	1/3 Anterior adenohypophysis 3/3 Posterior neurohypophysis
Prostate (2)	0/2	0/2
Salivary gland (3)	0/3	0/3
Skin (3)	0/3	1/3 Epithelium
Small intestine (2)	0/2	0/2
Spleen (3)	1/3 Macrophages 3/3 Littoral cell	0/3
Stomach (3)	0/3	0/3
Testis (3)	0/3	1/3 Leydig cells
Thymus (3)	3/3 Medullary epithelium	0/3
Thyroid (3)	0/3	0/3
Tonsil (3)	3/3 Crypt epithelium 3/3 Germinal center (immune cells)	0/3
Uterus (3)	0/3	0/3

Table 6: Summary of PD-L1 IHC 28-8 pharmDx Neoplastic Tissue Reactivity

Tumor Type	Location / Organ	PD-L1 positive/total (N=162)
Adenocarcinoma	Appendix	1/1
	Breast, DCIS	0/2
	Breast, invasive ductal	3/7
	Breast, invasive ductal metastatic to lymph node	1/1
	Bronchoalveolar carcinoma, lung	0/1
	Cervix, endocervical type	0/1
	Colon	2/5
	Colon, metastatic to liver	1/1
	Colon, mucinous	0/1
	Esophagus	1/1
	Gallbladder	2/4
	GI, metastatic to lung	0/1
	Head & neck, hard palate	0/1
	Lung	2/5
	Ovary	0/1
	Ovary, endometrioid	0/1
	Ovary, mucinous	0/1
	Ovary, serous	0/1
	Pancreas	1/2
	Pancreas, ductal	0/3
	Prostate	2/4
	Rectum	2/4
	Salivary/parotid gland	0/2
Small Intestine	0/2	

Tumor Type	Location / Organ	PD-L1 positive/total (N=162)
	Stomach	1/6
	Stomach, mucinous	0/1
	Thyroid, follicular	0/1
	Thyroid, follicular-papillary	0/1
	Thyroid, papillary	0/3
	Uterus, clear cell	1/1
	Uterus, endometrium	1/3
Adrenocortical carcinoma	Adrenal	0/1
Astrocytoma	Cerebrum	0/3
Basal cell carcinoma	Skin	0/1
Carcinoma	Nasopharyngeal, NPC	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	1/5
Islet cell tumor	Pancreas	0/1
Interstitialoma	Colon	0/1
	Rectum	0/1
	Small intestine	0/1
Large cell carcinoma	Lung	1/1
Liposarcoma	Abdominal cavity, mucinous	0/1
Lymphoma		
Anaplastic large cell	Lymph node	1/1
Diffuse B-cell	Lymph node	2/4
Hodgkin	Lymph node	2/2
Non-Hodgkin	Lymph node	1/1
Medullablastoma	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
Primitive neuroectodermal	Retroperitoneum	0/1
Renal cell carcinoma		
Papillary	Kidney	0/1
Clear cell	Kidney	0/6
Sarcoma		
Chondrosarcoma	Bone	0/1
Clear cell	Abdominal wall	0/1
Osteosarcoma	Bone	0/2
Leiomyosarcoma	Soft tissue, chest wall	0/1
	Bladder	0/1
Liposarcoma	Abdominal cavity, mucinous	0/1
Rhabdomyosarcoma	Soft tissue, embryonal	0/1
	Prostate	0/1
	Retroperitoneum	0/1
Synovial sarcoma	Pelvic cavity	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	1/2
Spermatocytoma	Testis	0/2
Squamous cell carcinoma	Metastatic esophageal squamous cell carcinoma to lymph node	1/1
	Cervix	2/4
	Esophagus	4/7
	Head & neck	0/2
	Lung	1/3
	Skin	1/2
Uterus	1/1	
Thymoma	Mediastinum	1/1
Transitional cell carcinoma	Bladder	3/6
	Kidney	0/1

Clinical Performance Evaluation

Clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in CA209057, a Phase 3, randomized, open-label study of nivolumab vs docetaxel in adult (≥ 18 years) subjects with advanced or metastatic non-squamous cell NSCLC after failure of prior platinum doublet -based chemotherapy. A total of 582 subjects were randomized at 112 sites in 22 countries (Argentina, Australia, Austria, Brazil, Canada, Chile, Czech Republic, France, Germany, Hong Kong, Hungary, Italy, Mexico, Norway, Peru, Poland, Romania, Russian Federation, Singapore, Spain, Switzerland, and United States). Subjects were randomized 1:1 and stratified according to 1) prior use of maintenance therapy vs. no use of maintenance therapy and 2) second-line vs. third-line therapy. Pre-study (baseline) tumor tissue specimens were collected prior to randomization and prior to first treatment to conduct pre-planned analyses of efficacy according to predefined baseline PD-L1 expression levels (secondary objective). The primary endpoint was overall survival (OS). Other Secondary endpoints were objective response rate (ORR), progression-free survival (PFS), and disease-related symptom improvement by 12 weeks, as measured by the Lung Symptom Cancer Scale (LCSS).

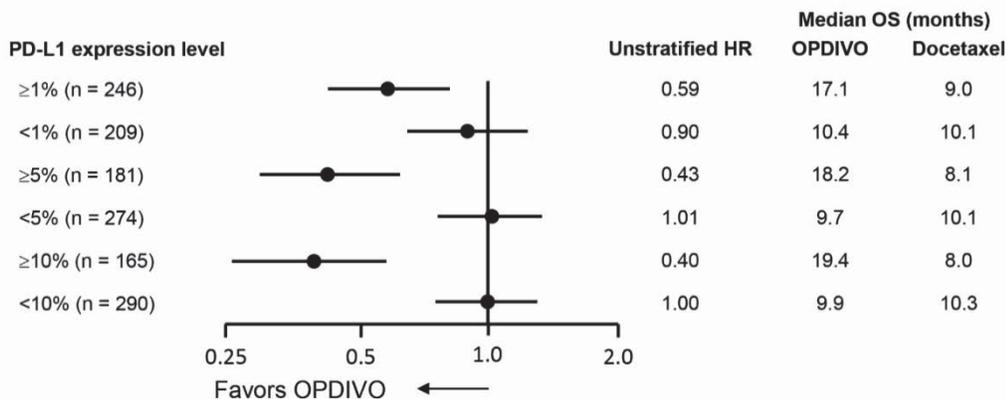
The baseline demographic and disease characteristics were generally balanced between randomized subjects in the nivolumab and docetaxel groups. The mean age was 62 years (range: 21 to 85) with 34% ≥ 65 years of age and 7% ≥ 75 years of age. The majority of patients were white (92%) and male (55%); baseline ECOG performance status was 0 (31%) or 1 (69%). Seventy-nine percent of patients were former/current smokers. Tumor specimens were collected from non-squamous NSCLC tumors, consistent with the inclusion requirements for the study. Frequencies of PD-L1 expression at each of the predefined baseline expression levels in all randomized subjects in CA209057 are presented in Table 7.

Table 7: Frequency of Pre-Study PD-L1 Expression in All Randomized Subjects - CA209057

Population PD-L1 Expression Category	Nivolumab 3 mg/kg N = 292	Docetaxel N = 290	Total N = 582
OVERALL	292	290	582
PD-L1 QUANTIFIABLE AT BASELINE (N(%))	231 (79.1)	224 (77.2)	455 (78.2)
BASELINE PD-L1 EXPRESSION $\geq 1\%$	123/231 (53.2)	123/224 (54.9)	246/455 (54.1)
BASELINE PD-L1 EXPRESSION $< 1\%$	108/231 (46.8)	101/224 (45.1)	209/455 (45.9)
BASELINE PD-L1 EXPRESSION $\geq 5\%$	95/231 (41.1)	86/224 (38.4)	181/455 (39.8)
BASELINE PD-L1 EXPRESSION $< 5\%$	136/231 (58.9)	138/224 (61.6)	274/455 (60.2)
BASELINE PD-L1 EXPRESSION $\geq 10\%$	86/231 (37.2)	79/224 (35.3)	165/455 (36.3)
BASELINE PD-L1 EXPRESSION $< 10\%$	145/231 (62.8)	145/224 (64.7)	290/455 (63.7)
PD-L1 Non-QUANTIFIABLE (N(%))	61 (20.9)	66 (22.8)	127 (21.8)

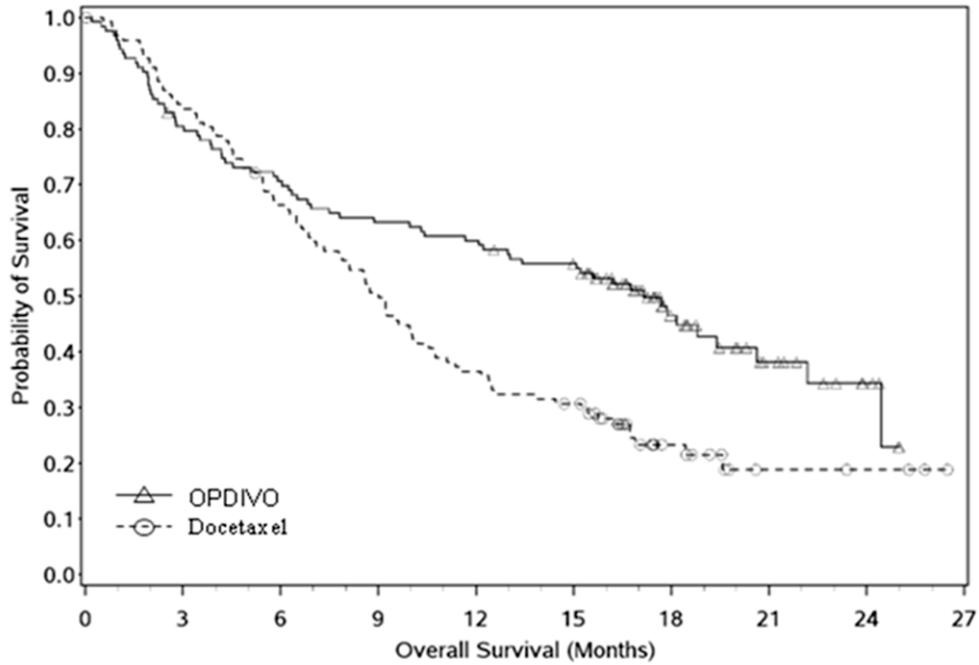
Patients with PD-L1 expression by all predefined expression levels in the OPDIVO[®] group were associated with enhanced survival compared to docetaxel, whereas survival was similar to docetaxel in patients with no PD-L1 expression. Meaningful differences in median OS were observed in nivolumab over docetaxel subgroups when analyzed by PD-L1 expression level. Median OS was 17.1, 18.2, and 19.4 months for nivolumab subjects compared to 9.0, 8.1, and 8.0 months for docetaxel subjects with $\geq 1\%$, $\geq 5\%$, and $\geq 10\%$ PD-L1 expression levels, respectively. There were no differences in OS between the treatment groups in subjects with $< 1\%$, $< 5\%$, and $< 10\%$ expression levels, with ranges of median OS of 9.7 to 10.4 months for nivolumab and 10.1 to 10.3 months for docetaxel. The unstratified hazard ratios (HR) and median overall survival (OS) are presented in Figure 1. The Kaplan-Meier plot for subgroups by PD-L1 expression level is shown in Figure 2 and Figure 3.

Figure 1: Forest Plot - OS Based on PD-L1 Expression - CA209057



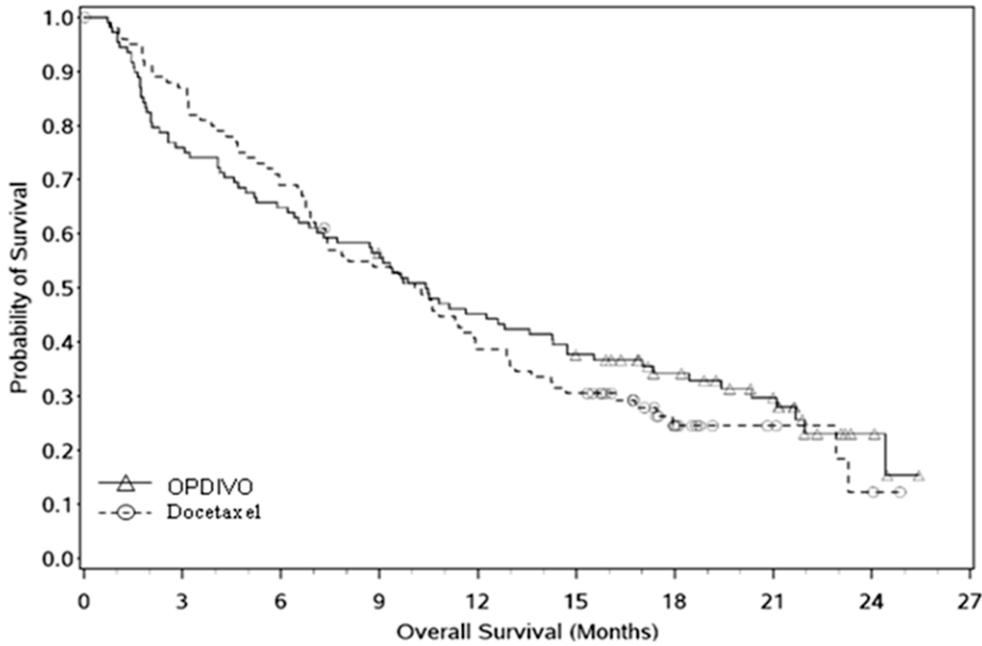
Note: The unstratified hazard ratio and the corresponding 95% CI were estimated in a Cox proportional hazards model using the randomized arm as a single covariate

Figure 2. Overall Survival: Patients with $\geq 1\%$ PD-L1 Expression - CA209057



Number at Risk										
OPDIVO	123	98	86	77	73	65	27	13	5	0
Docetaxel	123	102	80	61	44	36	13	4	3	0

Figure 3. Overall Survival: Patients with $< 1\%$ PD-L1 Expression - CA209057



Number at Risk										
OPDIVO	108	82	70	60	48	39	26	17	4	0
Docetaxel	101	87	69	53	38	30	13	5	2	0

Table 8: Troubleshooting

Problem	Probable Cause	Suggested Action
1. No staining of slides	1a. Programming error.	1a. Verify that the SK005 PD-L1 IHC 28-8 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 the 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.	4a. Use of incorrect microscope slides.	4a. Use Dako FLEX IHC Microscope Slides, (Code K8020), or charged slides (such as Fisherbrand Superfrost Plus).
	4b. Inadequate preparation of specimens.	4b. Cut sections should be placed in a 58 ± 2 °C oven for 1 hour prior to staining.
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 (10) (available from Dako).

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Catalogue number	Temperature limitation	In vitro diagnostic medical device
Manufacturer	Batch code	Contains sufficient for <N> tests
Use by	Consult instructions for use	Authorized representative in the European Community

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