Agilent Dako

CE

PD-L1 IHC 22C3 pharmDx

SK006

50 tests for use with Autostainer Link 48

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SK006
50 tests for use with Autostainer Link 48

1. 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), urothelial carcinoma, esophageal cancer, head and neck squamous cell carcinoma (HNSCC), triple-negative breast cancer (TNBC), cervical cancer, and melanoma tissues using EnVision FLEX visualization system on Autostainer Link 48.

PD-L1 protein expression in NSCLC is determined by using Tumor Proportion Score (TPS), which is the percentage of viable tumor cells showing partial or complete membrane staining at any intensity.

PD-L1 protein expression in urothelial carcinoma, esophageal cancer, TNBC, and cervical cancer is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100.

PD-L1 protein expression in HNSCC is determined by using CPS and/or TPS.

PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying patients for treatment with the therapies for the indications listed in Table 1.

Table 1. PD-L1 IHC 22C3 pharmDx companion diagnostic indications, PD-L1 expression levels, and therapies

Tumor Indication	PD-L1 Expression Level	Therapy
NSCLC	TPS ≥ 1%	KEYTRUDA® (pembrolizumab)*
	TPS ≥ 50%	
Urothelial Carcinoma	CPS ≥ 10	
Esophageal Cancer	CPS ≥ 10	
HNSCC	CPS ≥ 1	
	TPS ≥ 50%	
TNBC	CPS ≥ 10	
Cervical Cancer	CPS ≥ 1	
NSCLC	TPS ≥ 50%	LIBTAYO® (cemiplimab)**

*See the KEYTRUDA® product label for PD-L1 expression cutoff values and specific clinical circumstances guiding therapy.
**See the LIBTAYO® product label for specific clinical circumstances guiding therapy.

2. Summary and Explanation

2.1 KEYTRUDA (pembrolizumab)

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 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.[1] In syngeneic mouse tumor models, blocking PD-1 activity resulted in decreased tumor growth.[2]

2.1.1 NSCLC (KEYTRUDA)

Merck Sharp & Dohme LLC, Rahway, NJ, USA (hereinafter "MSD") sponsored clinical study, KEYNOTE-024 (KN024), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (TPS ≥ 50%) previously untreated metastatic NSCLC patients that may respond to KEYTRUDA treatment.[3] PD-L1 expressing (TPS

≥ 50%) metastatic NSCLC patients treated with KEYTRUDA displayed improved outcomes compared to standard of care chemotherapy. MSD sponsored clinical study, KEYNOTE-042 (KN042), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (TPS ≥ 1%) previously untreated locally advanced or metastatic NSCLC patients that may respond to KEYTRUDA treatment where the benefit shown in KN024 was confirmed.[4] Refer to 'Clinical performance evaluation: NSCLC (KEYTRUDA)' Section 16.3 for KN024 and KN042 study details.

MSD sponsored clinical study, KEYNOTE-010 (KN010), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (TPS ≥ 1%) previously treated advanced, metastatic NSCLC patients that may respond to KEYTRUDA treatment.[5] PD-L1 expressing (TPS ≥ 1%) advanced, metastatic NSCLC patients treated with KEYTRUDA displayed improved outcomes compared to docetaxel. Refer to 'Clinical performance evaluation: NSCLC (KEYTRUDA)' Section 16.3 for KN010 study details.

2.1.2 Urothelial carcinoma (KEYTRUDA)

MSD sponsored clinical study, KEYNOTE-052 (KN052), investigated the clinical performance of PD-L1 IHC 22C3 pharmDx in previously untreated, cisplatin ineligible, locally advanced or metastatic urothelial carcinoma patients that may respond to KEYTRUDA treatment.[6] Clinical validity was determined in PD-L1 expressing patients at CPS ≥ 10. Refer to 'Clinical performance evaluation: urothelial carcinoma (KEYTRUDA)' Section 16.6 for KN052 study details.

MSD sponsored clinical study, KEYNOTE-045 (KN045), investigated the clinical performance of PD-L1 IHC 22C3 pharmDx in locally advanced or metastatic urothelial carcinoma patients with disease progression on or after platinum-containing chemotherapy that may respond to KEYTRUDA treatment.[7] Refer to 'Clinical performance evaluation: urothelial carcinoma (KEYTRUDA)' Section 16.6 for KN045 study details.

2.1.3 Esophageal cancer (KEYTRUDA)

MSD sponsored clinical study, KEYNOTE-590 (KN590), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (CPS ≥ 10) patients with locally advanced unresectable or metastatic esophageal carcinoma or HER-2 negative gastroesophageal junction carcinoma that may respond to first-line treatment of KEYTRUDA in combination with platinum and fluoropyrimidine based chemotherapy.[8] Refer to 'Clinical performance evaluation: esophageal cancer (KEYTRUDA)' Section 16.8 for KN590 study details.

2.1.4 HNSCC (KEYTRUDA)

MSD sponsored clinical study, KEYNOTE-048 (KN048), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (CPS ≥ 1) patients with recurrent or metastatic HNSCC who had not previously received systemic therapy for recurrent or metastatic disease and who were considered incurable by local therapies, and who may respond to KEYTRUDA treatment.[9] Refer to 'Clinical performance evaluation: HNSCC (KEYTRUDA)' Section 16.10 for KN048 study details.

MSD sponsored clinical study, KEYNOTE-040 (KN040), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (TPS ≥ 50%) patients with recurrent or metastatic HNSCC who had disease progression on or after platinum-containing therapy administered for recurrent or metastatic HNSCC or following platinum-containing chemotherapy administered as part of induction, concurrent, or adjuvant therapy, and were not amenable to local therapy with curative intent, and who may respond to KEYTRUDA treatment.[10] Refer to 'Clinical performance evaluation: HNSCC (KEYTRUDA)' Section 16.10 for KN040 study details.

2.1.5 TNBC (KEYTRUDA)

MSD sponsored clinical study, KEYNOTE-355 (KN355), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (CPS ≥ 10) patients with previously untreated locally recurrent unresectable or metastatic TNBC, who have not received prior chemotherapy for metastatic disease, and who may respond to KEYTRUDA treatment in combination with chemotherapy.[11] Refer to 'Clinical performance evaluation: TNBC (KEYTRUDA)' Section 16.12 for KN355 study details.

2.1.6 Cervical cancer (KEYTRUDA)

MSD sponsored clinical study, KEYNOTE-826 (KN826), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (CPS ≥ 1) patients with persistent, recurrent, or metastatic cervical cancer who have not received prior systemic therapy, and who may respond to KEYTRUDA treatment in combination with platinum chemotherapy and paclitaxel, with or without bevacizumab.[12] Refer to 'Clinical performance evaluation: cervical cancer' Section 16.14 for KN826 study details.

2.1.7 Melanoma

PD-L1 IHC 22C3 pharmDx may be used to determine PD-L1 status in melanoma patients being considered for treatment.[13]

2.2 LIBTAYO (cemiplimab)

LIBTAYO is a human 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.[14]

2.2.1 NSCLC (LIBTAYO)

Regeneron Pharmaceuticals, Inc. sponsored clinical study, Study 1624, investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (TPS ≥ 50%) patients with locally advanced NSCLC who are not candidates for definitive chemoradiation, or with metastatic NSCLC, who may respond to LIBTAYO treatment.[15] Refer to 'Clinical performance evaluation: NSCLC (LIBTAYO)' Section 16.4 for Study 1624 details.

3. Principle of Procedure

PD-L1 IHC 22C3 pharmDx contains the 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 (HRP) 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.

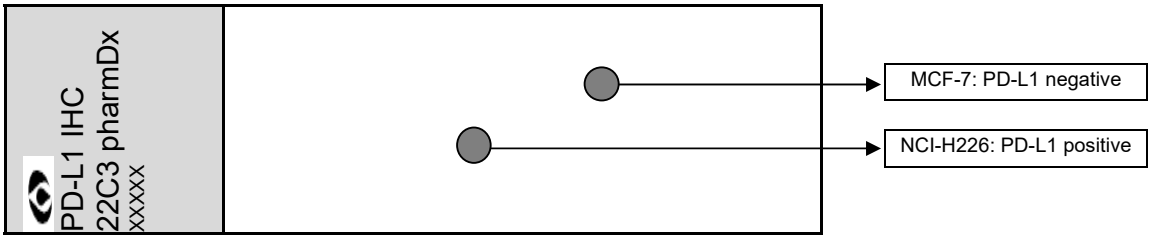
4. Materials Provided

Each kit includes 19.5 mL of PD-L1 primary antibody (approximately 3 µg/mL protein concentration) and contains the reagents necessary to perform 50 tests in up to 15 individual runs. The materials listed in this section are sufficient for 50 tests (50 slides incubated with primary antibody to PD-L1 and 50 slides incubated with the corresponding NCR; 100 slides in total). For larger tissue sections, three drop zones (3 x 150 µL) per slide may be used. Note that this will reduce the total number of tests per kit.

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 (IgG_1) 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_1 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 IHC 22C3 pharmDx Control Slides

Quantity	*Description*
	CONTROL SLIDES
	Each slide contains sections of two pelleted, FFPE cell lines: NCI-H226** with moderate PD-L1 protein expression and MCF-7 with negative PD-L1 protein expression.



**Dr. AF Gazdar and Dr. JD Minna at NIH are acknowledged for their contribution in developing NCI-H226 (ATCC Number: CRL-5826™).[16]

Note: All components 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 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.

5. Materials Required, but Not Supplied

PT Link Pre-treatment Module (Code PT100/PT101/PT200)
Autostainer Link 48 (Code AS480)
EnVision FLEX Wash Buffer (20x) (Code K8007)
EnVision FLEX Hematoxylin (Link) (Code K8008)
Distilled or de-ionized water (reagent-grade water)*
Timer
Positive and negative tissues to use as process controls (see 'Quality Control' Section 11)
Microscope slides: FLEX IHC Microscope Slides (Code K8020) or Superfrost Plus slides
Coverslips
Nonaqueous, permanent mounting medium and ancillary reagents required for mounting coverslips
Light microscope (4x–40x objective magnification)
pH meter (calibrated per manufacturer's recommendation)
Wash bottle

*Note: Not all sources of distilled or de-ionized water may be of sufficient quality for IHC reagent preparation. Agilent recommends reagent-grade distilled or de-ionized water [corresponding to Clinical Laboratory Reagent Water (CLRW) standard as specified by Clinical & Laboratory Standards Institute (CLSI)[17]], or water similar in quality to be used for reagent preparation.

6. Precautions

1) For in vitro diagnostic use.
2) For professional users.
3) This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, NaN_3 may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.[18]
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.[19]
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 small studies showed similar dynamic ranges of PD-L1 expression in primary and metastatic NSCLC and urothelial carcinoma specimen pairs. It is possible there may be differences in PD-L1 expression in primary tumors versus metastatic sites in the same patient as each tumor has unique heterogeneity.
12) Large tissue sections may require 3 x 150 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) Hazard information is available in the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) insert contained within the product package. Safety Data Sheets are available on www.agilent.com or on request.
17) Peroxidase-Blocking Reagent and Visualization Reagent-HRP: Safety Data Sheets are available on request.
18) For countries outside of the European Union, see the local KEYTRUDA product label for approved indications and expression cutoff values to guide therapy.
19) For countries outside of the European Union, see the local LIBTAYO product label for approved indications.

7. Storage

Store all components of PD-L1 IHC 22C3 pharmDx, including kit-supplied 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.

8. Specimen Preparation

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

8.1 Paraffin-embedded sections

FFPE tissue specimens 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, dehydrated, and cleared in a series of alcohols and xylene, followed by infiltration with melted paraffin. The paraffin temperature should not exceed 60 °C. NSCLC 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 FLEX IHC microscope slides (Code K8020) or Superfrost Plus slides, then placed in a 58 ± 2 °C oven for 1 hour.

8.2 Cut section storage recommendation

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

8.2.1 NSCLC cut section storage recommendation

Cut sections must be stained within 6 months when stored at 2–8 ºC (preferred), or at 25 ºC.

8.2.2 Urothelial carcinoma cut section storage recommendation

Cut sections must be stained within 6 months when stored at 2–8 °C (preferred), or at 25 °C.

8.2.3 Esophageal cancer cut section storage recommendation

Cut sections must be stained within 4.5 months when stored at 2–8 °C (preferred), or within 1 month when stored at 25 °C.

8.2.4 HNSCC cut section storage recommendation

Cut sections must be stained within 6 months when stored at 2–8 ºC (preferred), or within 4 months when stored at 25 ºC.

8.2.5 TNBC cut section storage recommendation

Cut sections must be stained within 7.5 months when stored at 2–8 °C (preferred), or within 4 months when stored at 25 °C.

8.2.6 Cervical cancer cut section storage recommendation

Cut sections must be stained within 2 months when stored at 2–8 ºC (preferred), or within 1 month when stored at 25 ºC.

8.2.7 Melanoma cut section storage recommendation

Cut sections must be stained within 4 months when stored at 2–8 ºC (preferred), or within 2 months when stored at 25 ºC.

9. 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 EnVision FLEX Target Retrieval Solution, Low pH (working solution) by diluting EnVision FLEX Target Retrieval Solution, Low pH (50x) 1:50 using distilled or de-ionized water; the pH of 1x EnVision FLEX Target Retrieval Solution, low pH must be 6.1 ± 0.2. Do not modify the pH of 1x EnVision FLEX Target Retrieval Solution, low pH after preparation under any circumstances. If a problem is suspected with the EnVision FLEX Target Retrieval Solution pH, please refer to 'Troubleshooting' Section 17 for more information. One 30 mL bottle of EnVision FLEX 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 EnVision FLEX Target Retrieval Solution after three uses and do not use after 5 days following dilution. Note, the EnVision FLEX Target Retrieval Solution, Low pH (50x), is a red colored solution. Please refer to 'Product-specific limitations' Section 15.2 for EnVision FLEX Target Retrieval Solution, low pH limitations in esophageal cancer specimens.

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 1x EnVision FLEX Wash Buffer by diluting EnVision FLEX Wash Buffer (20x) 1:20 using distilled or de-ionized water for the wash steps. Store unused 1x EnVision FLEX Wash Buffer at 2–8 ºC for no more than 1 month. Discard EnVision FLEX Wash 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 one drop of liquid DAB+ Chromogen per mL of DAB+ Substrate Buffer and mix*. Prepared DAB+ Substrate-Chromogen Solution 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 nine drops of DAB+ Chromogen.** Although the label states 7.2 mL, this is the useable volume and does not account for the "dead volume" (1.8 mL) 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 in this section. Addition of excess liquid DAB+ Chromogen to the DAB+ Substrate Buffer will result in deterioration of the positive signal.

10. Staining Procedure on the Autostainer Link 48 Solution

Procedural notes
The user should read these instructions carefully and become familiar with all components and instrumentation prior to use (see 'Precautions' Section 6).

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 after deparaffinization, rehydration, and target retrieval (3-in-1) procedure (specimen pretreatment) or at any time during the staining procedure. Dried tissue sections may display increased nonspecific staining (including nuclear staining).

Ensure that Autostainer slide racks are level prior to initiation of the IHC staining procedure. Level Autostainer slide racks are required for staining. Unlevel and/or warped Autostainer slide racks can result in uneven reagent distribution and improper pooling on the glass away from the specimen areas, which increases the risk for tissue drying and may lead to the appearance of nonspecific staining, on the primary antibody and/or NCR-stained slides. Perform level testing using dry untreated slides for each slide position in all Autostainer slide racks every 3 months or whenever the Autostainer Link 48 is moved or adjusted on the counter. Discard Autostainer slide racks that fail level testing in any slide position and/or have undergone ≥ 175 PT Link pretreatment cycles.

All of the required steps and incubation times for staining are preprogrammed in the DakoLink 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 DakoLink 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 or Agilent Pathology Support to obtain the protocols.

Step 1: Deparaffinization, rehydration and target retrieval (3-in-1) procedure (specimen pretreatment)
For details, please refer to the PT Link User Guide.
Set PT Link (Code PT100/PT101/PT200) 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 1x EnVision FLEX Target Retrieval Solution, Low pH (working solution) to cover the tissue sections.
►Preheat the 1x EnVision FLEX Target Retrieval Solution, Low pH to 65 °C.
►Immerse Autostainer racks containing mounted, FFPE tissue sections into the pre-heated 1x EnVision FLEX Target Retrieval Solution, Low pH (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 (1x), room temperature EnVision FLEX Wash Buffer (Code K8007).
►Incubate slides in diluted (1x), room temperature EnVision FLEX Wash Buffer for 5 minutes.

Step 2: Staining procedure
After deparaffinization, rehydration and target retrieval (3-in-1) procedure (specimen pretreatment), the Autostainer racks with slides should be placed one rack at a time on the Autostainer Link 48. Prior to initiating the staining procedure on the Autostainer Link 48, 1x EnVision FLEX Wash Buffer should be manually applied to the slides using a wash bottle for prevention of tissue drying. 1x EnVision FLEX Wash Buffer should not be applied directly on the tissue section, but applied sufficiently to the slide so that the tissue section is amply covered. Slides should remain wet prior to the initiation of the staining procedure. 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 DakoLink software. Ensure that the Autostainer Link 48 lid is properly closed to prevent reagent evaporation during the staining procedure.

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

Step 4: Mounting
Nonaqueous, permanent mounting medium 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).

11. Quality Control

Reagents in PD-L1 IHC 22C3 pharmDx have been quality controlled by immunohistochemistry using the target retrieval and staining procedures outlined in 'Staining Procedure on the Autostainer Link 48 Solution' Section 10. Deviations in the recommended procedures for tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results. Quality controls should be included in each staining run. These quality controls are specified in Table 20 and include: a lab-supplied H&E stained patient tissue specimen; kit-supplied Control Cell Line Slide; and lab-supplied positive and negative control tissues.[20] Consult the guidelines of the College of American Pathologists (CAP) Accreditation Program for Immunohistochemistry[21]; see also CLSI Quality Assurance for Immunohistochemistry, Approved Guideline for additional information.[22]

12. Assay Verification

Prior to initial use of a staining system in a diagnostic procedure, the user should verify the assay's performance by testing it on a series of lab-supplied tissues with known IHC performance characteristics representing known positive and negative tissues. Refer to the quality control procedures outlined in 'Quality Control' Section 11. These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Troubleshooting options for potential problems, their causes and suggested corrective actions are outlined in Table 59.

13. Staining and Scoring Interpretation

Refer to the specific indication for respective scoring interpretation information.

13.1 NSCLC – PD-L1 expression determined by Tumor Proportion Score

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

Slide evaluation should be performed by a pathologist using a light microscope. For evaluation of the immunohistochemical staining and scoring, an objective of 10–40x magnification is appropriate. Any perceptible membrane staining of tumor cells should be included in the scoring.

PD-L1 protein expression is determined by using TPS, which is the percentage of viable tumor cells showing partial or complete membrane staining at any intensity.

$$\text{TPS}\ (\%) = \frac{\#\ \text{PD} - \text{L1 staining cells (tumor cells)}}{\text{Total \# of viable tumor cells}} \times 100$$

Score partial or complete cell membrane staining (≥ 1+) that is perceived distinct from cytoplasmic staining. Cytoplasmic staining should be considered nonspecific 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 expression level.

Table 2 provides details about which tissue elements are included in and excluded from determining the TPS.

Table 2. TPS inclusion/exclusion criteria for NSCLC

Tissue Elements	Included in TPS Scoring for NSCLC	Excluded from TPS Scoring for NSCLC
Tumor Cells	• Convincing partial or complete cell membrane staining (at any intensity) of viable tumor cells	• Exclude any cytoplasmic staining
Immune Cells	• Not included	Exclude any staining of immune cells, such as: • Mononuclear inflammatory cells (large lymphocytes, monocytes, pulmonary macrophages) • Plasma cells • Neutrophils
Other	• Not included	Exclude any staining of: • Normal cells adjacent to tumor cells • Stromal cells (fibroblasts) • Necrotic cells and/or cellular debris • Anthracotic pigment

For each staining run, slides should be examined in the order presented in Table 20 ('Slide Evaluation' Section 14) to determine the validity of the staining run and enable assessment of the staining of the sample tissue. Examine patient specimens stained with PD-L1

and the NCR from PD-L1 IHC 22C3 pharmDx when evaluating PD-L1 expression. Specimens stained with NCR must have 0 specific staining. Nonspecific staining, including nuclear staining, should be ≤ 1+.

The specimen should be evaluated for categories of PD-L1 expression per Table 3. For treatment eligibility, refer to 'Intended Use' Section 1.

Table 3. NSCLC PD-L1 expression levels

Tumor Proportion Score			
PD-L1 Expression Levels	TPS < 1%	TPS ≥ 1%	TPS ≥ 50%

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

13.2 Urothelial carcinoma, esophageal cancer, TNBC, and cervical cancer – PD-L1 expression determined by Combined Positive Score

All viable tumor cells on the entire tissue section must be evaluated and included in the PD-L1 expression assessment.

PD-L1 protein expression is determined by CPS, which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. Distinction of viable tumor cells, lymphocytes, and macrophages is essential for accurate denominator estimation. Although the result of the calculation can exceed 100, the maximum score is defined as CPS 100. CPS is defined as follows:

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells (tumor cells, lymphocytes, macrophages)}}{\text{Total \# viable of tumor cells}} \text{ X } 100$$

Slide evaluation must be performed by a pathologist using a light microscope. For evaluation of the immunohistochemical staining, objectives of 10x and 20x magnification are appropriate. For determination of PD-L1 expression, an objective of 20x magnification is required.

By definition, PD-L1 staining cells are:

- Tumor cells with convincing partial or complete linear membrane staining (at any intensity) that is perceived distinct from cytoplasmic staining and
- Lymphocytes and macrophages (mononuclear inflammatory cells, MICs) within the tumor nests and/or adjacent supporting stroma with convincing membrane and/or cytoplasmic staining (at any intensity). MICs must be directly associated with the response against the tumor.

For each staining run, slides should be examined in the order presented in Table 20 ('Slide Evaluation' Section 14) to determine the validity of the staining run and enable assessment of the staining of the sample tissue. Examine patient specimens stained with PD-L1 and the NCR from PD-L1 IHC 22C3 pharmDx when evaluating PD-L1 expression. Specimens stained with NCR must have 0 specific staining. Nonspecific staining, including nuclear staining, should be ≤ 1+.

Refer to 'Urothelial carcinoma' Section 13.2.1, 'Esophageal cancer' Section 13.2.2, 'TNBC' Section 13.2.3, and 'Cervical cancer' Section 13.2.4 for tumor indication-specific information.

13.2.1 Urothelial carcinoma

A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide for the specimen to be considered adequate for PD-L1 evaluation.

Tables 4 and 5 provide details about which tissue elements are included in and excluded from the CPS numerator and denominator, respectively, in urothelial carcinoma.

Table 4. CPS numerator inclusion/exclusion criteria for urothelial carcinoma

Tissue Elements	Included in the Numerator	Excluded from the Numerator

Tumor Cells	• Convincing partial or complete linear membrane staining (at any intensity) of viable urothelial carcinoma tumor cells including:
	  • High grade papillary carcinoma
	  • Carcinoma in situ (CIS)
	  • Any lamina propria, muscularis, or serosal invasion
	  • Metastatic carcinoma
		• Nonstaining tumor cells
		• Tumor cells with only cytoplasmic staining
		• Low grade papillary carcinoma†

Immune Cells	• Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma:**
	  ○ Lymphocytes (including lymphocyte aggregates)
	  ○ Macrophages***
		• Nonstaining MICs
		• MICs (including lymphoid aggregates) associated with ulcers, chronic cystitis, and other processes not associated with the tumor
		• MICs associated with normal structures
		• Neutrophils, eosinophils, and plasma cells
		• BCG††-induced granulomas

Tissue Elements	Included in the Numerator	Excluded from the Numerator
	• Only MICs directly associated with the response to the tumor are scored.	
Other Cells	• Not included	• Normal cells • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

In **MICs, membrane and cytoplasmic staining are often indistinguishable due to high nuclear to cytoplasmic ratio. Therefore, membrane and/or cytoplasmic staining of MICs is included in the score.*
*****Adjacent MICs** are defined as being within the same 20x field as the tumor. However, MICs that are NOT directly associated with the response to the tumor should be excluded.*
****Macrophages and histiocytes are considered the same cells.*
†If the tumor consists entirely of low grade papillary carcinoma, the result should be flagged as such.
††Bacillus Calmette-Guérin

Table 5. CPS denominator inclusion/exclusion criteria for urothelial carcinoma

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	• All viable tumor cells including: ○ High grade papillary carcinoma ○ Carcinoma in situ (CIS) ○ Any lamina propria, muscularis, or serosal invasion ○ Metastatic carcinoma	• Any necrotic or nonviable tumor cells • Low grade papillary carcinoma††
Immune Cells	• Not included	• All immune cells of any type
Other Cells	• Not included	• Normal cells • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

††If the tumor consists entirely of low grade papillary carcinoma, the result should be flagged as such.

PD-L1 expression in the patient specimen is considered in two categories: CPS < 10 and CPS ≥ 10. See the KEYTRUDA® product label for specific clinical circumstances guiding PD-L1 testing.

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

13.2.2 Esophageal cancer

A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide for the specimen to be considered adequate for PD-L1 evaluation.

Tables 6 and 7 provide details about which tissue elements are included in and excluded from the CPS numerator and denominator, respectively, in esophageal cancer.

Table 6. CPS numerator inclusion/exclusion criteria for esophageal cancer

Tissue Elements	Included in the Numerator	Excluded from the Numerator
Tumor cells	• Convincing partial or complete cell membrane staining (at any intensity) of viable invasive esophageal tumor cells: adenocarcinoma (including intramucosal component) and squamous cell carcinoma	• Nonstaining tumor cells • Tumor cells with only cytoplasmic staining • Any noninvasive neoplasia including glandular and squamous dysplasia (e.g. high grade glandular dysplasia and squamous cell carcinoma in situ)
Immune cells	• Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma**, such as: ○ Lymphocytes (including lymphocyte aggregates) ○ Macrophages*** • Only MICs directly associated with the response to the tumor are scored.	• Nonstaining MICs • MICs associated with noninvasive neoplasia • MICs associated with benign structures • MICs (including lymphoid aggregates) not directly associated with the response to the tumor • Neutrophils, eosinophils and plasma cells
Other Cells	• Not included	• Benign epithelial cells • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

In **MICs membrane and cytoplasmic staining are often indistinguishable due to a high nuclear to cytoplasmic ratio. Therefore, membrane and/or cytoplasmic staining of MICs is included in the score.*
*****Adjacent MICs** are defined as being within the same 20x field as the tumor. However, MICs that are NOT directly associated with the response against the tumor should be excluded.*
****Macrophages and histiocytes are considered the same cells.*

Table 7. CPS denominator inclusion/exclusion criteria for esophageal cancer

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	• All viable invasive tumor cells	• Nonviable tumor cells • Noninvasive neoplasia
Immune Cells	• Not included	• All immune cells of any type
Other Cells	• Not included	• Benign cells • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

The specimen should be considered to have PD-L1 expression if CPS ≥ 10.

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

13.2.3 TNBC

A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide for the specimen to be considered adequate for PD-L1 evaluation.

Tables 8 and 9 provide details about which tissue elements are included in and excluded from the CPS numerator and denominator, respectively, in TNBC.

Table 8. CPS numerator inclusion/exclusion criteria for TNBC

Tissue Elements	Included in the Numerator	Excluded from the Numerator
Tumor Cells	• Convincing partial or complete linear membrane staining (at any intensity) of viable invasive tumor cells	• Nonstaining tumor cells • Tumor cells with only cytoplasmic staining • Carcinoma in situ (DCIS and LCIS)

Immune Cells	• Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma**:
	  ○ Lymphocytes (including lymphocyte aggregates)
	  ○ Macrophages***
	• Only MICs directly associated with the response to the tumor are scored.
		• Nonstaining MICs
		• MICs associated with DCIS and LCIS
		• MICs associated with benign structures
		• MICs (including lymphoid aggregates) not directly associated with the response to the tumor
		• Neutrophils, eosinophils, and plasma cells

Other Cells	• Not included	• Benign epithelial cells • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

In **MICs, membrane and cytoplasmic staining are often indistinguishable due to high nuclear to cytoplasmic ratio. Therefore, membrane and/or cytoplasmic staining of MICs is included in the score.*
*****Adjacent MICs** are defined as being within the same 20x field as the tumor. However, MICs that are NOT directly associated with the response against the tumor should be excluded.*
****Macrophages and histiocytes are considered the same cells.*

Table 9. CPS denominator inclusion/exclusion criteria for TNBC

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	• All viable invasive tumor cells	• Nonviable tumor cells • Carcinoma in situ (DCIS and LCIS)
Immune Cells	• Not included	• All immune cells
Other Cells	• Not included	• Benign Cells • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

The specimen should be considered to have PD-L1 expression if CPS ≥ 10.

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

13.2.4 Cervical cancer

A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide for the specimen to be considered adequate for PD-L1 evaluation.

Tables 10 and 11 provide details about which tissue elements are included in and excluded from the CPS numerator and denominator, respectively, in cervical cancer.

Table 10. CPS numerator inclusion/exclusion criteria for cervical cancer

Tissue Elements	Included in the Numerator	Excluded from the Numerator
Tumor cells	• Convincing partial or complete linear membrane staining (at any intensity) of viable invasive tumor cells	• Nonstaining tumor cells • Tumor cells with only cytoplasmic staining • Dysplasia • Carcinoma in situ (CIS)
Immune cells	• Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma**, such as: ○ Lymphocytes (including lymphocyte aggregates) ○ Macrophages*** • Only MICs directly associated with the response to the tumor are scored.	• Nonstaining MICs • MICs associated with dysplasia and CIS • MICs associated with benign cells including squamous or glandular mucosa, cervical polyps, and microglandular hyperplasia • MICs (including lymphoid aggregates) associated with ulcers, and other processes not associated with the response to the tumor such as cervicitis • Neutrophils, eosinophils and plasma cells
Other Cells	• Not included	• Benign cells including squamous or glandular mucosa, cervical polyps, and microglandular hyperplasia • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

** In **MICs**, membrane and cytoplasmic staining are often indistinguishable due to high nuclear to cytoplasmic ratio. Therefore, membrane and/or cytoplasmic staining of MICs is included in the score.*
******Adjacent MICs** are defined as being within the same 20x field as the tumor. However, MICs that are NOT directly associated with the response to the tumor should be excluded.*
****Macrophages and histiocytes are considered the same cells.*

Table 11. CPS denominator inclusion/exclusion criteria for cervical cancer

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	• All viable tumor cells	• Any necrotic or nonviable tumor cells • Carcinoma in situ (CIS) • Dysplasia
Immune Cells	• Not included	• All immune cells of any type
Other Cells	• Not included	• Benign cells including squamous or glandular mucosa, cervical polyps, and micrograndular hyperplasia • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

For cervical cancer, NSCLC tissue may be used as a positive and/or negative control if no cervical cancer control tissue is available.

The specimen should be considered to have PD-L1 expression if CPS ≥ 1.

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

13.3 HNSCC – PD-L1 expression determined by Combined Positive Score and/or Tumor Proportion Score

13.3.1 Combined Positive Score

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

PD-L1 protein expression is determined by CPS, which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. Distinction of viable tumor cells, lymphocytes, and macrophages is essential for accurate denominator estimation. Although the result of the calculation can exceed 100, the maximum score is defined as CPS 100. CPS is defined as follows:

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells (tumor cells, lymphocytes, macrophages)}}{\text{Total \# viable of tumor cells}} \times 100$$

Slide evaluation must be performed by a pathologist using a light microscope. For evaluation of the immunohistochemical staining, objectives of 10x and 20x magnification are appropriate. For determination of PD-L1 expression, an objective of 20x magnification is required.

By definition, PD-L1 staining cells are:

- Tumor cells with convincing partial or complete linear membrane staining (at any intensity) that is perceived distinct from cytoplasmic staining and
- Lymphocytes and macrophages (mononuclear inflammatory cells, MICs) within the tumor nests and/or adjacent supporting stroma with convincing membrane and/or cytoplasmic staining (at any intensity). MICs must be directly associated with the response against the tumor.

For each staining run, slides should be examined in the order presented in Table 20 ('Slide Evaluation' Section 14) to determine the validity of the staining run and enable assessment of the staining of the sample tissue. Examine patient specimens stained with PD-L1 and the NCR from PD-L1 IHC 22C3 pharmDx when evaluating PD-L1 expression. Specimens stained with NCR must have 0 specific staining. Nonspecific staining, including nuclear staining, should be ≤ 1+.

Tables 12 and 13 provide details about which tissue elements are included in and excluded from the CPS numerator and denominator, respectively, in HNSCC.

Table 12. CPS numerator inclusion/exclusion criteria for HNSCC

Tissue Elements	Included in the Numerator	Excluded from the Numerator
Tumor Cells	• Convincing partial or complete linear membrane staining (at any intensity) of viable, invasive tumor cells	• Nonstaining tumor cells • Tumor cells with only cytoplasmic staining • Carcinoma in situ (CIS)
Immune Cells	• Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma:** ○ Lymphocytes (including lymphocyte aggregates) ○ Macrophages*** • Only MICs directly associated with the response to the tumor are scored.	• Nonstaining MICs • MICs (including lymphoid aggregates) associated with ulcers or other inflammatory processes • MICs associated with carcinoma in situ • MICs associated with benign structures • Neutrophils, eosinophils and plasma cells
Other Cells	• Not included	• Benign cells • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

In **MICs, membrane and cytoplasmic staining are often indistinguishable due to high nuclear to cytoplasmic ratio. Therefore, membrane and/or cytoplasmic staining of MICs is included in the score.*
*****Adjacent MICs** are defined as being within the same 20x field as the tumor. However, MICs that are NOT directly associated with the response to the tumor should be excluded.*
****Macrophages and histiocytes are considered the same cells.*

Table 13. CPS denominator inclusion/exclusion criteria for HNSCC

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	• All viable invasive tumor cells	• Any necrotic or nonviable tumor cells • Carcinoma in situ (CIS)
Immune Cells	• Not included	• All immune cells of any type
Other Cells	• Not included	• Benign cells • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

The specimen should be considered to have PD-L1 expression if CPS ≥ 1.

Table 14. HNSCC PD-L1 expression levels – CPS

	Combined Positive Score		
PD-L1 Expression Levels	CPS < 1	CPS ≥ 1	CPS ≥ 20

Refer to 'Clinical performance evaluation: HNSCC (KEYTRUDA)' Section 16.10 for clinical performance information associated with PD-L1 expression levels. Refer to PD-L1 IHC 22C3 pharmDx HNSCC CPS Interpretation Manual for additional guidance.

13.3.2 Tumor Proportion Score

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

Slide evaluation should be performed by a pathologist using a light microscope. For evaluation of the immunohistochemical staining and scoring, an objective of 10–20x magnification is appropriate. Any perceptible membrane staining of tumor cells should be included in the scoring.

PD-L1 protein expression is determined by using TPS, which is the percentage of viable tumor cells showing partial or complete membrane staining at any intensity.

$$\text{TPS (\%)} = \frac{\text{\# PD-L1 staining cells (tumor cells)}}{\text{Total \# of viable tumor cells}} \times 100$$

Score partial or complete cell membrane staining (≥ 1+) that is perceived distinct from cytoplasmic staining. Cytoplasmic staining should be considered nonspecific 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 expression level.

Tables 15 and 16 provide details about which tissue elements are included in and excluded from determining the TPS numerator and denominator, respectively, in HNSCC.

Table 15. TPS numerator inclusion/exclusion criteria for HNSCC

Tissue Elements	Included in the Numerator	Excluded from the Numerator
Tumor Cells	• Convincing partial or complete linear membrane staining (at any intensity) of viable, invasive tumor cells	• Nonstaining viable, invasive tumor cells • Tumor cells with only cytoplasmic staining • Carcinoma in situ (CIS)
Other	• Not included	• Immune cells • Benign cells • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

Table 16. TPS denominator inclusion/exclusion criteria for HNSCC

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	• All viable invasive tumor cells	• Any necrotic or nonviable tumor cells • Carcinoma in situ (CIS)
Other Cells	• Not included	• Immune cells • Benign cells • Stromal cells (including fibroblasts) • Necrotic cells and/or cellular debris

For each staining run, slides should be examined in the order presented in Table 20 ('Slide Evaluation' Section 14) to determine the validity of the staining run and enable assessment of the staining of the sample tissue. Examine patient specimens stained with PD-L1 and the NCR from PD-L1 IHC 22C3 pharmDx when evaluating PD-L1 expression. Specimens stained with NCR must have 0 specific staining. Nonspecific staining, including nuclear staining, should be ≤ 1+.

The specimen should be considered to have PD-L1 expression if TPS ≥ 50%.

Table 17. HNSCC PD-L1 expression levels – TPS

Tumor Proportion Score		
PD-L1 Expression Levels	TPS < 50%	TPS ≥ 50%

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

13.4 Melanoma – PD-L1 expression determined by MEL Score

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

Slide evaluation should be performed by a pathologist using a light microscope. For evaluation of the immunohistochemical staining and scoring, an objective of 10–40x magnification is appropriate.

PD-L1 protein expression is determined by using Melanoma Score (MEL Score), which is the ratio of tumor and associated immune cells expressing PD-L1 at any intensity (weak, moderate, or strong staining), relative to all viable tumor cells and PD-L1 positive associated immune cells. The MEL Score will be reported as:

Table 18. MEL Score expression levels

MEL Score						
	MEL Score 0	**MEL Score 1**	**MEL Score 2**	**MEL Score 3**	**MEL Score 4**	**MEL Score 5**
% stained cells	0%	< 1%	≥ 1% – <10%	≥ 10% – < 33%	≥ 33% – < 66%	≥ 66%

Partial or complete membrane staining of tumor cells should be included in the MEL Score. Cytoplasmic staining of tumor cells should be considered nonspecific staining and is excluded from the assessment of PD-L1 expression.

Partial or complete cell membrane and/or cytoplasmic staining of tumor-associated immune cells should be included in the MEL Score. Tumor-associated immune cells are mononuclear inflammatory cells, which are intercalated within or are contiguous with tumor nests.

NOTE: When interpreting melanoma patient specimens, brown melanin pigmentation may be present. Baseline melanin content in both tumor cells and macrophages (melanophages) may be present and can lead to interpretive challenges. Melanin should be excluded when scoring cell membrane staining. It is highly recommended to do a comparison of the PD-L1 stained slide to a sequential section stained with NCR for identifying and excluding melanin content. If highly elevated melanin precludes scoring of cell membrane staining of tumor cells, the PD-L1 expression evaluation may be indeterminate.

Tumor specimens stained with the NCR must have 0 specific staining. Nonspecific staining, including nuclear staining, should be ≤1+.

Table 19 provides details about which elements are included in and excluded from determining the MEL Score.

Table 19. MEL score inclusion/exclusion criteria for melanoma

Tissue Elements	Included in MEL Score for Melanoma	Excluded from MEL Score for Melanoma
Tumor Cells	• Convincing partial or complete cell membrane staining (at any intensity) of viable tumor cells	• Exclude any cytoplasmic staining
Immune Cells	• Membrane and/or cytoplasmic staining (at any intensity) of immune cells or mononuclear inflammatory cells (MIC) within tumor nests and adjacent supporting stroma (tumor - infiltrating), such as: ○ Large lymphocytes (lymphocyte aggregates) ○ Monocytes	Exclude any staining of: • Immune cells within the stroma distinct from tumor nests • Plasma cells • Neutrophils
Other	• Not included	Exclude any staining of: • Normal cells adjacent to tumor cells • Stromal cells (fibroblasts) • Necrotic cells and/or cellular debris • Endogenous melanin

For each staining run, slides should be examined in the order presented in Table 20 ('Slide Evaluation' Section 14) 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 Melanoma Interpretation Manual for additional guidance.

14. Slide Evaluation

Table 20. Recommended order of slide evaluation

Specimens	Rationale	Requirements
1. H&E (Lab-supplied)	A hematoxylin and eosin (H&E) stain of the tissue specimen is evaluated first to assess tissue histology and preservation quality.	The PD-L1 IHC 22C3 pharmDx and H&E stain should be performed on serial sections from the same paraffin block of the specimen. Tissue specimens should be intact, well preserved, and should confirm tumor indication.
2. Control Cell Line Slide (Kit-supplied)	The Control Cell Line Slide stained with the PD-L1 primary antibody from PD-L1 IHC 22C3 pharmDx should be examined to ascertain that all reagents are functioning properly. The Control Cell Line Slide contains the PD-L1-positive cell line pellet and PD-L1-negative cell line pellet.	One Control Cell Line Slide should be stained with the PD-L1 Primary Antibody in each staining run. *NCI-H226 (PD-L1-positive control cell line) acceptance criteria:* • Cell membrane staining of ≥ 70% of cells. • ≥ 2+ average staining intensity of cells with membrane staining. • Nonspecific staining < 1+ intensity. *MCF-7 (PD-L1-negative control cell line) acceptance criteria:* • No cells with membrane staining.* • Nonspecific 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 cell membrane staining and/or nonspecific staining with ≥ 1+ intensity within the boundaries of the MCF-7 cell pellet are acceptable. If either of the Control Cell Lines does not meet these criteria, all results with the patient specimens should be considered invalid.

Specimens	Rationale	Requirements
3. Positive Control Tissue Slides (Lab-supplied)	The Positive Control Tissue Slides stained with both PD-L1 primary antibody and Negative Control Reagent should be examined next. These slides verify that the fixation method and epitope retrieval process are effective. 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.	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). Use well-preserved specimens for interpretation of staining results as necrotic or degenerated cells often demonstrate nonspecific staining. The tissues selected for use as the positive tissue controls should give weak to moderate positive staining when stained with PD-L1 to aid in detection of subtle changes in assay sensitivity. Two positive tissue control slides should be included in each staining run. **Slide stained with PD-L1:** Presence of brown cell membrane staining should be observed. Nonspecific staining, including nuclear staining, should be ≤1+. **Slide stained with Negative Control Reagent:** No membrane staining. Nonspecific staining, including nuclear staining, should be ≤ 1+. If the positive tissue controls fail to demonstrate appropriate positive staining, results with the test specimens should be considered invalid. See 'Cervical cancer' Section 13.2.4 for additional guidance on control tissue related to cervical cancer.
4. Negative Control Tissue Slides (Lab-supplied)	The Negative Control Tissue Slides (known to be PD-L1 negative) stained with both PD-L1 primary antibody and Negative Control Reagent should be examined next 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.	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). Two negative tissue control slides should be included in each staining run. **Slide stained with PD-L1:** No membrane staining in tumor cells. Nonspecific staining, including nuclear staining, should be ≤ 1+. **Slide stained with Negative Control Reagent:** No membrane staining. Nonspecific staining, including nuclear staining, should be ≤ 1+. If specific cell membrane staining occurs in the Negative Control Tissue Slides, results with the patient specimen should be considered invalid. See 'Cervical cancer' Section 13.2.4 for additional guidance on control tissue related to cervical cancer.
5. Tonsil Control Tissue (optional) (Lab-supplied)	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 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.
6. Patient tissue slide stained using the Negative Control Reagent	Examine patient specimens stained with the Negative Control Reagent from PD-L1 IHC 22C3 pharmDx. Negative Control Reagent is used in place of the primary antibody and aids in interpretation of specific staining at the antigen site.	Absence of cell membrane staining verifies the specific labeling of the target antigen by the primary antibody. Nonspecific staining, including nuclear staining, should be ≤ 1+.
7. Patient tissue slide stained using the PD-L1 primary antibody	Examine the entire slide of the patient specimens stained with the PD-L1 primary antibody from PD-L1 IHC 22C3 pharmDx last.	Positive staining intensity should be assessed within the context of any nonspecific staining observed on the patient's Negative Control Reagent slide in the same run. Nonspecific staining, including nuclear staining, should be ≤ 1+. As with any immunohistochemical test, a negative result means that the antigen was not detected, not necessarily that the antigen was absent in the cells/tissue assayed. 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 must be present for the specimen to be considered adequate for PD-L1 evaluation. Refer to 'Staining and Scoring Interpretation' Section 13 for scoring interpretation guidelines in PD-L1 expression.

15. Limitations

15.1 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) Tissue drying after specimen pretreatment may lead to appearance of > 1+ nonspecific staining, including nuclear staining, on the PD-L1 and/or NCR-stained slides.
4) Excessive or incomplete counterstaining may compromise proper interpretation of results.
5) The clinical interpretation of PD-L1 staining 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.
6) Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[23]
7) 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 Agilent Pathology Support with documented unexpected reactions.
8) False-positive results may be seen due to nonimmunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (cytochrome C).[20]
9) 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.

15.2 Product-specific limitations

1) False-negative results could be caused by degradation of the antigen in the tissues over time. Specimens should be stained within the cut section storage recommendations (refer to 'Cut section storage recommendation' Section 8.2).
2) For optimal and reproducible results, the PD-L1 protein requires target retrieval pretreatment when tissues are routinely fixed (10% 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 PD-L1 IHC 22C3 pharmDx on tissues with fixatives other than 10% neutral buffered formalin has not been validated.
6) Use of PD-L1 IHC 22C3 pharmDx on fine needle aspirates has not been validated.
7) Use of PD-L1 IHC 22C3 pharmDx on decalcified tissues has not been validated and is not recommended.
8) Clinicians should use caution when interpreting test results at the CPS ≥ 20 cutoff, because PD-L1 IHC 22C3 pharmDx failed to meet pre-specified acceptance criteria for positive percent agreement in two independent inter-site reproducibility studies and overall percent agreement in one inter-site reproducibility study conducted on HNSCC specimens at the CPS ≥ 20 cutoff. All pre-specified acceptance criteria were met in the independent inter-site reproducibility study conducted on HNSCC specimens at the CPS ≥ 1 cutoff.
9) Laboratories should pay particular attention to the pH of the 1x EnVision FLEX Target Retrieval Solution, Low pH for pretreatment of esophageal cancer specimens as pH 5.9 may affect PD-L1 staining performance.
10) The studies carried out to assess 1x EnVision FLEX Target Retrieval Solution, low pH use up to 3 times in esophageal cancer did not meet acceptance criteria for qualitative evaluation of PD-L1 expression status, therefore 1x EnVision FLEX Target Retrieval Solution, low pH reuse is not recommended for esophageal cancer specimens.

16. Performance Evaluation

16.1 Nonclinical performance evaluation: normal and neoplastic tissues

Normal tissues: Table 21 summarizes monoclonal mouse anti-PD-L1, Clone 22C3, immunoreactivity on the recommended panel of normal tissues. Cell 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. All tissues were FFPE 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 .[24,25]

Table 21. Summary of PD-L1 IHC 22C3 pharmDx normal tissue reactivity

Tissue Type (# tested)	Positive Cell Membrane Staining: Tissue Elements	Positive Cytoplasmic Staining: Tissue Elements	Nonspecific Staining
Adrenal (3)	0/3	1/3 Medullary cells	0/3
Bladder (3)	0/3	0/3	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

Tissue Type (# tested)	Positive Cell Membrane Staining: Tissue Elements	Positive Cytoplasmic Staining: Tissue Elements	Nonspecific Staining
Kidney (3)	1/3 Tubular epithelium	0/3	0/3
Liver (3)	1/3 Macrophages 1/3 Hepatocytes	0/3	0/3
Lung (3)	3/3 Alveolar macrophages	0/3	0/3
Mesothelial cells (3)	0/3	0/3	0/3
Muscle, cardiac (3)	0/3	0/3	0/3
Muscle, skeletal (3)	0/3	0/3	0/3
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 (3)	3/3 Epithelium	0/3	0/3
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: Table 22 summarizes monoclonal mouse anti-PD-L1, Clone 22C3, immunoreactivity on a panel of neoplastic tissues. Cell 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. All tissues were FFPE 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.[24-27]

Table 22. 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

Tumor Type	Location	PD-L1 positive/total N=159
Chordoma	Pelvic cavity	0/1
Embryonal carcinoma	Testis	0/1
Ependymoma	Brain	0/1
Gastrointestinal stromal tumor	Colon	0/1
	Rectum	0/1
	Small intestine	0/1
Glioblastoma	Brain	0/1
Hepatoblastoma	Liver	0/1
Hepatocellular carcinoma	Liver	0/5
Islet cell tumor	Pancreas	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
Clear cell	Kidney	0/6
Rhabdomyosarcoma	Soft tissue, embryonal	0/1
	Prostate	0/1
	Retroperitoneum	0/1
Seminoma	Testis	0/2
Signet ring cell adenocarcinoma	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
Urothelial carcinoma	Bladder	0/6
	Kidney	0/1

16.2 Nonclinical performance evaluation: NSCLC

The nonclinical studies were performed on FFPE NSCLC specimens.

Analytical sensitivity/specificity: NSCLC

Analytical sensitivity of PD-L1 IHC 22C3 pharmDx was tested on 127 unique cases of 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.

Precision: NSCLC

The precision of PD-L1 IHC 22C3 pharmDx was evaluated at Agilent. Average negative percent agreement (ANA), average positive percent agreement (APA), and overall percent agreement (OA) were computed with corresponding two sided 95% percentile bootstrap confidence intervals for the TPS ≥ 1% cutoff and TPS ≥ 50% cutoff. For studies which resulted in 100.0% agreement, negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with corresponding two-sided 95% Wilson score confidence intervals for the TPS ≥ 1% cutoff and TPS ≥ 50% cutoff.

Table 23. Precision of PD-L1 IHC 22C3 pharmDx tested at one site (TPS ≥ 1%)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-operator	TPS ≥ 1%	All 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression were tested by 6 analysts using 1 Autostainer Link 48 instrument.	NPA 100.0% (93.9–100.0%) PPA 100.0% (94.0–100.0%) OA 100.0% (96.9–100.0%)
Inter-instrument	TPS ≥ 1%	All 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression were tested using 6 Autostainer Link 48 instruments.	NPA 100.0% (94.0–100.0%) PPA 100.0% (94.0–100.0%) OA 100.0% (96.9–100.0%)
Inter-lot	TPS ≥ 1%	All 24 NSCLC specimens (13 PD-L1-negative and 11 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 3 replicates and 3 reagent lots using the Autostainer Link 48 instrument.	ANA 98.3% (95.9–100.0%) APA 97.9% (94.6–100.0%) OA 98.1% (95.3–100.0%)
Inter-day	TPS ≥ 1%	All 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression were tested on six nonconsecutive days using the Autostainer Link 48 instrument.	NPA 100.0% (94.0–100.0%) PPA 100.0% (94.0–100.0%) OA 100.0% (96.9–100.0%)
Intra-run (Repeatability)	TPS ≥ 1%	All 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 6 replicates within a run using the Autostainer Link 48 instrument.	NPA 100.0% (94.0–100.0%) PPA 100.0% (93.8–100.0%) OA 100.0% (96.8–100.0%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; ANA=Average Negative Percent Agreement; APA=Average Positive Percent Agreement; TPS=Tumor Proportion Score

Table 24. Precision of PD-L1 IHC 22C3 pharmDx tested at one site (TPS ≥ 50%)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-operator	TPS ≥ 50%	All 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression were tested by 6 analysts using 1 Autostainer Link 48 instrument.	ANA 94.0% (89.3–98.6%) APA 93.1% (85.8–98.6%) OA 93.6% (87.8–98.6%)
Inter-instrument	TPS ≥ 50%	All 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression were tested using 6 Autostainer Link 48 instruments.	NPA 100.0% (94.9–100.0%) PPA 100.0% (94.9–100.0%) OA 100.0% (97.4–100.0%)
Inter-lot	TPS ≥ 50%	All 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 3 replicates and 3 reagent lots using the Autostainer Link 48 instrument.	ANA 93.3% (88.5–97.1%) APA 93.7% (88.7–97.4%) OA 93.5% (88.9–97.2%)
Inter-day	TPS ≥ 50%	All 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 6 nonconsecutive days using the Autostainer Link 48 instrument.	NPA 100.0% (94.9–100.0%) PPA 100.0% (94.9–100.0%) OA 100.0% (97.4–100.0%)
Intra-run (Repeatability)	TPS ≥ 50%	All 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 6 replicates within a run using the Autostainer Link 48 instrument.	NPA 100.0% (94.0–100.0%) PPA 100.0% (95.6–100.0%) OA 100.0% (97.4–100.0%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement
ANA=Average Negative Percent Agreement; APA=Average Positive Percent Agreement; TPS=Tumor Proportion Score

External reproducibility: NSCLC

The reproducibility of PD-L1 IHC 22C3 pharmDx was evaluated at three external testing sites. Average percent agreements were calculated since no natural reference exists in reproducibility parameters such as site and observer. ANA, APA, and OA were computed with corresponding two-sided 95% percentile bootstrap confidence intervals for the TPS ≥ 1% cutoff and TPS ≥ 50% cutoff.

Table 25. Reproducibility of PD-L1 IHC 22C3 pharmDx tested at three external sites (TPS ≥ 1%)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	TPS ≥ 1%	All 36 NSCLC specimens (16 PD-L1-negative and 20 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days. Inter-site analysis was performed between 3 sites on a total of 2700 pair-wise comparisons.	ANA 94.8% (90.3–98.4%) APA 95.5% (91.2–98.7%) OA 95.2% (90.8–98.6%)
Intra-site	TPS ≥ 1%	All 36 NSCLC specimens (16 PD-L1-negative and 20 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days at each of 3 study sites. Intra-site analysis was	ANA 96.2% (94.1–97.5%) APA 96.7% (95.0–97.9%) OA 96.5% (95.2–97.4%)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
		performed for 3 sites on a total of 1080 pair-wise comparisons.	
Inter-observer	TPS ≥ 1%	All 62 NSCLC specimens (28 PD-L1-negative and 34 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Inter-observer analysis was performed between 3 sites on a total of 1674 pair-wise comparisons.	ANA 85.8% (79.3–91.8%) APA 88.2% (82.2–93.3%) OA 87.1% (81.0–92.6%)
Intra-observer	TPS ≥ 1%	All 62 NSCLC specimens (28 PD-L1-negative and 34 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Intra-observer analysis was performed for 3 sites on a total of 558 pair-wise comparisons.	ANA 93.7% (90.0–96.1%) APA 94.8% (91.6–96.7%) OA 94.3% (92.0–95.9%)

ANA=Average Negative Percent Agreement; APA=Average Positive Percent Agreement; OA=Overall Percent Agreement; TPS=Tumor Proportion Score

Table 26. Reproducibility of PD-L1 IHC 22C3 pharmDx tested at three external sites (TPS ≥ 50%)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	TPS ≥ 50%	All 36 NSCLC specimens (21 PD-L1-negative and 15 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days. Inter-site analysis was performed between 3 sites on a total of 2700 pair-wise comparisons.	ANA 90.3% (84.4–95.2%) APA 85.2% (75.6–92.9%) OA 88.3% (81.4–94.3%)
Intra-site	TPS ≥ 50%	All 36 NSCLC specimens (21 PD-L1-negative and 15 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days at each of 3 study sites. Intra-site analysis was performed for 3 sites on a total of 1080 pair-wise comparisons.	ANA 91.9% (88.8–94.8%) APA 87.6% (82.5–92.2%) OA 90.2% (86.3–93.7%)
Inter-observer	TPS ≥ 50%	All 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, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Inter-observer analysis was performed between 3 sites on a total of 1674 pair-wise comparisons.	ANA 92.6% (87.8–96.7%) APA 92.8% (88.1–96.8%) OA 92.7% (88.1–96.8%)
Intra-observer	TPS ≥ 50%	All 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, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Intra-observer analysis was performed for 3 sites on a total of 558 pair-wise comparisons.	ANA 96.4% (94.0–98.5%) APA 96.5% (94.3–98.6%) OA 96.4% (94.3–98.6%)

ANA=Average Negative Percent Agreement; APA=Average Positive Percent Agreement; OA=Overall Percent Agreement; TPS=Tumor Proportion Score

16.3 Clinical performance evaluation: NSCLC (KEYTRUDA)

KEYNOTE-042: Controlled trial of NSCLC patients naïve to treatment

The safety and efficacy of pembrolizumab were also investigated in KEYNOTE-042, a multicenter, controlled study for the treatment of previously untreated locally advanced or metastatic NSCLC.[4] The study design was similar to that of KEYNOTE-024 , except that patients had PD-L1 expression with a TPS ≥ 1% based on PD-L1 IHC 22C3 pharmDx. Patients were randomized (1:1) to receive pembrolizumab at a dose of 200 mg every 3 weeks (n=637) or investigator's choice platinum-containing chemotherapy (n=637; including pemetrexed+carboplatin or paclitaxel+carboplatin; patients with non-squamous NSCLC could receive pemetrexed maintenance). Assessment of tumor status was performed every 9 weeks for the first 45 weeks, and every 12 weeks thereafter.

Among the 1,274 patients in KEYNOTE-042, 599 (47%) had tumors that expressed PD-L1 with TPS ≥ 50% based on PD-L1 IHC 22C3 pharmDx. The baseline characteristics of these 599 patients included: median age 63 years (45% age 65 or older); 69% male; 63% White and 32% Asian; 17% Hispanic or Latino; and ECOG performance status 0 and 1 in 31% and 69%, respectively. Disease characteristics were squamous (37%) and non-squamous (63%); stage IIIA (0.8%); stage IIIB (9%); stage IV (90%); and treated brain metastases (6%).

The primary efficacy outcome measure was OS. Secondary efficacy outcome measures were PFS and ORR (as assessed by BICR using RECIST 1.1). The trial demonstrated a statistically significant improvement in OS for patients whose tumors expressed PD-L1 TPS ≥ 1% randomized to pembrolizumab monotherapy compared to chemotherapy (HR 0.82; 95% CI 0.71, 0.93 at the final analysis) and in patients whose tumors expressed PD-L1 TPS ≥ 50% randomized to pembrolizumab monotherapy compared to chemotherapy.

Table 27 summarizes key efficacy measures for the TPS ≥ 50% population at the final analysis performed at a median follow-up of 15.4 months. The Kaplan-Meier curve for OS for the TPS ≥ 50% population based on the final analysis is shown in Figure 1.

Table 27. Efficacy results (PD-L1 TPS ≥ 50%) in KEYNOTE-042

Endpoint	Pembrolizumab 200 mg every 3 weeks n=299	Chemotherapy n=300
OS		
Number (%) of patients with event	180 (60%)	220 (73%)
Hazard ratio* (95% CI)	0.70 (0.58, 0.86)	
p-Value†	0.0003	
Median in months (95% CI)	20.0 (15.9, 24.2)	12.2 (10.4, 14.6)
PFS		
Number (%) of patients with event	238 (80%)	250 (83%)
Hazard ratio* (95% CI)	0.84 (0.70, 1.01)	
Median in months (95% CI)	6.5 (5.9, 8.5)	6.4 (6.2, 7.2)
Objective response rate		
ORR % (95% CI)	39% (34, 45)	32% (27, 38)
Complete response %	1%	0.3%
Partial response %	38%	32%
Response duration‡		
Median in months (range)	22.0 (2.1+, 36.5+)	10.8 (1.8+, 30.4+)
% with duration ≥ 18 months	57%	34%

* Hazard ratio (pembrolizumab compared to chemotherapy) based on the stratified Cox proportional hazard model
† Based on stratified log-rank test
‡ Based on patients with a best objective response as confirmed complete or partial response

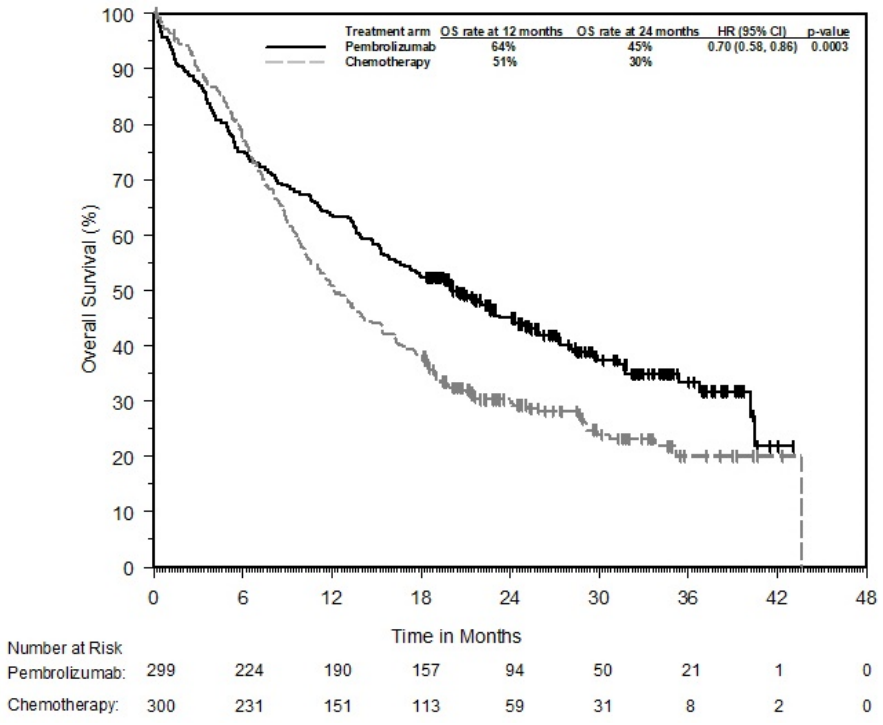


Figure 1. Kaplan-Meier curve for overall survival by treatment arm in KEYNOTE-042 (patients with PD-L1 expression TPS ≥ 50%, intent to treat population)

The results of a post-hoc exploratory subgroup analysis indicated a trend towards reduced survival benefit of pembrolizumab compared to chemotherapy, during both the first 4 months and throughout the entire duration of treatment, in patients who were never-smokers. However, due to the exploratory nature of this subgroup analysis, no definitive conclusions can be drawn.

KEYNOTE-024: Controlled study of NSCLC patients naïve to treatment
The safety and efficacy of pembrolizumab were investigated in KEYNOTE-024, a multicenter, open label, controlled study for the treatment of previously untreated metastatic NSCLC.[3] Patients had PD-L1 expression with a TPS ≥ 50% based on PD-L1 IHC 22C3

pharmDx. Patients were randomized (1:1) to receive pembrolizumab at a dose of 200 mg every 3 weeks (n=154) or investigator's choice platinum containing chemotherapy (n=151; including pemetrexed+carboplatin, pemetrexed+cisplatin, gemcitabine+cisplatin, gemcitabine+carboplatin, or paclitaxel+carboplatin; patients with non-squamous NSCLC could receive pemetrexed maintenance). Patients were treated with pembrolizumab until unacceptable toxicity or disease progression. Treatment could continue beyond disease progression if the patient was clinically stable and was considered to be deriving clinical benefit by the investigator. Patients without disease progression could be treated for up to 24 months. The study excluded patients with EGFR or ALK genomic tumor aberrations; autoimmune disease that required systemic therapy within 2 years of treatment; a medical condition that required immunosuppression; or who had received more than 30 Gy of thoracic radiation within the prior 26 weeks. Assessment of tumor status was performed every 9 weeks. Patients on chemotherapy who experienced independently verified progression of disease were able to crossover and receive pembrolizumab.

Among the 305 patients in KEYNOTE-024, baseline characteristics were: median age 65 years (54% age 65 or older); 61% male; 82% White, 15% Asian; and ECOG performance status 0 and 1 in 35% and 65%, respectively. Disease characteristics were squamous (18%) and non-squamous (82%); M1 (99%); and brain metastases (9%).

The primary efficacy outcome measure was PFS as assessed by blinded independent central review (BICR) using RECIST 1.1. Secondary efficacy outcome measures were OS and ORR (as assessed by BICR using RECIST 1.1). Table 28 summarizes key efficacy measures for the entire intent to treat (ITT) population. PFS and ORR results are reported from an interim analysis at a median follow up of 11 months. OS results are reported from the final analysis at a median follow up of 25 months.

Table 28. Efficacy results in KEYNOTE-024

Endpoint	Pembrolizumab 200 mg every 3 weeks n=154	Chemotherapy n=151
PFS		
Number (%) of patients with event	73 (47%)	116 (77%)
Hazard ratio* (95% CI)	0.50 (0.37, 0.68)	
p-Value†	< 0.001	
Median in months (95% CI)	10.3 (6.7, NA)	6.0 (4.2, 6.2)
OS		
Number (%) of patients with event	73 (47%)	96 (64%)
Hazard ratio* (95% CI)	0.63 (0.47, 0.86)	
p-Value†	0.002	
Median in months (95% CI)	30.0 (18.3, NA)	14.2 (9.8, 19.0)
Objective response rate		
ORR % (95% CI)	45% (37, 53)	28% (21, 36)
Complete response %	4%	1%
Partial response %	41%	27%
Response duration‡		
Median in months (range)	Not reached (1.9+, 14.5+)	6.3 (2.1+, 12.6+)
% with duration ≥ 6 months	88%§	59%¶

* Hazard ratio (pembrolizumab compared to chemotherapy) based on the stratified Cox proportional hazard model
† Based on stratified log-rank test
‡ Based on patients with a best objective response as confirmed complete or partial response
§ Based on Kaplan-Meier estimates; includes 43 patients with responses of 6 months or longer
¶ Based on Kaplan-Meier estimates; includes 16 patients with responses of 6 months or longer
NA = not available

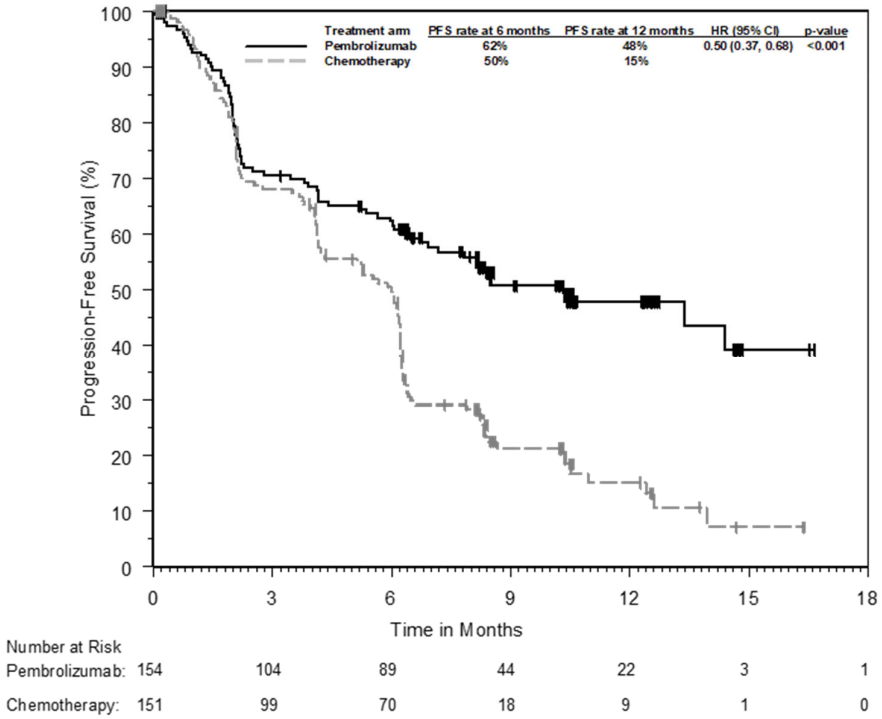


Figure 2. Kaplan-Meier curve for progression-free survival by treatment arm in KEYNOTE-024 (intent to treat population)

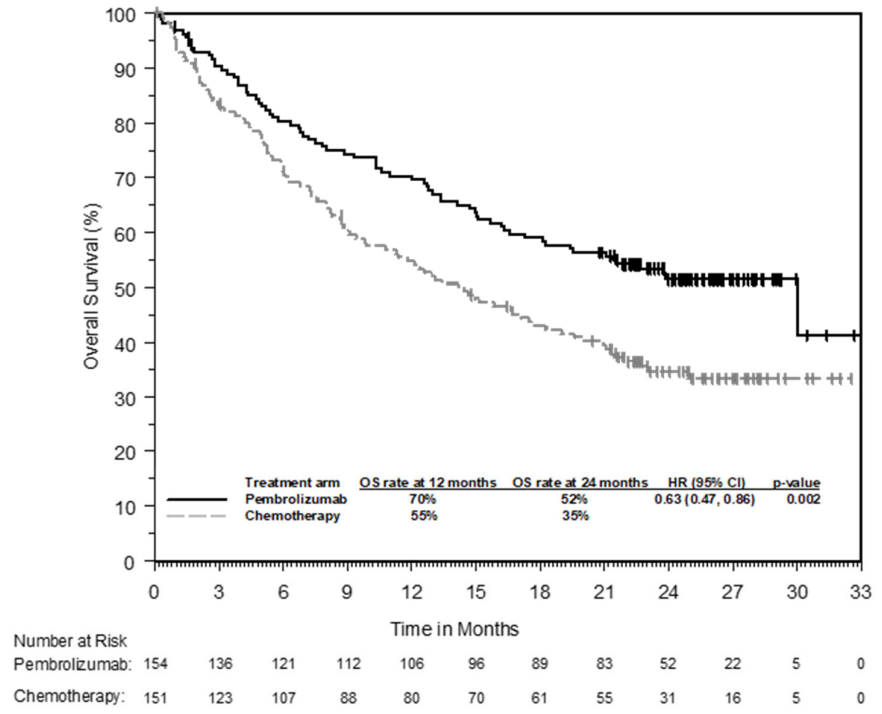


Figure 3. Kaplan-Meier curve for overall survival by treatment arm in KEYNOTE-024 (intent to treat population)

In a subgroup analysis, a reduced survival benefit of pembrolizumab compared to chemotherapy was observed in the small number of patients who were never-smokers; however, due to the small number of patients, no definitive conclusions can be drawn from these data.

KEYNOTE-010: Controlled trial of NSCLC patients previously treated with chemotherapy

The clinical benefit of PD-L1 IHC 22C3 pharmDx was investigated in KEYNOTE-010, a multicenter, open-label, randomized clinical study conducted to assess the safety and efficacy of KEYTRUDA in patients with advanced NSCLC previously treated with platinum-containing chemotherapy.[5] Patients had PD-L1 expression with a TPS ≥ 1% based on a Clinical Trial Assay version of PD-L1 IHC 22C3 pharmDx (CTA). Patients with EGFR activation mutation or ALK translocation also had disease progression on approved therapy for these mutations prior to receiving pembrolizumab. Patients were randomized (1:1:1) to receive pembrolizumab at a dose of 2 (n=344) or 10 mg/kg (n=346) every 3 weeks or docetaxel at a dose of 75 mg/m^2 every 3 weeks (n=343) until disease progression or unacceptable toxicity. The trial excluded patients with autoimmune disease; a medical condition that required immunosuppression; or who had received more than 30 Gy of thoracic radiation within the prior 26 weeks. Assessment of tumor status was performed every 9 weeks. The primary efficacy outcome measures were OS and PFS as assessed by BICR using RECIST 1.1.

Based on the CTA, a total of 1,033 NSCLC patients were randomized in the study. To evaluate the clinical utility of PD-L1 IHC 22C3 pharmDx, archived clinical study samples were retrospectively tested at a US based reference laboratory with PD-L1 IHC 22C3 pharmDx. Out of the 1,033 patients, tumor tissue from 529 patients was retrospectively tested with PD-L1 IHC 22C3 pharmDx. Specimens from 413 patients had PD-L1 expression TPS ≥ 1% and samples from 94 patients did not have PD-L1 expression (TPS < 1%). In these 413 patients with PD-L1 expression TPS ≥ 1%, 163 patient specimens had PD-L1 expression TPS ≥ 50%.

The level of agreement achieved between the CTA and PD-L1 IHC 22C3 pharmDx is shown in Table 29.

Table 29. CTA vs. PD-L1 IHC 22C3 pharmDx agreement

Agreement Rates	PD-L1 Cutoff	Negative Percent Agreement (95% Confidence Interval (CI))	Positive Percent Agreement (95% Confidence Interval (CI))
CTA vs. PD-L1 IHC 22C3 pharmDx	TPS ≥ 1%	94.5% [91.4%–96.6%]	80.0% [76.9–82.8%]
	TPS ≥ 50%	98.3% [97.1%–99.0%]	73.2% [67.9%–77.9%]

Among randomized patients having PD-L1 expression TPS ≥ 1% by PD-L1 IHC 22C3 pharmDx, the demographic and other baseline characteristics were well balanced between the treatment arms. The median age was 63 years (44% age 65 or older). The majority of patients were White (77%) and male (58%); baseline ECOG performance status was 0 (29%) or 1 (71%). Seventy-eight percent (78%) of patients were former/current smokers. Twenty-two percent (22%) of patients had squamous histology and 69% had non-squamous histology. The baseline and demographic characteristics were similarly well balanced across pembrolizumab and docetaxel arms in the overall clinical study.

Efficacy results are summarized in Tables 30 and 31. KEYTRUDA demonstrated durable clinical benefit in NSCLC patients with PD-L1 expression TPS ≥ 1%, which was enhanced in patients with PD-L1 expression TPS ≥ 50%, as determined by PD-L1 IHC 22C3 pharmDx. The magnitude of benefit was comparable to that in the overall clinical trial. Tables 30 and 31 summarize key efficacy measures in the overall population with PD-L1 expression TPS ≥ 1% and in the subpopulation with PD-L1 expression TPS ≥ 50% for the overall clinical study (PD-L1 expression TPS ≥ 1% based on CTA) and in the population with PD-L1 expression determined by PD-L1 IHC 22C3 pharmDx. The Kaplan-Meier curve for OS (TPS ≥ 1%), as determined by PD-L1 IHC 22C3 pharmDx, is shown in Figure 4. Efficacy results were similar for the 2 mg/kg and 10 mg/kg KEYTRUDA arms.

Table 30. Response to KEYTRUDA in previously treated NSCLC patients: overall clinical study and patients with PD-L1 expression TPS ≥ 1% as determined by PD-L1 IHC 22C3 pharmDx

Endpoint	KEYTRUDA 2 mg/kg every 3 weeks		KEYTRUDA 10 mg/kg every 3 weeks		Docetaxel 75 mg/m^2 every 3 weeks	
	Clinical Trial	PD-L1 IHC 22C3 pharmDx	Clinical Trial	PD-L1 IHC 22C3 pharmDx	Clinical Trial	PD-L1 IHC 22C3 pharmDx
Number of patients	344	140	346	142	343	131
OS						
Deaths (%)	172 (50%)	59 (42%)	156 (45%)	59 (42%)	193 (56%)	67 (51%)
Hazard ratio* (95% CI)	0.71 (0.58, 0.88)	0.54 (0.37, 0.78)	0.61 (0.49, 0.75)	0.57 (0.39, 0.82)	–	–
p-Value†	<0.001	<0.001	<0.001	0.00115	–	–
Median in months (95% CI)	10.4 (9.4, 11.9)	11.8 (9.6, NA)	12.7 (10.0, 17.3)	12.0 (8.7, NA)	8.5 (7.5, 9.8)	7.5 (6.3, 9.9)
PFS‡						
Events (%)	266 (77%)	97 (69%)	255 (74%)	103 (73%)	257 (75%)	94 (72%)
Hazard ratio* (95% CI)	0.88 (0.73, 1.04)	0.68 (0.50, 0.92)	0.79 (0.66, 0.94)	0.79 (0.59, 1.06)	–	–
p-Value†	0.068	0.00578	0.005	0.05767	–	–
Median in months (95% CI)	3.9 (3.1, 4.1)	4.9 (4.1, 6.2)	4.0 (2.6, 4.3)	4.0 (2.2, 4.6)	4.0 (3.1, 4.2)	3.8 (2.2, 4.2)
Overall response rate‡						
ORR %§ (95% CI)	18% (14, 23)	24% (17, 32)	18% (15, 23)	20% (14, 28)	9% (7, 13)	5% (2, 11)

* Hazard ratio (KEYTRUDA compared to docetaxel) based on the stratified Cox proportional hazard model
† Based on stratified Log rank test
‡ Assessed by BICR using RECIST 1.1
§ All responses were partial responses

Table 31. Response to KEYTRUDA in previously treated NSCLC patients: overall clinical study and patients with PD-L1 expression TPS ≥ 50% as determined by PD-L1 IHC 22C3 pharmDx

Endpoint	KEYTRUDA 2 mg/kg every 3 weeks		KEYTRUDA 10 mg/kg every 3 weeks		Docetaxel 75 mg/m² every 3 weeks	
	Clinical Trial	PD-L1 IHC 22C3 pharmDx	Clinical Trial	PD-L1 IHC 22C3 pharmDx	Clinical Trial	PD-L1 IHC 22C3 pharmDx
Number of patients	139	56	151	60	152	47
OS						
Deaths (%)	58 (42%)	18 (32%)	60 (40%)	19 (32%)	86 (57%)	25 (53%)
Hazard ratio* (95% CI)	0.54 (0.38, 0.77)	0.45 (0.24, 0.84)	0.50 (0.36, 0.70)	0.29 (0.15 0.56)	–	–
p-Value†	<0.001	0.00541	<0.001	<0.001	–	–
Median in months (95% CI)	14.9 (10.4, NA)	Not reached (9.3, NA)	17.3 (11.8, NA)	Not reached (8.3, NA)	8.2 (6.4, 10.7)	7.2 (4.4, 8.3)
PFS‡						
Events (%)	89 (64%)	33 (59%)	97 (64%)	34 (57%)	118 (78%)	33 (70%)
Hazard ratio* (95% CI)	0.58 (0.43, 0.77)	0.47 (0.28, 0.80)	0.59 (0.45, 0.78)	0.41 (0.24, 0.70)	–	–
p-Value†	<0.001	0.00221	<0.001	<0.001	–	–
Median in months (95% CI)	5.2 (4.0, 6.5)	5.9 (4.2, 9.0)	5.2 (4.1, 8.1)	4.8 (2.8, NA)	4.1 (3.6, 4.3)	3.9 (2.0, 4.3)
Overall response rate‡						
ORR %§ (95% CI)	30% (23, 39)	37% (25, 52)	29% (22, 37)	28% (18, 41)	8% (4, 13)	4% (1, 15)

* Hazard ratio (KEYTRUDA compared to docetaxel) based on the stratified Cox proportional hazard model
† Based on stratified Log rank test
‡ Assessed by BICR using RECIST 1.1
§ All responses were partial responses

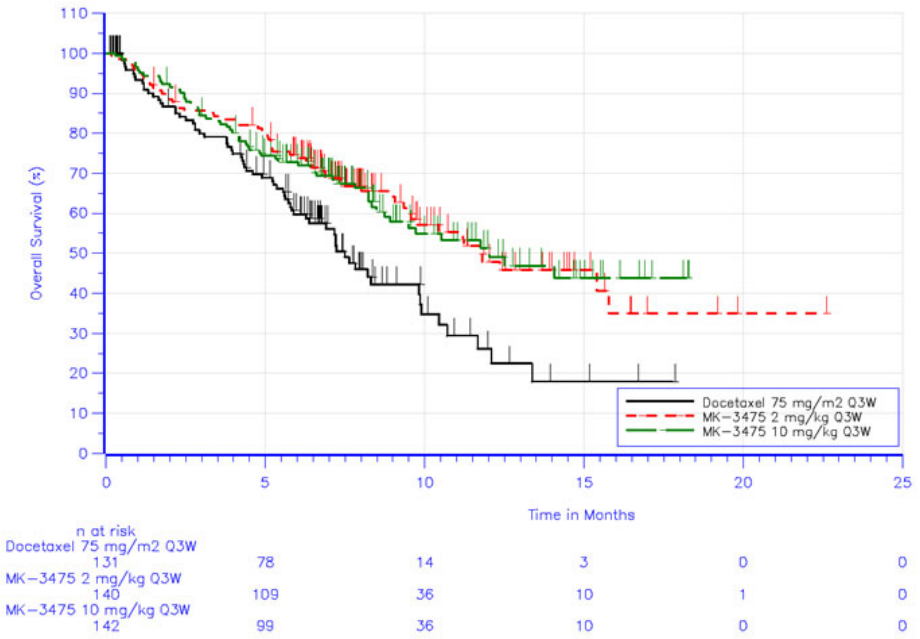


Figure 4. Kaplan-Meier curve for overall survival by treatment arm in KEYNOTE-010 (TPS ≥ 1% by PD-L1 IHC 22C3 pharmDx, intent to treat population)

Additional robustness analyses were conducted to consider the potential impact of missing data arising from patients with PD-L1 expression TPS ≥ 1% by PD-L1 IHC 22C3 pharmDx, but who may have had no PD-L1 expression (TPS < 1%) by the CTA. Patients with such test results are part of the intended use/ intent to diagnose (ITD)/ population of PD-L1 IHC 22C3 pharmDx; however, they were excluded from the clinical trial due to no PD-L1 expression upon CTA screening. To account for these missing data, a sensitivity analysis was conducted to understand the plausible range for the hazard ratio (HR) estimated based on PD-L1 IHC 22C3 pharmDx in the TPS ≥ 1% and TPS ≥ 50% subpopulations under an ITD framework to verify the consistency with the observed HR based on enrollment with the CTA. The HR sensitivity analysis results showed that the HR estimates are robust to any assumed attenuation of the treatment effect under the ITD framework.

16.4 Clinical performance evaluation: NSCLC (LIBTAYO)

Regeneron Study 1624: First-line treatment of locally advanced NSCLC in patients who are not candidates for definitive chemoradiation, or metastatic NSCLC

The efficacy and safety of LIBTAYO compared with platinum-doublet chemotherapy in patients with locally advanced NSCLC who were not candidates for definitive chemoradiation, or with metastatic NSCLC who had tumor PD-L1 expression of TPS ≥ 50% using PD-L1 IHC 22C3 pharmDx were evaluated in Study 1624, a randomized, open-label, multi-center trial.[15]

The trial was designed to enroll patients with tumor PD-L1 expression of TPS ≥ 50%. A total of 710 patients (Intent-To-Treat [ITT] population) were enrolled and an analysis was performed on a population (n=563) who had PD-L1 expression of TPS ≥ 50% using PD-L1 IHC 22C3 pharmDx according to the product labeling.

The study excluded patients with EGFR, ALK or ROS1 genomic tumor aberrations, ECOG performance score (PS) ≥ 2, medical conditions that required systemic immunosuppression, uncontrolled infection with hepatitis B (HBV) or hepatitis C (HCV) or human immunodeficiency virus (HIV), history of interstitial lung disease, who were never smokers or who had an autoimmune disease that required systemic therapy within 2 years of treatment. Treatment of brain metastases was permitted, and patients could be enrolled if they had been adequately treated and had neurologically returned to baseline for at least 2 weeks prior to randomization. Radiological confirmation of stability or response was not required.

Randomization was stratified by histology (non-squamous vs squamous) and geographic region (Europe vs Asia vs Rest of world). Patients were randomized (1:1) to receive LIBTAYO 350 mg intravenously (IV) every 3 weeks for up to 108 weeks or a platinum-doublet chemotherapy regimen for 4 to 6 cycles followed by optional pemetrexed maintenance for patients with non-squamous histology who received a pemetrexed containing regimen.

Treatment with LIBTAYO continued until RECIST 1.1-defined progressive disease, unacceptable toxicity, or up to 108 weeks. Patients who experienced independent review committee (IRC)-assessed RECIST 1.1-defined progressive disease on LIBTAYO therapy were permitted to continue treatment with LIBTAYO (up to an additional 108 weeks) with the addition of 4 cycles of histology-specific chemotherapy until further progression was observed. Of the 150 patients in the population with TPS ≥ 50% randomized to receive chemotherapy who had IRC-assessed RECIST 1.1-defined disease progression, 107 (71.3%) patients crossed over to treatment with LIBTAYO. Assessment of tumor status was performed every 9 weeks. The major efficacy outcome measures were overall survival (OS) and progression-free survival (PFS). An additional efficacy outcome measure was objective response rate (ORR).

The study population characteristics of patients with PD-L1 expression of TPS ≥ 50% are included in Table 32.

Table 32. Summary of baseline patient and disease characteristics in the population with TPS ≥ 50%

	LIBTAYO N=283	**Chemotherapy N=280**
Patient Characteristics		
Median Age, Years (min, max)	63 (31, 79)	64 (40, 84)
Age < 65 Years, n (%)	157 (55)	147 (53)
Age ≥ 65 Years, n (%)	126 (45)	133 (48)
Gender: Male n (%)	248 (88)	231 (83)
Race: White n (%)	243 (86)	240 (86)
ECOG Performance Status n (%)		
0	77 (27)	75 (27)
1	206 (73)	205 (73)
History of brain metastasis (%)	12	12
Disease Characteristics		
Extent of Disease n (%)		
Locally Advanced	45 (16)	42 (15)
Metastatic	238 (84)	238 (85)
Histological Subtype n (%)		
Squamous	122 (43)	121 (43)
Non-squamous	161 (57)	159 (57)

In the ITT population, baseline patient and disease characteristics were consistent with those in the population with TPS ≥ 50%.

In the population with TPS ≥ 50%, the trial demonstrated statistically significant improvement in OS and PFS for patients randomized to LIBTAYO as compared with chemotherapy. Results were similar to the efficacy results for the ITT population.

Efficacy results for the population with PD-L1 expression of TPS ≥ 50% are presented in Table 33 and in Figures 5 and 6.

Table 33. Efficacy results from Study 1624 in non-small cell lung cancer in the population with TPS ≥ 50%

	TPS ≥ 50% Population (N=563)	
Endpoints[a]	**LIBTAYO 350 mg every 3 weeks n=283**	**Chemotherapy n=280**
Overall Survival (OS)		
Number of deaths (%)	70 (24.7)	105 (37.5)
Median in months (95% CI)[b]	NR (17.9, NE)	14.2 (11.2, 17.5)
Hazard ratio (95% CI)[c]	0.57 (0.42, 0.77)	
p-Value[d]	0.0002	
OS rate at 12 months (95% CI)[b]	72.4 (65.6, 78.1)	53.9 (46.2, 61.1)
Progression-free Survival (PFS)		
Number of events (%)	147 (51.9)	197 (70.4)
Median in months (95% CI)[b]	8.2 (6.1, 8.8)	5.7 (4.5, 6.2)
Hazard ratio (95% CI)[c]	0.54 (0.43, 0.68)	
p-Value[d]	<0.0001	
PFS rate at 12 months (95% CI)[b]	40.7 (33.7, 47.5)	7.1 (3.6, 12.1)
Objective Response Rate (ORR) (%)[e,f]		
ORR (95% CI)	39.2 (33.5, 45.2)	20.4 (15.8, 25.6)
Complete response (CR) rate	2.1	1.1
Partial response (PR) rate	37.1	19.3
Duration of Response (DOR)[e]		
Median (months)[b]	16.7	6.0
Range (months)	1.9+, 23.3+	1.3+, 14.5+
Patients with observed DOR ≥ 6 months (%)	73 (65.8)	23 (40.4)

CI: confidence interval; NE: Not evaluable; NR: Not reached
+: Ongoing response
[a] Median duration of follow-up: Cemiplimab: 10.8 months; Chemotherapy: 10.9 months
[b] Based on Kaplan-Meier estimates
[c] Based on stratified proportional hazards model
[d] Based on a two-sided p-value
[e] Not a pre-specified endpoint
[f] Based on Clopper-Pearson exact confidence interval

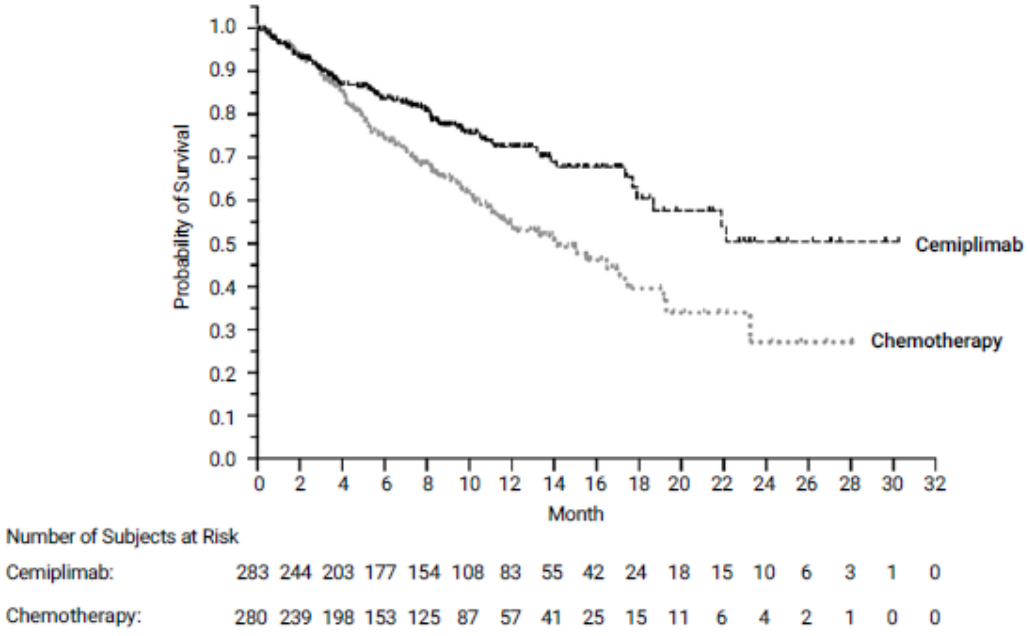


Figure 5. Kaplan-Meier curve for OS in the TPS ≥ 50% population

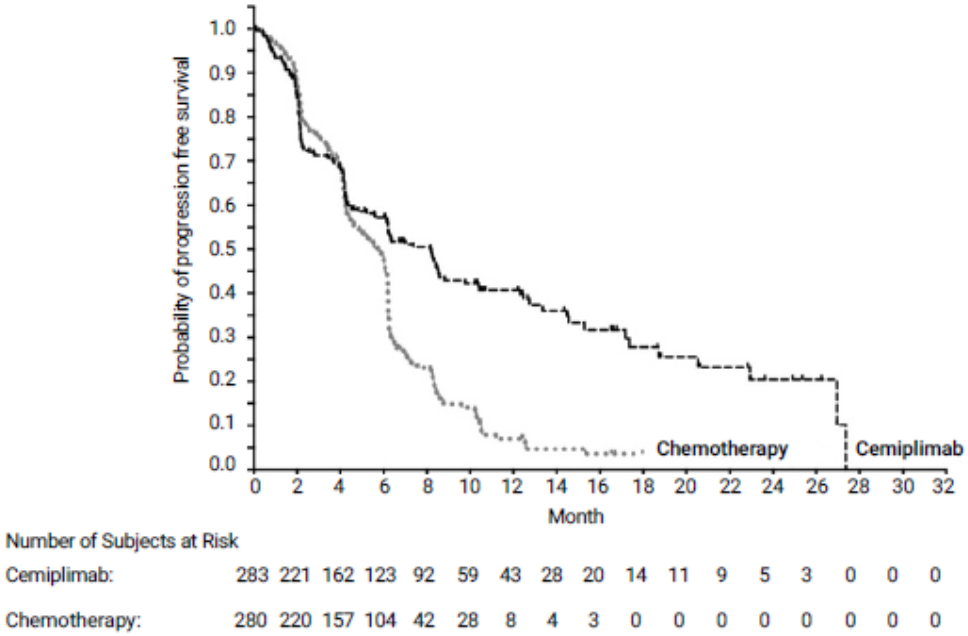


Figure 6. Kaplan-Meier curve for PFS in the TPS ≥ 50% population

16.5 Nonclinical performance evaluation: urothelial carcinoma

The nonclinical studies were performed on FFPE urothelial carcinoma specimens.

Analytical sensitivity/specificity: urothelial carcinoma
Analytical sensitivity of PD-L1 IHC 22C3 pharmDx was tested on 103 FFPE urothelial carcinoma specimens (staged III to IV) using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a range of CPS 0–100, where 37% of the specimens had PD-L1 expression with a CPS ≥ 10.

Precision: urothelial carcinoma
The precision of PD-L1 IHC 22C3 pharmDx was evaluated at Agilent. Inter-operator, inter-instrument, inter-lot, and inter-day were tested in combined precision. For the precision studies, average negative percent agreement (ANA), average positive percent agreement (APA), and overall percent agreement (OA) were computed with corresponding two sided 95% percentile bootstrap confidence intervals for the CPS ≥ 10 cutoff as shown in Table 34.

Table 34. Precision of PD-L1 IHC 22C3 pharmDx in urothelial carcinoma, tested at one site (CPS ≥ 10)

Precision Endpoint	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Intra-run (Repeatability)	CPS ≥ 10	All 32 urothelial carcinoma specimens (17 PD-L1-negative and 15 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 5 replicates within a run using the Autostainer Link 48 instrument.	ANA 96.6% (93.4–100.0%) APA 95.8% (91.3–100.0%) OA 96.2% (92.5–100.0%)
Combined Precision (Inter-operator, inter-instrument, inter-lot, and inter-day as combined variables)	CPS ≥ 10	All 46 urothelial carcinoma specimens (26 PD-L1-negative and 20 PD-L1-positive) with a range of PD-L1 IHC expression were tested by 3 operators, using 3 Autostainer Link 48 instruments and 3 reagent lots, over 3 nonconsecutive days.	ANA 94.7% (88.9–98.7%) APA 93.5% (87.1–98.4%) OA 94.2% (88.4–98.6%)

ANA=Average Negative Percent Agreement; APA=Average Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

External reproducibility: urothelial carcinoma
The reproducibility of PD-L1 IHC 22C3 pharmDx was evaluated at three external sites. Negative percent agreement (NPA), positive percent agreement (PPA) and overall percent agreement (OA) were computed with corresponding two sided 95% Wilson score confidence intervals for the CPS ≥ 10 cutoff.

Table 35. Reproducibility of PD-L1 IHC 22C3 pharmDx in urothelial carcinoma, tested at three external sites (CPS ≥ 10)

Reproducibility Endpoint	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	CPS ≥ 10	All 36 urothelial carcinoma specimens (20 PD-L1-negative and 16 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days. Inter-site analysis was performed between 3 sites on a total of 539 pair-wise comparisons.	NPA 94.0% (90.7–96.2%) PPA 84.6% (79.5–88.6%) OA 89.8% (86.9–92.1%)
Intra-site	CPS ≥ 10	All 36 urothelial carcinoma specimens (20 PD-L1-negative and 16 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days at each of 3 study sites. Intra-site analysis was performed for 3 sites on a total of 539 pair-wise comparisons.	NPA 96.2% (93.5–97.8%) PPA 95.0% (91.3–97.2%) OA 95.7% (93.7–97.1%)
Inter-observer	CPS ≥ 10	All 60 urothelial carcinoma specimens (29 PD-L1-negative and 31 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of three study sites, on 3 nonconsecutive days. Inter-observer analysis was performed between 3 sites on a total of 540 pair-wise comparisons.	NPA 97.3% (94.6–98.7%) PPA 90.7% (86.7–93.6%) OA 93.9% (91.5–95.6%)
Intra-observer	CPS ≥ 10	All 60 urothelial carcinoma specimens (29 PD-L1-negative and 31 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Intra-observer analysis was performed for 3 sites on a total of 540 pair-wise comparisons.	NPA 95.7% (92.7–97.6%) PPA 96.1% (93.0–97.9%) OA 95.9% (93.9–97.3%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

16.6 Clinical performance evaluation: urothelial carcinoma (KEYTRUDA)

KEYNOTE-052: Open label trial in urothelial carcinoma patients ineligible for cisplatin-containing chemotherapy

The safety and efficacy of pembrolizumab were investigated in KEYNOTE-052, a multicenter, open-label study for the treatment of locally advanced or metastatic urothelial carcinoma in patients who were not eligible for cisplatin-containing chemotherapy.[6] Patients received pembrolizumab at a dose of 200 mg every 3 weeks until unacceptable toxicity or disease progression. Treatment could continue beyond progression if the patient was clinically stable and was considered to be deriving clinical benefit by the investigator. Patients without disease progression could be treated for up to 24 months. The study excluded patients with autoimmune disease or a medical condition that required immunosuppression. Assessment of tumor status was performed at 9 weeks after the first dose, then every 6 weeks through the first year, followed by every 12 weeks thereafter.

PD-L1 status was determined using PD-L1 IHC 22C3 pharmDx. Data from the first 100 patients enrolled, the training set, were used to determine the CPS ≥ 10 cutoff. Data from the remaining 270 patients, the validation set, were used to clinically validate the CPS ≥ 10 cutoff.

Among the 370 patients, 30% (n = 110) had tumors that expressed PD-L1 with CPS ≥ 10. Baseline characteristics of these patients were: median age 73 years, 68% male, and 87% White. Eighty-two percent had M1 disease, and 18% had M0 disease. Eighty-one percent had a primary tumor in the lower tract, and 18% of patients had a primary tumor in the upper tract. Seventy-six percent of patients had visceral metastases, including 11% with liver metastases. Reasons for cisplatin ineligibility included: 45% with baseline creatinine clearance of <60 mL/min, 37% with ECOG performance status of 2, 10% with ECOG 2 and baseline creatinine clearance of <60 mL/min, and 8% with other reasons (Class III heart failure, Grade 2 or greater peripheral neuropathy, and Grade 2 or greater hearing loss). Ninety percent of patients were treatment naïve, and 10% received prior adjuvant or neoadjuvant platinum-based chemotherapy.

Among the 270 patients in the validation set, 30% (n = 80) had tumors that expressed PD-L1 with CPS ≥ 10. Baseline characteristics of these patients were: median age 72 years, 68% male, and 86% White. Seventy-nine percent had M1 disease, and 21% had M0 disease. Seventy-nine percent had a primary tumor in the lower tract, and 20% of patients had a primary tumor in the upper tract. Seventy-eight percent of patients had visceral metastases, including 8% with liver metastases. Reasons for cisplatin ineligibility included: 41% with baseline creatinine clearance of <60 mL/min, 43% with ECOG performance status of 2, 11% with ECOG 2 and baseline creatinine clearance of <60 mL/min, and 5% with other reasons (Class III heart failure, Grade 2 or greater peripheral neuropathy, and Grade 2 or greater hearing loss). Ninety percent of patients were treatment naïve, and 10% received prior adjuvant or neoadjuvant platinum-based chemotherapy.

The primary efficacy outcome measure was ORR as assessed by BICR using RECIST 1.1. Secondary efficacy outcome measures included duration of response, and OS. Table 36 summarizes the efficacy results for patients with CPS ≥ 10 in the overall study and in the validation set subgroup.

Table 36 summarizes the key efficacy measures (ORR and OS) for the study population.

Table 36. ORR and OS by PD-L1 expression

Endpoint	Subjects with CPS ≥ 10 in Overall Study (N=110)	Subjects with CPS ≥ 10 in Validation Set (N=80)
Objective Response Rate*		
ORR %, (95% CI)	47% (38, 57)	51% (40, 63)
Complete Response	19%	21%
Partial Response	28%	30%
Response Duration		
Median in months (range)	Not reached (1.4+ - 26.5+)	Not reached (1.4+ - 22.8+)
OS		
Median in months (95% CI)	19 (12.2,)	Not Reached (11.6,)
12-month OS rate	61%	61%

* BICR using RECIST 1.1

In patients with PD-L1 CPS < 10 in the overall study (N=251), the ORR was 21% (95%CI:16, 26). The median OS in months and 12-month OS rate was 10 (8, 12) and 42% respectively.

In patients with PD-L1 CPS < 10 in the validation set (N=185), the ORR was 22% (95% CI: 16, 29). The median OS in months and 12-month OS rate was 11 (8, 13) and 44% respectively.

KEYNOTE-045: Controlled trial of urothelial carcinoma patients previously treated with chemotherapy

The safety and efficacy of pembrolizumab were evaluated in KEYNOTE-045, a multicenter, randomized (1:1), controlled study for the treatment of locally advanced or metastatic urothelial carcinoma in patients with disease progression on or after platinum-containing chemotherapy.[7] Patients must have received first line platinum-containing regimen for locally advanced/metastatic disease or as neoadjuvant/adjuvant treatment, with recurrence/progression ≤12 months following completion of therapy. Patients were randomized (1:1) to receive either KEYTRUDA 200 mg every 3 weeks (n=270) or investigator's choice of any of the following chemotherapy regimens all given intravenously every 3 weeks (n=272): paclitaxel 175 mg/m^2 (n=84), docetaxel 75 mg/m^2 (n=84), or vinflunine 320 mg/m^2 (n=87). Patients were treated with pembrolizumab until unacceptable toxicity or disease progression. Treatment could continue beyond progression if the patient was clinically stable and was considered to be deriving clinical benefit by the investigator. Patients without disease progression could be treated for up to 24 months.

Among the 542 randomized patients in KEYNOTE-045, baseline characteristics were: median age 66 years (range: 26 to 88), 58% age 65 or older; 74% male; 72% White and 23% Asian; 56% ECOG performance status of 1 and 1% ECOG PS of 2; and 96% M1 disease and 4% M0 disease. Eighty-seven percent of patients had visceral metastases, including 34% with liver metastases. Eighty-six percent had a primary tumor in the lower tract and 14% had a primary tumor in the upper tract. Fifteen percent of patients had disease progression following prior platinum-containing neoadjuvant or adjuvant chemotherapy. Twenty-one percent had received 2 prior systemic regimens in the metastatic setting. Seventy-six percent of patients received prior cisplatin, 23% had prior carboplatin, and 1% was treated with other platinum-based regimens. PD-L1 status was determined using PD-L1 IHC 22C3 pharmDx. Thirty percent of the patients had tumors that expressed PD-L1 with a Combined Positive Score (CPS) of ≥ 10.

Table 37 summarizes the key efficacy measures for the intention-to-treat (ITT) population. The Kaplan-Meier curve is shown in Figure 7. The study demonstrated statistically significant improvements in OS and ORR for patients randomized to pembrolizumab as compared to chemotherapy. There was no statistically significant difference between pembrolizumab and chemotherapy with respect to PFS.

Further analysis was performed in KEYNOTE-045 in patients who had PD L1 Combined Positive Score (CPS) ≥ 10 and in patients who had CPS < 10 in both pembrolizumab- and chemotherapy-treated arms (see Table 38).[7]

Table 37. Efficacy results in KEYNOTE-045

Endpoint	Pembrolizumab 200 mg every 3 weeks n=270	Chemotherapy n=272
OS		
Number (%) of patients with event	155 (57%)	179 (66%)
Hazard ratio* (95% CI)	0.73 (0.59, 0.91)	
p-Value†	0.002	
Median in months (95% CI)	10.3 (8.0, 11.8)	7.4 (6.1, 8.3)
PFS‡		
Number (%) of patients with event	218 (81%)	219 (81%)
Hazard ratio* (95% CI)	0.98 (0.81, 1.19)	
p-Value†	0.416	
Median in months (95% CI)	2.1 (2.0, 2.2)	3.3 (2.3, 3.5)
Objective Response Rate‡		
ORR % (95% CI)	21% (16, 27)	11% (8, 16)
Complete Response	7%	3%
Partial Response	14%	8%
p-Value§,	0.001	
Response duration‡,¶		
Median in months (range)	Not reached (1.6+, 15.6+)	4.3 (1.4+, 15.4+)
Number (%#) of patients with duration ≥6 months	41 (78%)	7 (40%)
Number (%#) of patients with duration ≥12 months	14 (68%)	3 (35%)

* Hazard ratio (pembrolizumab compared to chemotherapy) based on the stratified Cox proportional hazard model
† Based on stratified Log rank test
‡ Assessed by BICR using RECIST 1.1
§ Based on method by Miettinen and Nurminen
¶ Based on patients with a best overall response as confirmed complete or partial response
# Based on Kaplan-Meier estimation

Table 38. Overall survival in KEYNOTE-045 by PD-L1 expression

PD-L1 Expression	Pembrolizumab	Chemotherapy	
	OS by PD-L1 Expression Number of Events (number of patients)		Hazard Ratio* (95% CI)
CPS < 10	106 (186)	116 (176)	0.80 (0.61, 1.05)
CPS ≥ 10	44 (74)	60 (90)	0.57 (0.37, 0.88)

*Hazard ratio (pembrolizumab compared to chemotherapy) based on the stratified Cox proportional hazard model
+16 patients (10 from Permbrolizumab and 6 from Chemotherapy) had unknown PD-L1 status

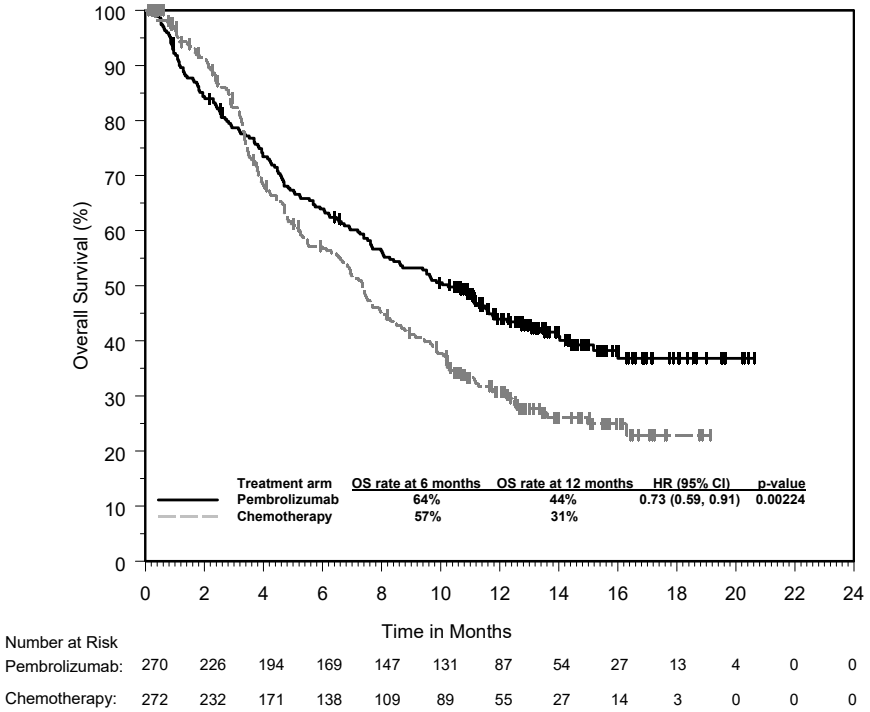


Figure 7. Kaplan-Meier curve for OS by treatment arm in KEYNOTE-045 (ITT)

16.7 Nonclinical performance evaluation: esophageal cancer

The nonclinical studies were performed on FFPE esophageal cancer specimens (studies were conducted with both squamous and adenocarcinoma specimens).

Analytical sensitivity/specificity: esophageal cancer

Analytical sensitivity of PD-L1 IHC 22C3 pharmDx was tested on 100 FFPE esophageal cancer specimens. Assessment of PD-L1 expression demonstrated staining across a range of CPS 0–100, where 34% of the specimens had PD-L1 expression with a CPS ≥ 10. Two specimens were not evaluable due to containing fewer than 100 viable tumor cells.

Precision: esophageal cancer

The precision of PD-L1 IHC 22C3 pharmDx was evaluated at Agilent. Inter-operator, inter-instrument, inter-lot, and inter-day were tested in combined precision. For the precision studies, negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with corresponding two-sided 95% percentile bootstrap confidence intervals for the CPS ≥ 10 cutoff as shown in Table 39.

Table 39. Precision of PD-L1 IHC 22C3 pharmDx in esophageal cancer, tested at one site (CPS ≥ 10)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Combined Precision (Inter-operator, inter-instrument, inter-lot, and inter-day as combined variables)	CPS ≥ 10	All 32 esophageal cancer specimens (15 PD-L1-negative and 17 PD-L1-positive) with a range of PD-L1 IHC expression were tested by 3 operators, using 3 Autostainer Link 48 instruments and 3 reagent lots, over 3 nonconsecutive days.	NPA 97.8% (93.3–100.0%) PPA 98.0% (94.1–100.0%) OA 97.9% (94.8–100.0%)
Intra-run precision (Repeatability)	CPS ≥ 10	All 32 esophageal cancer specimens (21 PD-L1-negative and 11 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 5 replicates within a run using the Autostainer Link 48 instrument.	NPA 98.1% (95.2–100.0%) PPA 92.7% (83.6–100.0%) OA 96.2% (93.1–98.8%)
Inter-observer precision	CPS ≥ 10	All 59 esophageal cancer specimens (28 PD-L1-negative and 31 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days.	NPA 95.1% (90.5–98.8%) PPA 92.4% (87.5–96.8%) OA 93.7% (90.3–96.8%)
Intra-observer precision	CPS ≥ 10	All 60 esophageal cancer specimens (29 PD-L1-negative and 31 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days.	NPA 96.2% (93.4–98.8%) PPA 98.5% (96.5–100.0%) OA 97.3% (95.6–98.9%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

External reproducibility: esophageal cancer
The reproducibility of PD-L1 IHC 22C3 pharmDx was evaluated at three external sites using esophageal cancer specimens. NPA, PPA, and OA were computed with corresponding two-sided 95% percentile bootstrap confidence intervals for the CPS ≥ 10 cutoff.

Table 40. Reproducibility of PD-L1 IHC 22C3 pharmDx in esophageal cancer, tested at three external sites (CPS ≥ 10)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-Site	CPS ≥ 10	All 36 esophageal cancer specimens (23 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days. Inter-site analysis was performed between 3 sites on a total of 540 comparisons to majority call.	NPA 99.7% (99.1–100.0%) PPA 99.0% (96.9–100.0%) OA 99.4% (98.5–100.0%)
Intra-Site	CPS ≥ 10	All 36 esophageal cancer specimens (23 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days. Intra-site analysis was performed for 3 sites on a total of 540 comparisons to majority call.	NPA 99.7% (99.1–100.0%) PPA 99.0% (96.9–100.0%) OA 99.4% (98.5–100.0%)
Inter-observer	CPS ≥ 10	All 60 esophageal cancer specimens (31 PD-L1-negative and 29 PD-L1 positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Inter-observer analysis was performed between 3 sites on a total of 540 comparisons to majority call.	NPA 97.1% (94.3–99.3%) PPA 87.4% (81.6–92.7%) OA 92.4% (89.3–95.4%)
Intra-observer	CPS ≥ 10	All 60 esophageal cancer specimens (31 PD-L1-negative and 29 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Intra-observer analysis was performed for 3 sites on a total of 540 comparisons to majority call.	NPA 97.1% (95.2–98.7%) PPA 97.0% (94.8–98.8%) OA 97.0% (95.6–98.3%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

16.8 Clinical performance evaluation: esophageal cancer (KEYTRUDA)

KEYNOTE-590: Controlled study of combination therapy in esophageal carcinoma patients naïve to treatment

The efficacy of pembrolizumab in combination with chemotherapy was investigated in KEYNOTE-590, a multicenter, randomized, double-blind, placebo-controlled study in patients with locally advanced unresectable or metastatic esophageal carcinoma or gastroesophageal junction carcinoma (Siewert type I).[8] Patients with active autoimmune disease, a medical condition that required immunosuppression, or known HER-2 positive GEJ adenocarcinoma patients were ineligible for the study. Randomization was stratified by tumor histology (squamous cell carcinoma vs. adenocarcinoma), geographic region (Asia vs. ex-Asia), and ECOG performance status (0 vs. 1).

Patients were randomized (1:1) to one of the following treatment arms:

- Pembrolizumab 200 mg on Day 1 of each three-week cycle in combination with cisplatin 80 mg/m^2 IV on Day 1 of each three-week cycle for up to six cycles and 5-FU 800 mg/m^2 IV per day on Day 1 to Day 5 of each three-week cycle, or per local standard for 5-FU administration
- Placebo on Day 1 of each three-week cycle in combination with cisplatin 80 mg/m^2 IV on Day 1 of each three-week cycle for up to six cycles and 5-FU 800 mg/m^2 IV per day on Day 1 to Day 5 of each three-week cycle, or per local standard for 5-FU administration.

Treatment with pembrolizumab or chemotherapy continued until unacceptable toxicity or disease progression or a maximum of 24 months. Patients randomized to pembrolizumab were permitted to continue beyond the first RECIST v1.1-defined disease progression if clinically stable until the first radiographic evidence of disease progression was confirmed at least 4 weeks later with repeat imaging. Assessment of tumor status was performed every 9 weeks.

Among the 749 patients in KEYNOTE-590, 383 (51%) had tumors that expressed PD-L1 with a CPS ≥ 10 based on PD-L1 IHC 22C3 pharmDx. The baseline characteristics of these 383 patients were: median age of 63 years (range: 28 to 89), 41% age 65 or older; 82% male; 34% White and 56% Asian; 43% and 57% had an ECOG performance status of 0 and 1, respectively. Ninety-three percent had M1 disease. Seventy-five percent had a tumor histology of squamous cell carcinoma, and 25% had adenocarcinoma.

The primary efficacy outcome measures were OS and PFS as assessed by the investigator according to RECIST 1.1 in squamous cell histology, CPS ≥ 10, and in all patients. The study demonstrated a statistically significant improvement in OS and PFS for all pre-specified study populations. In all patients randomized to pembrolizumab in combination with chemotherapy, compared to chemotherapy the OS HR was 0.73 (95% CI 0.62-0.86) and the PFS HR was 0.65 (95% CI 0.55-0.76). Secondary efficacy outcome measures were ORR and DoR, according to RECIST 1.1 as assessed by the investigator. Table 41 summarizes key efficacy measures from the pre-specified analysis in patients whose tumors expressed PD-L1 with a CPS ≥ 10 in KEYNOTE-590 performed at a median follow-up time of 13.5 months (range: 0.5 to 32.7 months). The Kaplan-Meier curve for OS and PFS are shown in Figures 8 and 9, respectively.

Table 41. Efficacy Results for pembrolizumab plus chemotherapy in KEYNOTE-590 with PD-L1 expression (CPS ≥ 10)

Endpoint	Pembrolizumab Cisplatin Chemotherapy 5-FU n=186	Standard Treatment* n=197
OS		
Number (%) of patients with event	124 (66.7%)	165 (83.8%)
Median in months† (95% CI)	13.5 (11.1, 15.6)	9.4 (8.0, 10.7)
Hazard ratio‡ (95% CI)	0.62 (0.49, 0.78)	
p-Value§	< 0.0001	
PFS¶		
Number of patients with event (%)	140 (75.3)	174 (88.3)
Median in months† (95% CI)	7.5 (6.2, 8.2)	5.5 (4.3, 6.0)
Hazard ratio‡ (95% CI)	0.51 (0.41, 0.65)	
p-Value§	< 0.0001	
Objective response rate¶		
Objective response rate§ (95% CI)	51.1 (43.7, 58.5)	26.9 (20.8, 33.7)
Complete response	5.9%	2.5%
Partial response	45.2%	24.4%
p-Value#	< 0.0001	
Response duration¶, Þ		
Median in months (range)	10.4 (1.9, 28.9+)	5.6 (1.5+, 25.0+)
% with duration ≥ 6 months†	80.2%	47.7%
% with duration ≥ 12 months†	43.7%	23.2%
% with duration ≥ 18 months†	33.4%	10.4%

* Cisplatin and 5-FU
† Based on Kaplan-Meier estimation
‡ Based on the stratified Cox proportional hazard model
§ One-sided p-Value based on log-rank test stratified by geographic region (Asia versus Rest of the World) and tumor histology (Adenocarcinoma versus Squamous Cell Carcinoma) and ECOG performance status (0 versus 1)
¶ Assessed by investigator using RECIST 1.1
# One-sided p-Value for testing. H0: difference in % = 0 versus H1: difference in % > 0
Þ Best objective response as confirmed complete response or partial response.

A total of 32 patients aged ≥ 75 years for PD-L1 CPS ≥ 10 were enrolled in KEYNOTE-590 (18 in the pembrolizumab combination and 14 in the control). Data about efficacy and safety of pembrolizumab in combination with chemotherapy are too limited in this patient population.

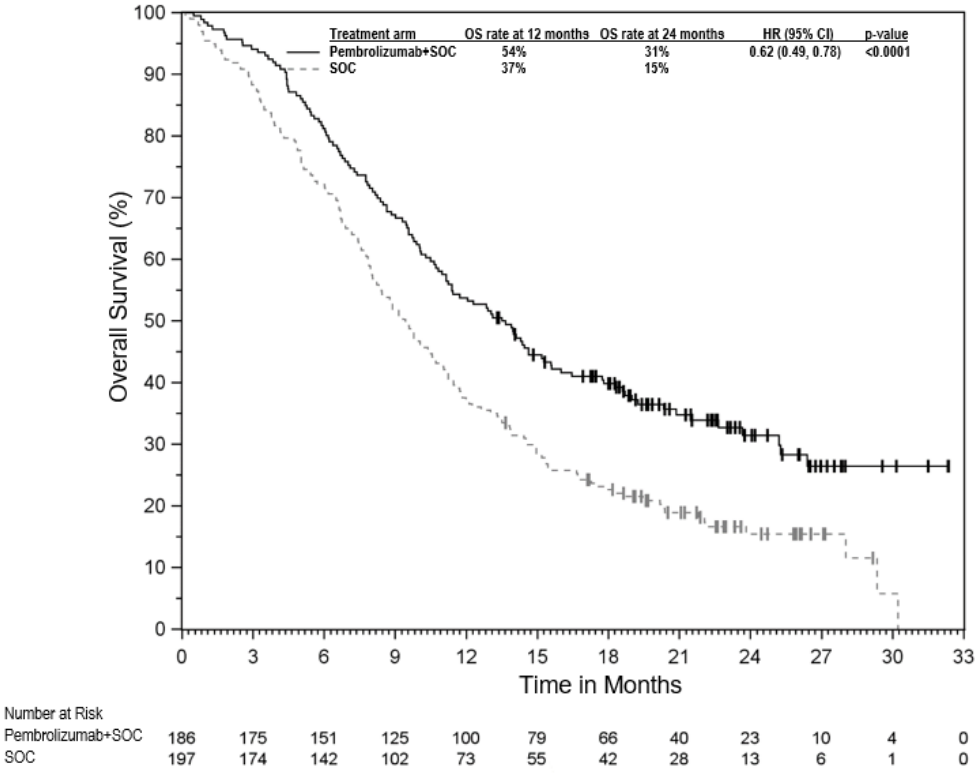


Figure 8. Kaplan- Meier curve for overall survival by treatment arm in KEYNOTE-590 with PD-L1 expression (CPS ≥ 10)

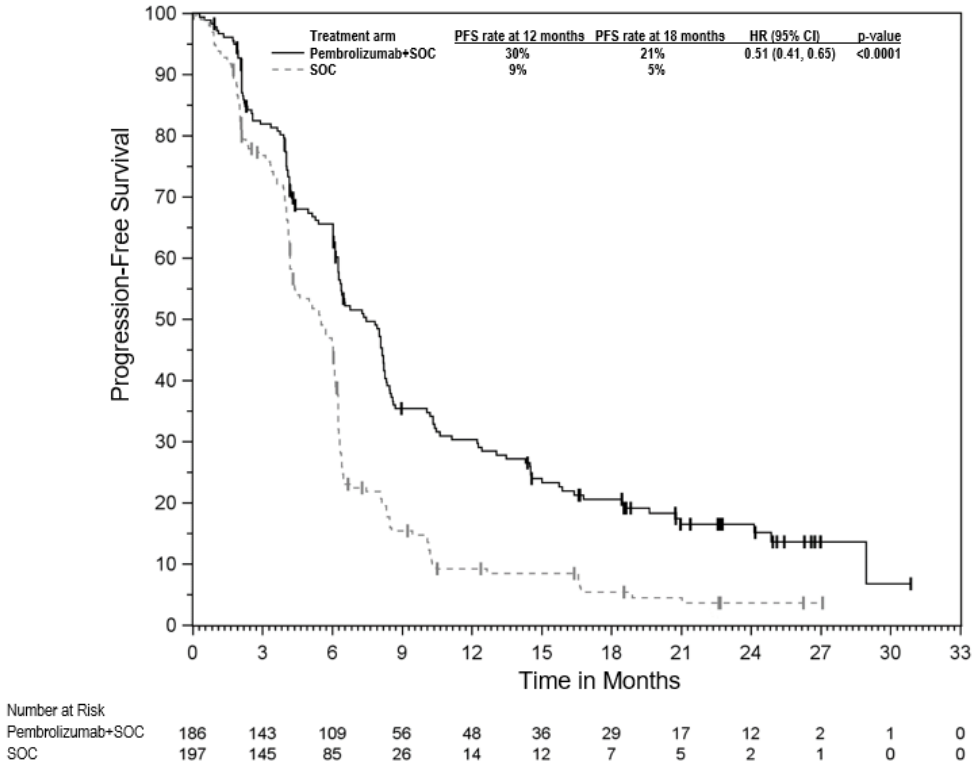


Figure 9. Kaplan-Meier curve for progression-free survival by treatment arm in KEYNOTE-590 with PD-L1 expression (CPS ≥ 10)

16.9 Nonclinical performance evaluation: HNSCC

The nonclinical studies were performed on FFPE HNSCC specimens.

Analytical sensitivity/specificity: HNSCC

Analytical sensitivity of PD-L1 IHC 22C3 pharmDx was tested on 112 FFPE HNSCC specimens (staged I to IV) using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a range of CPS 0–100, where 72% of the specimens had PD-L1 expression with a CPS ≥ 1 and 45% of the specimens had PD-L1 expression with a CPS ≥ 20. In a second, independent study, analytical sensitivity of PD-L1 IHC 22C3 pharmDx was tested on 109 FFPE HNSCC specimens (staged I to IV) using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a range of TPS 0–100%, where 26% of the specimens had PD-L1 expression with a TPS ≥ 50%.

Precision: HNSCC

The precision of PD-L1 IHC 22C3 pharmDx was evaluated at Agilent. Inter-operator, inter-instrument, inter-lot, and inter-day were tested in combined precision. For the precision studies, negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with corresponding two-sided 95% percentile bootstrap confidence intervals for the CPS ≥ 1, CPS ≥ 20, and TPS ≥ 50% cutoffs as shown in Tables 42-44, respectively. For studies with agreement parameters which resulted in 100.0% agreement, two-sided 95% confidence intervals were calculated using the Wilson Score method for the CPS ≥ 1, CPS ≥ 20 and TPS ≥ 50% cutoffs.

Table 42. Precision of PD-L1 IHC 22C3 pharmDx in HNSCC, tested at one site (CPS ≥ 1)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Combined Precision(Inter-operator, inter-instrument, inter-lot, and inter-day as combined variables)	CPS ≥ 1	All 34 HNSCC specimens (12 PD-L1-negative and 22 PD-L1-positive) with a range of PD-L1 IHC expression were tested by 5 operators, using 5 Autostainer Link 48 instruments and 5 reagent lots, over 5 days.	NPA 100.0% (94.0–100.0%) PPA 99.1% (97.3–100.0%) OA 99.4% (98.2–100.0%)
Intra-run precision (Repeatability)	CPS ≥ 1	All 34 HNSCC specimens (16 PD-L1-negative and 18 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 5 replicates within a run using the Autostainer Link 48 instrument.	NPA 98.8% (96.2–100.0%) PPA 97.8% (94.4–100.0%) OA 98.2% (95.9–100.0%)
Inter-observer precision	CPS ≥ 1	All 24 HNSCC specimens (11 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days with a minimum 2-week washout period in between each read.	NPA 88.9% (78.8–98.0%) PPA 99.1% (97.4–100.0%) OA 94.4% (89.8–98.6%)
Intra-observer precision	CPS ≥ 1	All 24 HNSCC specimens (11 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days with a minimum 2-week washout period in between each read.	NPA 98.8% (96.0–100.0%) PPA 95.4% (92.3–98.4%) OA 96.7% (94.0–99.1%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

Table 43. Precision of PD-L1 IHC 22C3 pharmDx in HNSCC, tested at one site (CPS ≥ 20)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Combined Precision(Inter-operator, inter-instrument, inter-lot, and inter-day as combined variables)	CPS ≥ 20	All 34 HNSCC specimens (17 PD-L1-negative and 17 PD-L1-positive) with a range of PD-L1 IHC expression were tested by 5 operators, using 5 Autostainer Link 48 instruments and 5 reagent lots, over 5 days.	NPA 100.0% (95.7–100.0%) PPA 96.5% (90.6–100.0%) OA 98.2% (95.3–100.0%)
Intra-run precision (Repeatability)	CPS ≥ 20	All 34 HNSCC specimens (18 PD-L1-negative and 16 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 5 replicates within a run using the Autostainer Link 48 instrument.	NPA 97.7% (92.9–100.0%) PPA 98.7% (96.2–100.0%) OA 98.2% (95.2-100.0%)
Inter-observer precision	CPS ≥ 20	All 48 HNSCC specimens (27 PD-L1-negative and 21 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days with a minimum 2-week washout period in between each read.	NPA 96.3% (91.8–100.0%) PPA 93.1% (87.3–97.9%) OA 94.9% (91.4–97.9%)
Intra-observer precision	CPS ≥ 20	All 48 HNSCC specimens (27 PD-L1-negative and 21 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days with a minimum 2-week washout period in between each read.	NPA 98.0% (95.9–99.6%) PPA 96.8% (94.4–98.9%) OA 97.5% (95.8–98.8%)

NPA= Negative Percent Agreement; PPA= Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

Table 44. Precision of PD-L1 IHC 22C3 pharmDx in HNSCC, tested at one site (TPS ≥ 50%)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Combined Precision (Inter-operator, inter-instrument, and inter-day as combined variables)	TPS ≥ 50%	All 32 HNSCC specimens (15 PD-L1-negative and 17 PD-L1-positive) with a range of PD-L1 IHC expression were tested by 3 operators, using 3 Autostainer Link 48 instruments, over 3 days.	NPA 97.7% (93.0–100.0%) PPA 98.0% (94.1–100.0%) OA 97.9% (94.7–100.0%)
Intra-run precision (Repeatability)	TPS ≥ 50%	All 32 HNSCC specimens (19 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 5 replicates within a run on the Autostainer Link 48 instrument.	NPA 97.9% (94.7–100.0%) PPA 96.9% (90.8–100.0%) OA 97.5% (94.4–100.0%)
Inter-observer precision	TPS ≥ 50%	All 50 HNSCC specimens (26 PD-L1-negative and 24 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days with a minimum 2-week washout period in between each read.	NPA 92.5% (86.2–98.3%) PPA 96.8% (92.1–100.0%) OA 94.6% (90.7–98.0%)
Intra-observer precision	TPS ≥ 50%	All 50 HNSCC specimens (26 PD-L1-negative and 24 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days with a minimum 2-week washout period in between each read.	NPA 96.8% (93.2–99.5%) PPA 97.3% (95.0–99.5%) OA 97.1% (94.9–98.9%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; TPS=Tumor Proportion Score

External reproducibility: HNSCC

The reproducibility of PD-L1 IHC 22C3 pharmDx was evaluated at three external sites. NPA, PPA, and OA were computed with corresponding two-sided 95% percentile bootstrap confidence intervals for the CPS ≥ 1 and CPS ≥ 20 cutoffs as shown in Tables 45 and 46, respectively.

Table 45. Reproducibility of PD-L1 IHC 22C3 pharmDx in HNSCC, tested at three external sites (CPS ≥ 1)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	CPS ≥ 1	All 38 HNSCC specimens (19 PD-L1 negative and 19 PD-L1 positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days. Inter-site analysis was performed between 3 sites on a total of 570 comparisons to majority call.	NPA 96.8% (92.6–100.0%) PPA 93.3% (86.7–98.6%) OA 95.1% (91.2–98.2%)
Intra-site	CPS ≥ 1	All 38 HNSCC specimens (19 PD-L1 negative and 19 PD-L1 positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days at each of 3 study sites. Intra-site analysis was performed for 3 sites on a total of 570 comparisons to majority call.	NPA 95.7% (91.3–99.0%) PPA 97.0% (94.5–98.9%) OA 96.3% (93.5–98.6%)
Inter-observer	CPS ≥ 1	All 62 HNSCC 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, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Inter-observer analysis was performed between 3 sites on a total of 556 comparisons to majority call.	NPA 94.0% (89.3–97.8%) PPA 97.2% (94.4–99.3%) OA 95.7% (93.0–98.0%)
Intra-observer	CPS ≥ 1	All 62 HNSCC 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, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Intra-observer analysis was performed for 3 sites on a total of 555 comparisons to majority call.	NPA 97.3% (95.4–98.9%) PPA 98.3% (96.8–99.7%) OA 97.8% (96.8–98.9%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

Table 46. Reproducibility of PD-L1 IHC 22C3 pharmDx in HNSCC, tested at three external sites (CPS ≥ 20)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	CPS ≥ 20	All 38 HNSCC specimens (25 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days. Inter-site analysis was performed between 3 sites on a total of 570 comparisons to majority call.	NPA 95.5% (92.0–98.4%) PPA 81.0% (71.3–90.3%) OA 90.5% (86.5–94.4%)
Intra-site	CPS ≥ 20	All 38 HNSCC specimens (25 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC	NPA 96.9% (94.6–98.8%) PPA 90.6% (86.3–94.9%)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
		expression were tested on 5 nonconsecutive days at each of 3 study sites. Intra-site analysis was performed for 3 sites on a total of 570 comparisons to majority call.	OA 94.9% (92.8–96.8%)
Inter-observer	CPS ≥ 20	All 62 HNSCC specimens (31 PD-L1-negative and 31 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Inter-observer analysis was performed between 3 sites on a total of 556 comparisons to majority call.	NPA 93.1% (87.2–97.8%) PPA 91.0% (85.7–95.7%) OA 92.1% (88.2–95.5%)
Intra-observer	CPS ≥ 20	All 62 HNSCC specimens (31 PD-L1-negative and 31 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Intra-observer analysis was performed for 3 sites on a total of 555 comparisons to majority call.	NPA 96.8% (94.5–98.7%) PPA 97.8% (96.0–99.3%) OA 97.3% (95.9–98.6%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

Note: Study results failed to meet pre-specified acceptance criteria for inter-site PPA for CPS ≥ 20 in two independent studies and inter-site OA for CPS ≥ 20 in one study.

16.10 Clinical performance evaluation: HNSCC (KEYTRUDA)

KEYNOTE–048: Controlled trial of monotherapy and combination therapy in HNSCC patients naïve to treatment in the recurrent or metastatic setting

The efficacy of pembrolizumab was investigated in KEYNOTE–048, a multicenter, randomized, open-label, active-controlled study in patients with histologically confirmed metastatic or recurrent HNSCC of the oral cavity, pharynx or larynx, who had not previously received systemic therapy for recurrent or metastatic disease and who were considered incurable by local therapies.[9] Patients with nasopharyngeal carcinoma, active autoimmune disease that required systemic therapy within 2 years of treatment or a medical condition that required immunosuppression were ineligible for the study. Randomization was stratified by tumor PD-L1 expression (TPS ≥ 50% or < 50%), HPV status (positive or negative), and ECOG PS (0 vs. 1). Patients were randomized 1:1:1 to one of the following treatment arms:

- KEYTRUDA 200 mg every 3 weeks
- KEYTRUDA 200 mg every 3 weeks, carboplatin AUC 5 mg/mL/min every 3 weeks or cisplatin 100 mg/m^2 every 3 weeks, and 5-FU 1,000 mg/m^2/d 4 days continuous every 3 weeks (maximum of 6 cycles of platinum and 5-FU)
- Cetuximab 400 mg/m^2 load then 250 mg/m^2 once weekly, carboplatin AUC 5 mg/mL/min every 3 weeks or cisplatin 100 mg/m^2 every 3 weeks, and 5-FU 1,000 mg/m^2/d 4 days continuous every 3 weeks (maximum of 6 cycles of platinum and 5-FU)

Treatment with pembrolizumab continued until RECIST 1.1-defined progression of disease as determined by the investigator, unacceptable toxicity, or a maximum of 24 months. Administration of pembrolizumab was permitted beyond RECIST-defined disease progression if the patient was clinically stable and considered to be deriving clinical benefit by the investigator. Assessment of tumor status was performed at Week 9 and then every 6 weeks for the first year, followed by every 9 weeks through 24 months.

Among the 882 patients in KEYNOTE-048, 754 (85%) had tumors that expressed PD-L1 with a CPS ≥ 1 based on PD-L1 IHC 22C3 pharmDx. The baseline characteristics of these 754 patients included: median age of 61 years (range: 20 to 94); 36% age 65 or older; 82% male; 74% White and 19% Asian; 61% ECOG performance status of 1; and 77% former/current smokers. Disease characteristics were: 21% HPV positive and 95% had Stage IV disease (Stage IVa 21%, Stage IVb 6%, and Stage IVc 69%).

The primary efficacy outcome measures were OS and PFS (assessed by BICR according to RECIST 1.1). The trial demonstrated a statistically significant improvement in OS for all patients randomized to pembrolizumab in combination with chemotherapy compared to standard treatment (HR 0.72; 95% CI 0.60-0.87) and in patients whose tumors expressed PD-L1 CPS ≥ 1 randomized to pembrolizumab monotherapy compared to standard treatment. Tables 47 and 48 summarize key efficacy results for pembrolizumab in patients whose tumors expressed PD-L1 with a CPS ≥ 1 in KEYNOTE-048 at the final analysis performed at a median follow-up of 13 months for pembrolizumab in combination with chemotherapy and at a median follow-up of 11.5 months for pembrolizumab monotherapy. Kaplan Meier curves for OS based on the final analysis are shown in Figures 10 and 11.

Table 47. Efficacy results for pembrolizumab plus chemotherapy in KEYNOTE–048 with PD-L1 expression (CPS ≥ 1)

Endpoint	Pembrolizumab + Platinum Chemotherapy + 5-FU n=242	Standard Treatment* n=235
OS		
Number (%) of patients with event	177 (73%)	213 (91%)
Median in months (95% CI)	13.6 (10.7, 15.5)	10.4 (9.1, 11.7)
Hazard ratio† (95% CI)	0.65 (0.53, 0.80)	
p-Value‡	0.00002	
PFS		
Number (%) of patients with event	212 (88%)	221 (94%)
Median in months (95% CI)	5.1 (4.7, 6.2)	5.0 (4.8, 6.0)
Hazard ratio† (95% CI)	0.84 (0.69, 1.02)	
p-Value‡	0.03697	
ORR		
Objective response rate§ (95% CI)	36% (30.3, 42.8)	36% (29.6, 42.2)
Complete response	7%	3%
Partial response	30%	33%
p-Value¶	0.4586	
Duration of Response		
Median in months (range)	6.7 (1.6+, 39.0+)	4.3 (1.2+, 31.5+)
% with duration ≥6 months	54%	34%

* Cetuximab, platinum, and 5-FU
† Based on the stratified Cox proportional hazard model
‡ Based on stratified log-rank test
§ Response: Best objective response as confirmed complete response or partial response
¶ Based on Miettinen and Nurminen method stratified by ECOG (0 vs. 1), HPV status (positive vs. negative) and PD-L1 status (strongly positive vs. not strongly positive)

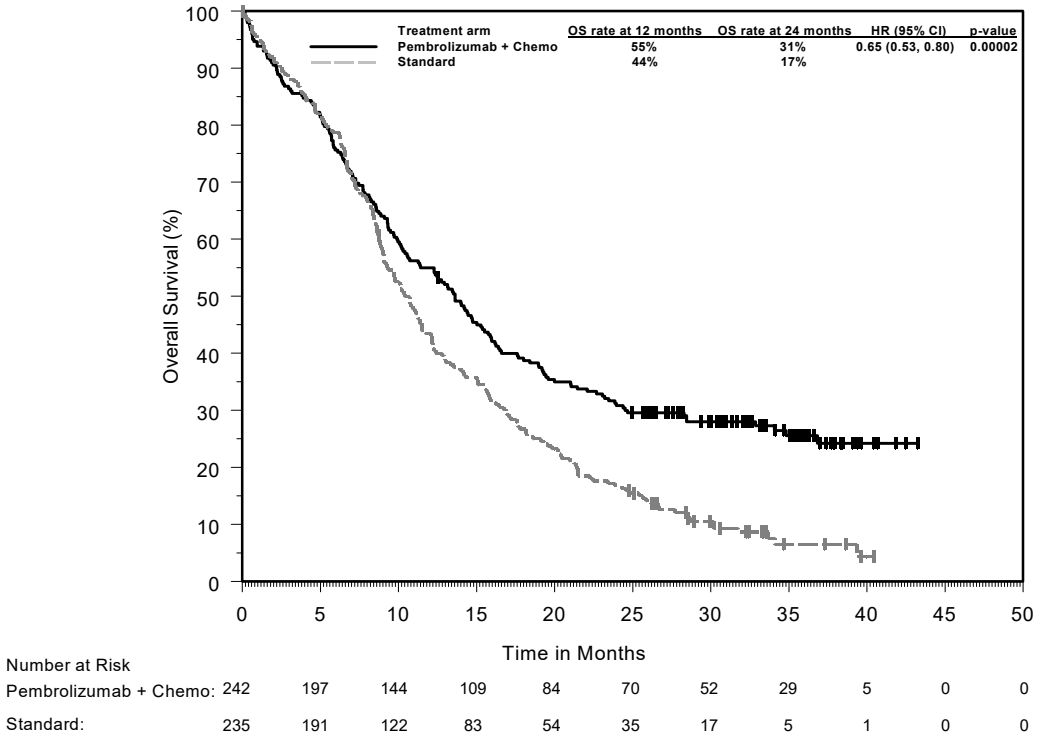


Figure 10. Kaplan-Meier curve for overall survival for pembrolizumab plus chemotherapy in KEYNOTE–048 with PD-L1 expression (CPS ≥ 1)

Table 48. Efficacy results for pembrolizumab as monotherapy in KEYNOTE–048 with PD-L1 expression (CPS ≥ 1)

Endpoint	Pembrolizumab n=257	Standard Treatment* n=255
OS		
Number (%) of patients with event	197 (77%)	229 (90%)
Median in months (95% CI)	12.3 (10.8, 14.3)	10.3 (9.0, 11.5)
Hazard ratio† (95% CI)	0.74 (0.61, 0.90)	
p-Value‡	0.00133	
PFS		
Number (%) of patients with event	228 (89%)	237 (93%)
Median in months (95% CI)	3.2 (2.2, 3.4)	5.0 (4.8, 6.0)
Hazard ratio† (95% CI)	1.13 (0.94, 1.36)	
p-Value‡	0.89580	
ORR		
Objective response rate§ (95% CI)	19.1% (14.5, 24.4)	35% (29.1, 41.1)
Complete response	5%	3%
Partial response	14%	32%
p-Value¶	1.0000	
Duration of Response		
Median in months (range)	23.4 (1.5+, 43.0+)	4.5 (1.2+, 38.7+)
% with duration ≥6 months	81%	36%

* Cetuximab, platinum, and 5-FU
† Based on the stratified Cox proportional hazard model
‡ Based on stratified log-rank test
§ Response: Best objective response as confirmed complete response or partial response
¶ Based on Miettinen and Nurminen method stratified by ECOG (0 vs. 1), HPV status (positive vs. negative) and PD-L1 status (strongly positive vs. not strongly positive)

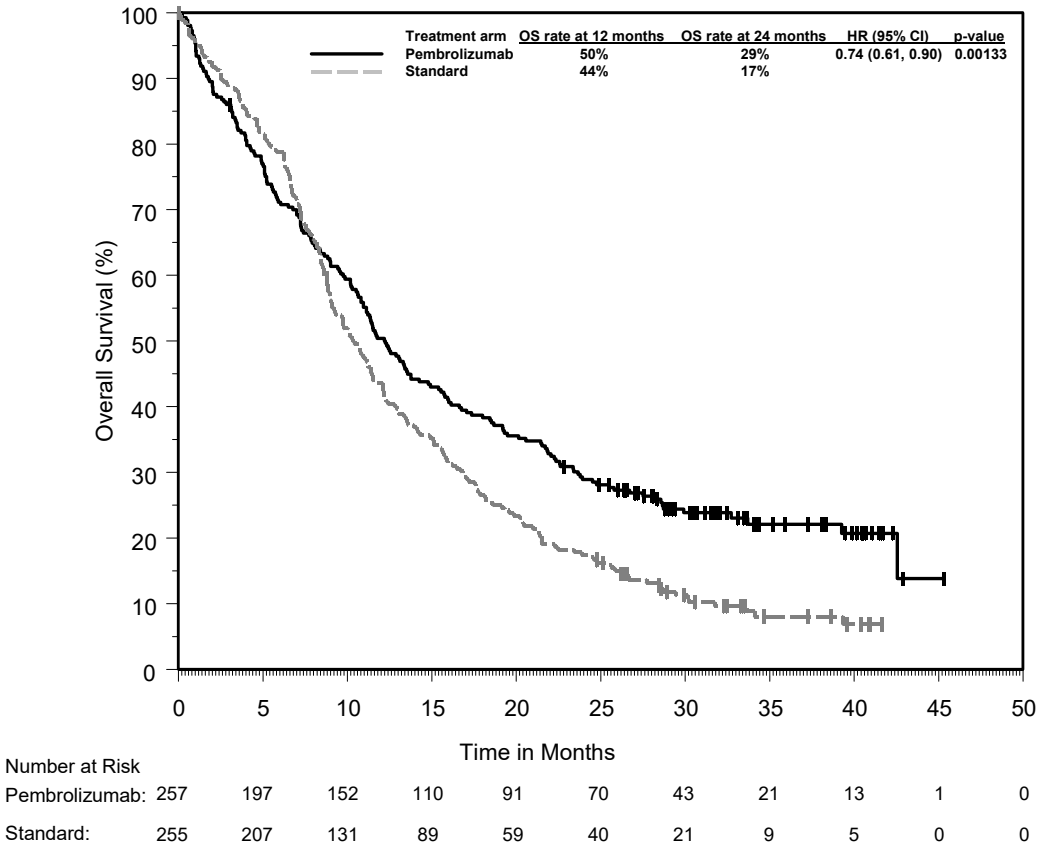


Figure 11. Kaplan-Meier curve for overall survival for pembrolizumab as monotherapy in KEYNOTE-048 with PD-L1 expression (CPS ≥ 1)

An analysis was performed in KEYNOTE-048 in patients whose tumors expressed PD-L1 CPS ≥ 20 [pembrolizumab plus chemotherapy: n=126 (49%) vs. standard treatment: n=110 (43%) and pembrolizumab monotherapy: n=133 (52%) vs. standard treatment: n=122 (48%)] (see Table 49).

Table 49. Efficacy results for pembrolizumab plus chemotherapy and pembrolizumab as monotherapy by PD-L1 expression in KEYNOTE-048 (CPS ≥ 20)

Endpoint	Pembrolizumab + Platinum Chemotherapy + 5-FU n=126	Standard Treatment* n=110	Pembrolizumab Monotherapy n=133	Standard Treatment* n=122
OS				
Number (%) of patients with event	84 (66.7)	98 (89.1)	94 (70.7)	108 (88.5)
Median in months (95% CI)	14.7 (10.3, 19.3)	11.0 (9.2, 13.0)	14.8 (11.5, 20.6)	10.7 (8.8, 12.8)
Hazard ratio† (95% CI)	0.60 (0.45, 0.82)		0.58 (0.44, 0.78)	
p-Value‡	0.00044		0.00010	
OS rate at 6 months (95% CI)	74.6 (66.0, 81.3)	80.0 (71.2, 86.3)	74.4 (66.1, 81.0)	79.5 (71.2, 85.7)
OS rate at 12 months (95% CI)	57.1 (48.0, 65.2)	46.1 (36.6, 55.1)	56.4 (47.5, 64.3)	44.9 (35.9, 53.4)
OS rate at 24 months (95% CI)	35.4 (27.2, 43.8)	19.4 (12.6, 27.3)	35.3 (27.3, 43.4)	19.1 (12.7, 26.6)
PFS				
Number (%) of patients with event	106 (84.1)	104 (94.5)	115 (86.5)	114 (93.4)
Median in months (95% CI)	5.8 (4.7, 7.6)	5.3 (4.9, 6.3)	3.4 (3.2, 3.8)	5.3 (4.8, 6.3)
Hazard ratio† (95% CI)	0.76 (0.58, 1.01)		0.99 (0.76, 1.29)	
p-Value‡	0.02951		0.46791	
PFS rate at 6 months (95% CI)	49.4 (40.3, 57.9)	47.2 (37.5, 56.2)	33.0 (25.2, 41.0)	46.6 (37.5, 55.2)
PFS rate at 12 months (95% CI)	23.9 (16.7, 31.7)	14.0 (8.2, 21.3)	23.5 (16.6, 31.1)	15.1 (9.3, 22.2)
PFS rate at 24 months (95% CI)	14.6 (8.9, 21.5)	5.0 (1.9, 10.5)	16.8 (10.9, 23.8)	6.1 (2.7, 11.6)
ORR				
Objective response rate§ (95% CI)	42.9 (34.1, 52.0)	38.2 (29.1, 47.9)	23.3 (16.4, 31.4)	36.1 (27.6, 45.3)
Duration of Response				
Number of responders	54	42	31	44
Median in months (range)	7.1 (2.1+, 39.0+)	4.2 (1.2+, 31.5+)	22.6 (2.7+, 43.0+)	4.2 (1.2+, 31.5+)

* Cetuximab, platinum, and 5-FU
† Based on the stratified Cox proportional hazard model
‡ Based on stratified log-rank test
§ Response: Best objective response as confirmed complete response or partial response

An exploratory subgroup analysis was performed in KEYNOTE-048 in patients whose tumors expressed PD-L1 CPS ≥ 1 to < 20 [pembrolizumab plus chemotherapy: n=116 (45%) vs. standard treatment: n=125 (49%) and pembrolizumab monotherapy: n=124 (48%) vs. standard treatment: n=133 (52%)] (see Table 50).

Table 50. Efficacy results for pembrolizumab plus chemotherapy and pembrolizumab as monotherapy by PD-L1 expression in KEYNOTE-048 (CPS ≥ 1 to < 20)

Endpoint	Pembrolizumab + Platinum Chemotherapy + 5-FU n=116	Standard Treatment* n=125	Pembrolizumab Monotherapy n=124	Standard Treatment* n=133
OS				
Number (%) of patients with event	93 (80.2)	115 (92.0)	103 (83.1)	121 (91.0)
Median in months (95% CI)	12.7 (9.4, 15.3)	9.9 (8.6, 11.5)	10.8 (9.0, 12.6)	10.1 (8.7, 12.1)
Hazard ratio† (95% CI)	0.71 (0.54, 0.94)		0.86 (0.66, 1.12)	
OS rate at 6 months (95% CI)	76.7 (67.9, 83.4)	77.4 (69.0, 83.8)	67.6 (58.6, 75.1)	78.0 (70.0, 84.2)
OS rate at 12 months (95% CI)	52.6 (43.1, 61.2)	41.1 (32.4, 49.6)	44.0 (35.1, 52.5)	42.4 (33.9, 50.7)
OS rate at 24 months (95% CI)	25.9 (18.3, 34.1)	14.5 (9.0, 21.3)	22.0 (15.1, 29.6)	15.9 (10.3, 22.6)
PFS				
Number (%) of patients with event	106 (91.4)	117 (93.6)	113 (91.1)	123 (92.5)
Median in months (95% CI)	4.9 (4.2, 5.3)	4.9 (3.7, 6.0)	2.2 (2.1, 2.9)	4.9 (3.8, 6.0)
Hazard ratio† (95% CI)	0.93 (0.71, 1.21)		1.25 (0.96, 1.61)	
PFS rate at 6 months (95% CI)	40.1 (31.0, 49.0)	40.0 (31.2, 48.5)	24.2 (17.1, 32.0)	41.4 (32.8, 49.7)
PFS rate at 12 months (95% CI)	15.1 (9.1, 22.4)	11.3 (6.4, 17.7)	17.5 (11.4, 24.7)	12.1 (7.2, 18.5)
PFS rate at 24 months (95% CI)	8.5 (4.2, 14.7)	5.0 (1.9, 10.1)	8.3 (4.3, 14.1)	6.3 (2.9, 11.5)
ORR				
Objective response rate‡ (95% CI)	29.3 (21.2, 38.5)	33.6 (25.4, 42.6)	14.5 (8.8, 22.0)	33.8 (25.9,42.5)
Duration of Response				
Number of responders	34	42	18	45
Median in months (range)	5.6 (1.6+, 25.6+)	4.6 (1.4+, 31.4+)	NR (1.5+, 38.9+)	5.0 (1.4+, 38.7+)

* Cetuximab, platinum, and 5-FU
† Based on the stratified Cox proportional hazard model
‡ Response: Best objective response as confirmed complete response or partial response

KEYNOTE-040: Controlled trial in HNSCC patients previously treated with platinum-containing chemotherapy

The safety and efficacy of pembrolizumab were investigated in KEYNOTE-040, a multicenter, open-label, randomized, controlled study for the treatment of histologically confirmed recurrent or metastatic HNSCC of the oral cavity, pharynx or larynx in patients who had disease progression on or after platinum-containing chemotherapy administered for recurrent or metastatic HNSCC or following platinum-containing chemotherapy administered as part of induction, concurrent, or adjuvant therapy, and were not amenable to local therapy with curative intent.[10] Patients were stratified by PD-L1 expression (TPS ≥ 50%), HPV status and ECOG performance status and then randomized (1:1) to receive either pembrolizumab 200 mg every 3 weeks (n=247) or one of three standard treatments (n=248): methotrexate 40 mg/m^2 once weekly (n=64), docetaxel 75 mg/m^2 once every 3 weeks (n=99), or cetuximab 400 mg/m^2 loading dose and then 250 mg/m^2 once weekly (n=71). Treatment could continue beyond progression if the patient was clinically stable and was considered to be deriving clinical benefit by the investigator. The study excluded patients with nasopharyngeal carcinoma, active autoimmune disease that required systemic therapy within 2 years of treatment, a medical condition that required immunosuppression, or who were previously treated with 3 or more systemic regimens for recurrent and/or metastatic HNSCC. Assessment of tumor status was performed at 9 weeks, then every 6 weeks through week 52, followed by every 9 weeks through 24 months.

Among the 495 patients in KEYNOTE-040, 129 (26%) had tumors that expressed PD-L1 with a TPS ≥ 50% based on PD-L1 IHC 22C3 pharmDx. The baseline characteristics of these 129 patients included: median age 62 years (40% age 65 or older); 81% male; 78% White, 11% Asian, and 2% Black; 23% and 77% with an ECOG performance status 0 or 1, respectively; and 19% with HPV positive tumors. Sixty-seven percent of patients had M1 disease and the majority had Stage IV disease (Stage IV 32%, Stage IVa 14%, Stage IVb 4%, and Stage IVc 44%). Sixteen percent (16%) had disease progression following platinum-containing neoadjuvant or adjuvant chemotherapy, and 84% had received 1-2 prior systemic regimens for metastatic disease.

The primary efficacy outcome was OS in the ITT population. The initial analysis resulted in a HR for OS of 0.82 (95% CI: 0.67, 1.01) with a one-sided p-value of 0.0316. The median OS was 8.4 months for pembrolizumab compared to 7.1 months for standard treatment. Table 51 summarizes the key efficacy measures for the TPS ≥ 50% population. The Kaplan-Meier curve for OS for the TPS ≥ 50% population is shown in Figure 12.

Table 51. Efficacy of pembrolizumab 200 mg every 3 weeks in HNSCC patients with TPS ≥ 50% who were previously treated with platinum chemotherapy in KEYNOTE-040

Endpoint	Pembrolizumab 200 mg every 3 weeks n=64	Standard Treatment* n=65
OS		
Number (%) of patients with event	41 (64)	56 (86)
Hazard ratio† (95% CI)	0.53 (0.35, 0.81)	
p-Value‡	0.001	
Median in months (95% CI)	11.6 (8.3, 19.5)	6.6 (4.8, 9.2)
PFS§		
Number (%) of patients with event	52 (81)	58 (89)
Hazard ratio† (95% CI)	0.58 (0.39, 0.86)	
p-Value‡	0.003	
Median in months (95% CI)	3.5 (2.1, 6.3)	2.1 (2.0, 2.4)
Rate (%) at 6 months (95% CI)	40.1 (28.1, 51.9)	17.1 (8.8, 27.7)
Overall response rate§		
ORR% (95% CI)	26.6 (16.3, 39.1)	9.2 (3.5, 19.0)
p-Value¶	0.0009	
Complete response	5%	2%
Partial response	22%	8%
Stable disease	23%	23%
Response duration§,#		
Median in months (range)	Not reached (2.7, 13.8+)	6.9 (4.2, 18.8)
Number (%Þ) of patients with duration ≥ 6 months	9 (66)	2 (50)

* Methotrexate, docetaxel, or cetuximab
† Hazard ratio (pembrolizumab compared to standard treatment) based on the stratified Cox proportional hazard model
‡ One-sided p-Value based on log-rank test
§ Assessed by BICR using RECIST 1.1
¶ Based on method by Miettinen and Nurminen
# Based on patients with a best overall response as confirmed complete or partial response
Þ Based on Kaplan-Meier estimation

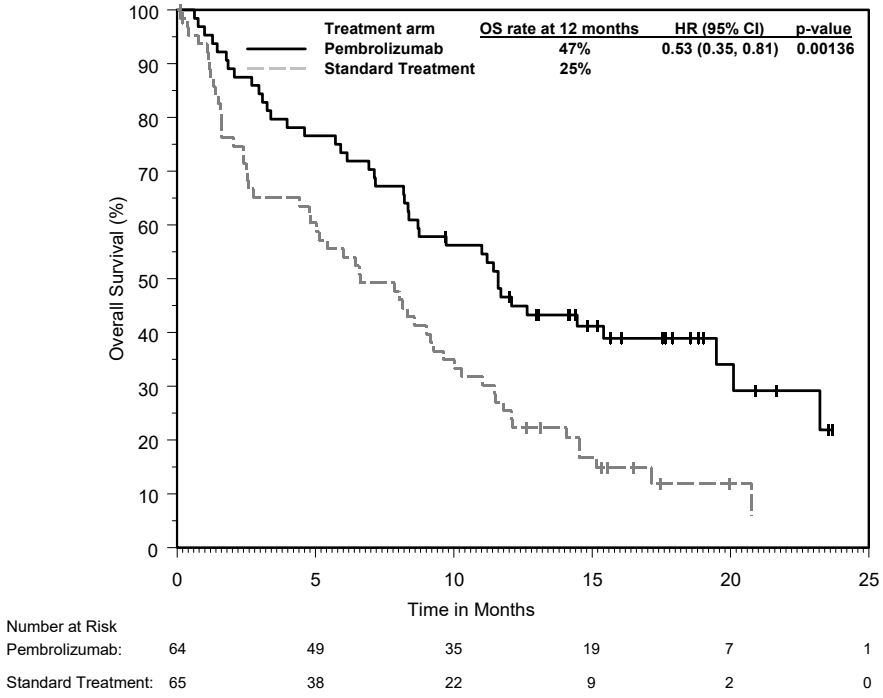


Figure 12. Kaplan-Meier curve for overall survival by treatment arm in KEYNOTE-040 patients with PD-L1 expression (TPS ≥ 50%)

16.11 Nonclinical performance evaluation: TNBC

The nonclinical studies were performed on FFPE TNBC specimens.

Analytical sensitivity/specificity: TNBC

Analytical sensitivity of PD-L1 IHC 22C3 pharmDx was tested on 100 FFPE TNBC specimens (staged I to IV) using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a range of CPS 0–100, where 30% of the specimens had PD-L1 expression with a CPS ≥ 10.

Precision: TNBC

The precision of PD-L1 IHC 22C3 pharmDx was evaluated at Agilent. Inter-operator, inter-instrument, inter-lot, and inter-day were tested in combined precision. For the precision studies, negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with corresponding two–sided 95% percentile bootstrap confidence intervals for the CPS ≥ 10 cutoff as shown in Table 52. For studies which resulted in 100.0% agreement, NPA, PPA, and OA were computed with corresponding two-sided 95% Wilson score confidence intervals for the CPS ≥ 10 cutoff.

Table 52. Precision of PD-L1 IHC 22C3 pharmDx in TNBC, tested at one site (CPS ≥ 10)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Combined Precision(Inter-operator, inter-instrument, inter-lot, and inter-day as combined variables)	CPS ≥ 10	All 33 TNBC specimens (21 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression were tested by 3 operators, using 3 Autostainer Link 48 instruments and 3 reagent lots, over 3 nonconsecutive days.	NPA 100.0% (94.3–100.0%) PPA 100.0% (90.4–100.0%) OA 100.0% (96.3–100.0%)
Intra-run precision* (Repeatability)	CPS ≥ 10	All 33 TNBC specimens (16 PD-L1-negative and 17 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 5 replicates within a run using the Autostainer Link 48 instrument.	NPA 100.0% (95.4–100.0%) PPA 94.0% (86.9–100.0%) OA 96.9% (93.3–100.0%)
Inter-observer precision	CPS ≥ 10	All 48 TNBC specimens (31 PD-L1-negative and 17 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days.	NPA 93.2% (87.5–97.8%) PPA 92.2% (85.6–97.4%) OA 92.8% (88.4–96.8%)
Intra-observer precision	CPS ≥ 10	All 48 TNBC specimens (31 PD-L1-negative and 17 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days.	NPA 98.5% (97.0–99.6%) PPA 94.5% (90.9–98.0%) OA 97.0% (95.4–98.6%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

External reproducibility: TNBC
The reproducibility of PD-L1 IHC 22C3 pharmDx was evaluated at three external sites. NPA, PPA, and OA were computed with two-sided 95% percentile bootstrap confidence intervals.

Table 53. Reproducibility of PD-L1 IHC 22C3 pharmDx in TNBC, tested at three external sites (CPS ≥ 10)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	CPS ≥ 10	All 40 TNBC specimens (19 PD-L1-negative and 21 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days. Inter-site analysis was performed for 3 sites on a total of 600 comparisons to majority call.	NPA 93.0% (85.3–100.0%) PPA 92.1% (86.3–97.1%) OA 92.5% (87.8–96.7%)
Intra-site	CPS ≥ 10	All 40 TNBC specimens (19 PD-L1-negative and 21 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days at each of 3 study sites. Intra-site analysis was performed for 3 sites on a total of 600 comparisons to majority call.	NPA 97.1% (94.3–99.3%) PPA 94.4% (90.0–98.1%) OA 95.7% (92.7–98.2%)
Inter-observer	CPS ≥ 10	All 60 TNBC specimens (26 PD-L1-negative and 34 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Inter-observer analysis was performed between 3 sites on a total of 540 comparisons to majority call.	NPA 97.0% (93.6–100.0%) PPA 95.4% (91.2–98.7%) OA 96.1% (93.3–98.5%)
Intra-observer	CPS ≥ 10	All 60 TNBC specimens (26 PD-L1-negative and 34 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Intra-observer analysis was performed for 3 sites on a total of 540 comparisons to majority call.	NPA 98.7% (96.6–100.0%) PPA 96.7% (94.6–98.7%) OA 97.6% (96.1–98.9%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

16.12 Clinical performance evaluation: TNBC (KEYTRUDA)

KEYNOTE355: Controlled study of combination therapy in TNBC patients previously untreated for metastatic disease

The efficacy of pembrolizumab in combination with paclitaxel, nab-paclitaxel, or gemcitabine and carboplatin was investigated in KEYNOTE-355, a randomized, double-blind, multicenter, placebo-controlled study.[11] Key eligibility criteria were locally recurrent unresectable or metastatic TNBC, regardless of tumor PD-L1 expression, not previously treated with chemotherapy in the advanced setting. Patients with active autoimmune disease that required systemic therapy within 2 years of treatment or a medical condition that required immunosuppression were ineligible. Randomization was stratified by chemotherapy treatment (paclitaxel or nab-paclitaxel vs. gemcitabine and carboplatin), tumor PD-L1 expression (CPS ≥ 1 vs. CPS < 1), and prior treatment with the same class of chemotherapy in the neoadjuvant setting (yes vs. no). Patients were randomized (2:1) to one of the following treatment arms via intravenous infusion:

- Pembrolizumab 200 mg on Day 1 every 3 weeks in combination with nab-paclitaxel 100 mg/m^2 on Days 1, 8 and 15 every 28 days, or paclitaxel 90 mg/m^2 on Days 1, 8, and 15 every 28 days, or gemcitabine 1,000 mg/m^2 and carboplatin AUC 2 mg/mL/min on Days 1 and 8 every 21 days.
- Placebo on Day 1 every 3 weeks in combination with nab-paclitaxel 100 mg/m^2 on Days 1, 8 and 15 every 28 days, or paclitaxel 90 mg/m^2 on Days 1, 8, and 15 every 28 days, or gemcitabine 1,000 mg/m^2 and carboplatin AUC 2 mg/mL/min on Days 1 and 8 every 21 days.

Treatment with pembrolizumab or placebo, both in combination with chemotherapy, continued until RECIST 1.1-defined progression of disease as determined by the investigator, unacceptable toxicity, or a maximum of 24 months. Chemotherapy could continue per standard of care. Administration of pembrolizumab was permitted beyond RECIST-defined disease progression if the patient was clinically stable and deriving clinical benefit as determined by the investigator. Assessment of tumor status was performed at Weeks 8, 16, and 24, then every 9 weeks for the first year, and every 12 weeks thereafter.

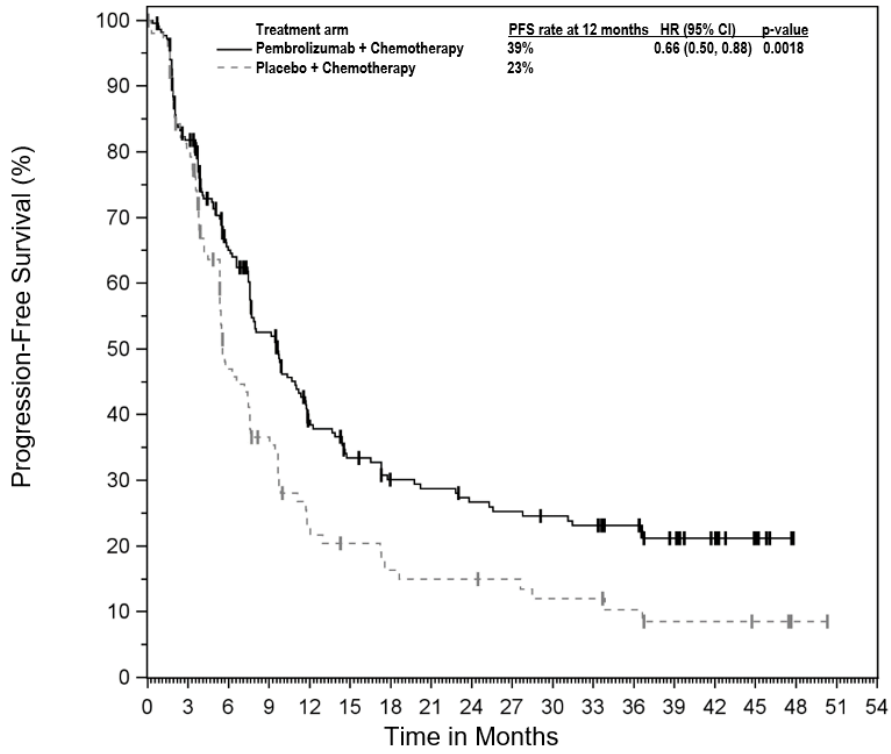
Among the 847 patients randomized in KEYNOTE-355, 636 (75%) had tumors that expressed PD-L1 with a CPS ≥ 1 and 323 (38%) had tumor PD-L1 expression CPS ≥ 10 based on PD-L1 IHC 22C3 pharmDx. The baseline characteristics of the 323 patients with tumor PD-L1 expression CPS ≥ 10 included: median age of 53 years (range: 22 to 83); 20% age 65 or older; 100% female; 69% White, 20% Asian, and 5% Black; ECOG performance status of 0 (61%) and 1 (39%); 67% were post-menopausal status; 3% had a history of brain metastases; and 20% had disease-free interval of < 12 months.

The dual primary efficacy outcome measures were PFS as assessed by BICR using RECIST 1.1 and OS. Secondary efficacy outcome measures were ORR and response duration as assessed by BICR using RECIST 1.1. The study demonstrated a statistically significant improvement in PFS at its pre-specified interim analysis (HR 0.65; 95% CI 0.49, 0.86; p-Value 0.0012) and OS at final analysis for patients with tumor PD-L1 expression CPS ≥ 10 randomized to the pembrolizumab in combination with chemotherapy arm compared with placebo in combination with chemotherapy. Table 54 summarizes key efficacy measures and Figures 13 and 14 show the Kaplan-Meier curves for PFS and OS based on the final analysis with a median follow-up time of 20.2 months (range: 0.3 to 53.1 months) for patients with tumor PD-L1 expression CPS ≥ 10.

Table 54. Efficacy results in KEYNOTE-355 patients with CPS ≥ 10

Endpoint	Pembrolizumab with chemotherapy* n=220	Placebo with chemotherapy* n=103
PFS†		
Number (%) of patients with event	144 (65%)	81 (79%)
Hazard ratio‡ (95% CI)	0.66 (0.50, 0.88)	
p-Value§	0.0018	
Median in months (95% CI)	9.7 (7.6, 11.3)	5.6 (5.3, 7.5)
OS		
Number (%) of patients with event	155 (70%)	84 (82%)
Hazard ratio‡ (95% CI)	0.73 (0.55, 0.95)	
p-Value¶	0.0093	
Median in months (95% CI)	23.0 (19.0, 26.3)	16.1 (12.6, 18.8)
Objective response rate†		
ORR % (95% CI)	53% (46, 60)	41% (31, 51)
Complete response	17%	14%
Partial response	36%	27%
Response duration†		
Median in months (range)	12.8 (1.6+, 45.9+)	7.3 (1.5, 46.6+)
% with duration ≥ 6 months#	82%	60%
% with duration ≥ 12 months#	56%	38%

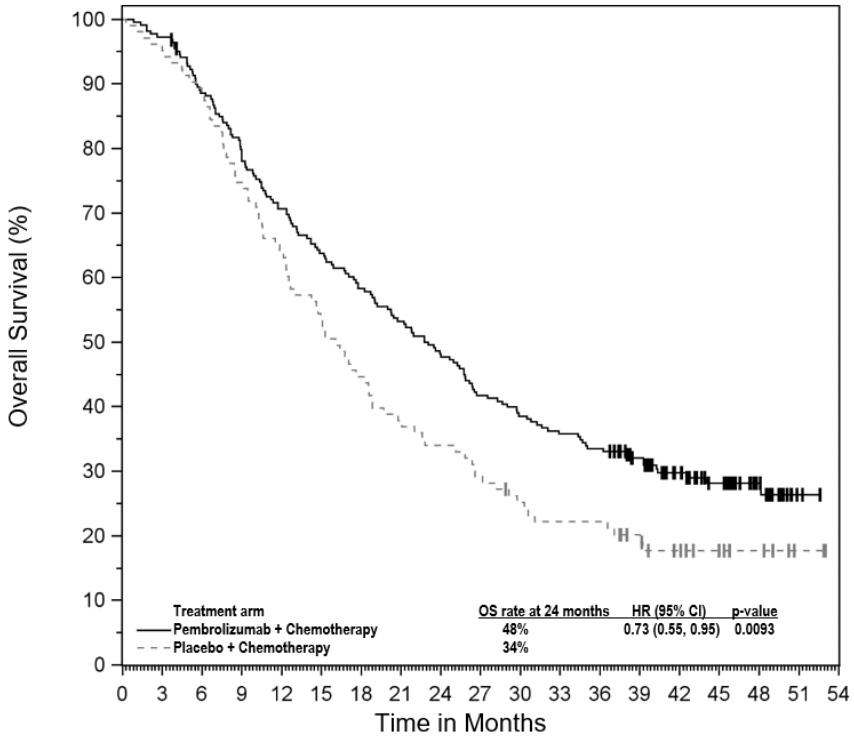
* Chemotherapy: paclitaxel, nab-paclitaxel, or gemcitabine and carboplatin
† Assessed by BICR using RECIST 1.1
‡ Based on Cox regression model with Efron's method of tie handling with treatment as a covariate stratified by chemotherapy on study (taxane vs. gemcitabine and carboplatin) and prior treatment with same class of chemotherapy in the neoadjuvant setting (yes vs. no)
§ Nominal p-Value based on log-rank test stratified by chemotherapy on study (taxane vs. gemcitabine and carboplatin) and prior treatment with same class of chemotherapy in the neoadjuvant setting (yes vs. no). At the pre-specified interim analysis of PFS (median follow-up time of 19.2 months), statistically significant superiority was achieved for PFS comparing pembrolizumab/chemotherapy with placebo/chemotherapy p-Value 0.0012.
¶ One-sided p-Value based on log-rank test stratified by chemotherapy on study (taxane vs. gemcitabine and carboplatin) and prior treatment with same class of chemotherapy in the neoadjuvant setting (yes vs. no). OS results met the pre-specified efficacy boundary of 0.0113 for statistical significance.
# From product-limit (Kaplan-Meier) method for censored data
\+ Denotes there is no progressive disease by the time of last disease assessment



Number at Risk

Pembrolizumab + Chemotherapy	220	173	122	95	63	52	44	42	38	36	34	32	27	19	13	6	0	0	0
Placebo + Chemotherapy	103	80	41	30	18	15	12	11	11	10	8	8	6	4	4	3	1	0	0

Figure 13. Kaplan-Meier curve for progression-free survival by treatment arm in KEYNOTE-355 patients with PD-L1 expression (CPS ≥ 10)



Number at Risk

Pembrolizumab + Chemotherapy	220	214	193	171	154	139	127	116	105	91	84	78	73	59	43	31	17	2	0
Placebo + Chemotherapy	103	98	91	77	66	55	46	39	35	30	25	22	22	17	12	8	6	2	0

Figure 14. Kaplan-Meier curve for overall survival by treatment arm in KEYNOTE-355 patients with PD-L1 expression (CPS ≥ 10)

16.13 Nonclinical performance evaluation: cervical cancer

The nonclinical studies were performed on FFPE cervical cancer specimens (studies were conducted with both squamous and adenocarcinoma specimens).

Analytical sensitivity/specificity: cervical cancer
Analytical sensitivity of PD-L1 IHC 22C3 pharmDx was analyzed for 130 FFPE cervical cancer specimens. Assessment of PD-L1 expression demonstrated staining across a range of CPS 0--100, where 68% of the specimens had PD-L1 expression with a CPS ≥ 1.

Precision: cervical cancer
The precision of PD-L1 IHC 22C3 pharmDx was evaluated at Agilent Technologies. Inter-operator, inter-instrument, and inter-day were tested in combined precision. For the precision studies, negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with corresponding two-sided 95% percentile bootstrap confidence intervals for the CPS ≥ 1 cutoff as shown in Table 55. For studies with agreement parameters which resulted in 100.0% agreement, two-sided 95% confidence intervals were calculated using the Wilson Score method for the CPS ≥ 1 cutoff.

Table 55. Precision of PD-L1 IHC 22C3 pharmDx in cervical cancer, tested at one site (CPS ≥ 1)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Combined Precision(Inter-operator, inter-instrument, and inter-day as combined variables)	CPS ≥ 1	All 36 cervical cancer specimens (18 PD-L1-negative and 18 PD-L1-positive) with a range of PD-L1 IHC expression were tested by 3 operators, using 3 Autostainer Link 48 instruments, over 3 nonconsecutive days.	NPA 100.0% (93.2–100.0%) PPA 96.2% (90.2–100.0%) OA 98.1% (95.2–100.0%)
Intra-run precision* (Repeatability)	CPS ≥ 1	All 30 cervical cancer specimens (15 PD-L1-negative and 15 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 5 replicates within a run using the Autostainer Link 48 instrument.	NPA 100.0% (95.0–100.0%) PPA 98.7% (96.0–100.0%) OA 99.3% (97.9–100.0%)
Inter-observer precision	CPS ≥ 1	All 50 cervical cancer specimens (24 PD-L1-negative and 26 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days with a minimum 2-week washout period in between each read.	NPA 99.5% (98.6–100.0%) PPA 98.3% (96.2–100.0%) OA 98.9% (97.6–99.8%)
Intra-observer precision	CPS ≥ 1	All 50 cervical cancer specimens (24 PD-L1-negative and 26 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists over 3 nonconsecutive days with a minimum 2-week washout period in between each read.	NPA 99.1% (97.7–100.0%) PPA 99.1% (97.8–100.0%) OA 99.1% (98.2–99.8%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

External reproducibility: cervical cancer
The reproducibility of PD-L1 IHC 22C3 pharmDx was evaluated at three external sites. NPA, PPA, and OA were computed with corresponding two-sided 95% percentile bootstrap confidence intervals for the CPS ≥ 1 cutoff as shown in Table 56.

Table 56. Reproducibility of PD-L1 IHC 22C3 pharmDx in cervical cancer, tested at three external sites (CPS ≥ 1)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	CPS ≥ 1	All 38 cervical cancer specimens (19 PD-L1 negative and 19 PD-L1 positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days. Inter-site analysis was performed between 3 sites on a total of 570 comparisons to majority call.	NPA 97.5% (94.7–99.6%) PPA 98.9% (97.2–100.0%) OA 98.2% (96.7–99.5%)
Intra-site	CPS ≥ 1	All 38 cervical cancer specimens (19 PD-L1 negative and 19 PD-L1 positive) with a range of PD-L1 IHC expression were tested on 5 nonconsecutive days at each of 3 study sites. Intra-site analysis was performed for 3 sites on a total of 570 comparisons to majority call.	NPA 98.6% (96.8–100.0%) PPA 98.3% (96.1–100.0%) OA 98.4% (97.0–99.5%)
Inter-observer	CPS ≥ 1	All 52 cervical cancer specimens (24 PD-L1-negative and 28 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Inter-observer analysis was performed between 3 sites on a total of 468 comparisons to majority call.	NPA 98.1% (94.9–100.0%) PPA 98.8% (96.8–100.0%) OA 98.5% (96.8–99.8%)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Intra-observer	CPS ≥ 1	All 52 cervical cancer specimens (24 PD-L1-negative and 28 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, 1 at each of 3 study sites, on 3 nonconsecutive days. Intra-observer analysis was performed for 3 sites on a total of 468 comparisons to majority call.	NPA 98.6% (96.8–100.0%) PPA 99.2% (98.0–100.0%) OA 98.9% (97.6–99.8%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement; CPS=Combined Positive Score

16.14 Clinical performance evaluation: cervical cancer (KEYTRUDA)

KEYNOTE-826: Controlled study of combination therapy in patients with persistent, recurrent, or metastatic cervical cancer

The efficacy of pembrolizumab in combination with paclitaxel and cisplatin or paclitaxel and carboplatin, with or without bevacizumab, was investigated in KEYNOTE-826, a multicenter, randomized, double-blind, placebo-controlled study that enrolled 617 patients with persistent, recurrent, or first-line metastatic cervical cancer who had not been treated with chemotherapy except when used concurrently as a radio-sensitizing agent.[12] Patients were enrolled regardless of tumor PD-L1 expression status. Patients with autoimmune disease that required systemic therapy within 2 years of treatment or a medical condition that required immunosuppression were ineligible. Randomization was stratified by metastatic status at initial diagnosis, investigator decision to use bevacizumab, and PD-L1 status (CPS < 1 vs. CPS 1 to < 10 vs. CPS ≥ 10). Patients were randomized (1:1) to one of the two treatment groups:

- Treatment Group 1: Pembrolizumab 200 mg plus chemotherapy with or without bevacizumab
- Treatment Group 2: Placebo plus chemotherapy with or without bevacizumab

The investigator selected one of the following four treatment regimens prior to randomization:

1. Paclitaxel 175 mg/m2 + cisplatin 50 mg/m^2
2. Paclitaxel 175 mg/m2 + cisplatin 50 mg/m^2 + bevacizumab 15 mg/kg
3. Paclitaxel 175 mg/m2 + carboplatin AUC 5 mg/mL/min
4. Paclitaxel 175 mg/m2 + carboplatin AUC 5 mg/mL/min + bevacizumab 15 mg/kg

All study medications were administered as an intravenous infusion. All study treatments were administered on Day 1 of each 3-week treatment cycle. Cisplatin could be administered on Day 2 of each 3-week treatment cycle. The option to use bevacizumab was by investigator choice prior to randomization. Treatment with pembrolizumab continued until RECIST v1.1-defined progression of disease, unacceptable toxicity, or a maximum of 24 months. Administration of pembrolizumab was permitted beyond RECIST-defined disease progression if the patient was clinically stable and considered to be deriving clinical benefit by the investigator. Assessment of tumor status was performed at Week 9 and then every 9 weeks for the first year, followed by every 12 weeks thereafter.

Of the 617 enrolled patients, 548 patients (89%) had tumors expressing PD-L1 with a CPS ≥ 1 based on PD-L1 IHC 22C3 pharmDx. Among these 548 enrolled patients with tumors expressing PD-L1, 273 patients were randomized to pembrolizumab in combination with chemotherapy with or without bevacizumab, and 275 patients were randomized to placebo in combination with chemotherapy with or without bevacizumab. The baseline characteristics of these 548 patients were: median age of 51 years (range: 22 to 82), 16% age 65 or older; 59% White, 18% Asian, and 1% Black; 37% Hispanic or Latino; 56% and 43% ECOG performance status of 0 or 1, respectively; 63% received bevacizumab as study treatment; 21% with adenocarcinoma and 5% with adenosquamous histology; for patients with persistent or recurrent disease with or without distant metastases, 39% had received prior chemoradiation only and 17% had received prior chemoradiation plus surgery.

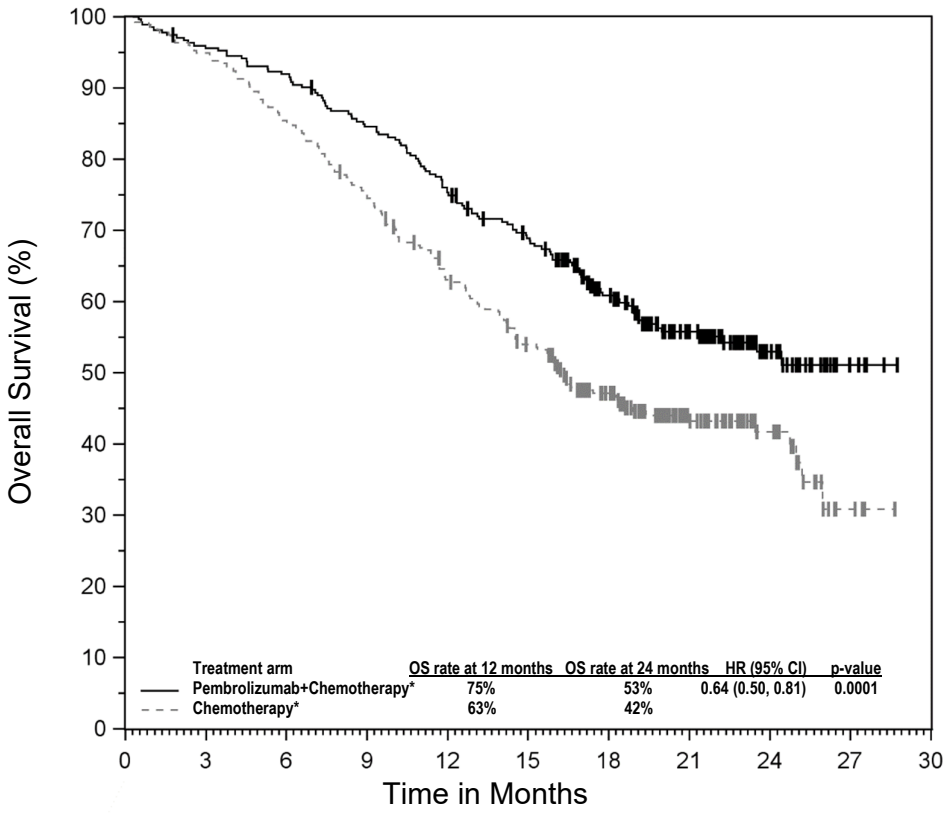
The primary efficacy outcome measures were OS and PFS as assessed by investigator according to RECIST v1.1. Secondary efficacy outcome measures were ORR and DoR, according to RECIST v1.1, as assessed by investigator.

The study demonstrated statistically significant improvements in OS and PFS for patients randomized to pembrolizumab in combination with chemotherapy with or without bevacizumab compared to placebo in combination with chemotherapy with or without bevacizumab at a pre-specified interim analysis in the overall population. The median follow-up time was 17.2 months (range: 0.3 to 29.4 months). Table 57 summarizes key efficacy measures for patients whose tumors expressed PD-L1 with a CPS ≥ 1 in KEYNOTE-826 from the pre-specified interim analysis. The Kaplan-Meier curves for OS and PFS are shown in Figures 15 and 16.

Table 57. Efficacy results in KEYNOTE-826 for patients with PD-L1 expression (CPS ≥ 1)

Endpoint	Pembrolizumab 200 mg every 3 weeks plus Chemotherapy* with or without bevacizumab n=273	Placebo plus Chemotherapy* with or without bevacizumab n=275
OS		
Number of patients with event (%)	118 (43)	154 (56)
Median in months (95% CI)	NR (19.8, NR)	16.3 (14.5, 19.4)
Hazard ratio† (95% CI)	0.64 (0.50, 0.81)	
p-Value‡	0.0001	
PFS		
Number of patients with event (%)	157 (58)	198 (72)
Median in months (95% CI)	10.4 (9.7, 12.3)	8.2 (6.3, 8.5)
Hazard ratio† (95% CI)	0.62 (0.50, 0.77)	
p-Value§	< 0.0001	
Objective response rate		
ORR¶ (95% CI)	68% (62, 74)	50% (44, 56)
Complete response rate	23%	13%
Partial response rate	45%	37%
Duration of response		
Median in months (range)	18.0 (1.3+, 24.2+)	10.4 (1.5+, 22.0+)
% of patients with duration ≥ 12 months#	56	46

* Chemotherapy (paclitaxel and cisplatin or paclitaxel and carboplatin)
† Based on the stratified Cox proportional hazard model
‡ Based on stratified log-rank test (compared to an alpha boundary of 0.00549)
§ Based on stratified log-rank test (compared to an alpha boundary of 0.00144)
¶ Response: Best objective response as confirmed complete response or partial response
# Based on Kaplan-Meier estimation
NR = not reached

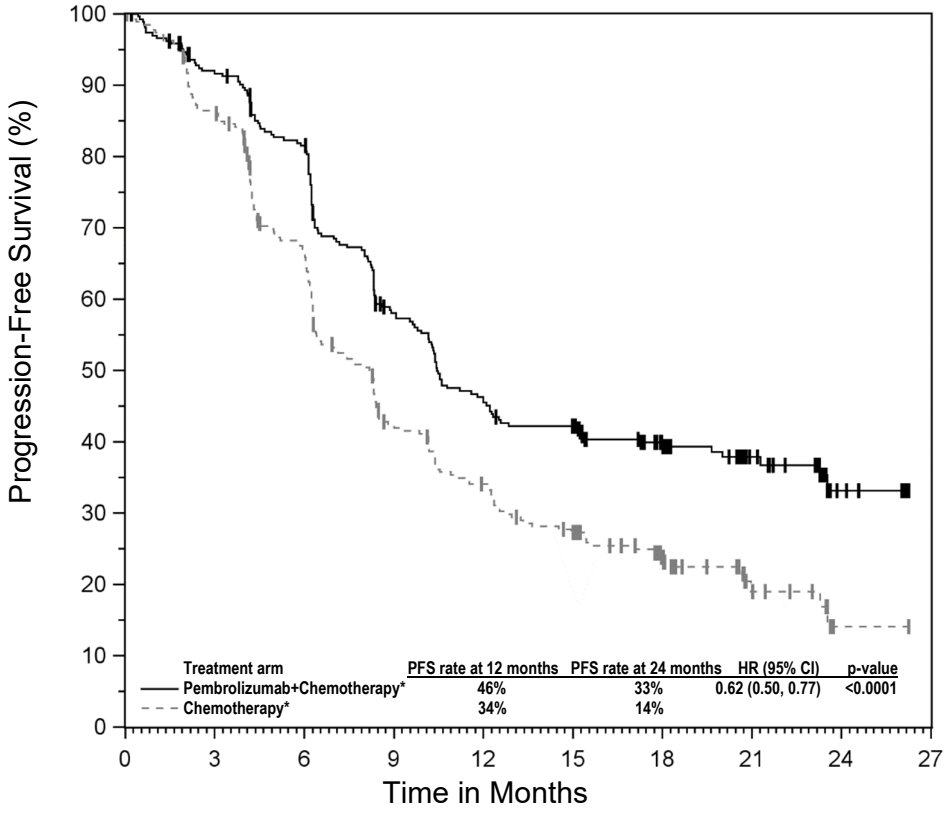


Number at Risk

Pembrolizumab+Chemotherapy*	273	260	250	229	204	181	132	82	34	6	0
Chemotherapy*	275	261	235	206	168	140	100	55	25	4	0

* Chemotherapy (paclitaxel and cisplatin or paclitaxel and carboplatin) with or without bevacizumab

Figure 15. Kaplan-Meier curve for overall survival by treatment arm in KEYNOTE-826 patients with PD-L1 expression (CPS ≥ 1)



Number at Risk

Pembrolizumab+Chemotherapy*	273	238	208	143	112	101	66	34	10	0
Chemotherapy*	275	229	170	103	81	63	38	13	1	0

* Chemotherapy (paclitaxel and cisplatin or paclitaxel and carboplatin) with or without bevacizumab

Figure 16. Kaplan-Meier curve for progression free survival by treatment arm in KEYNOTE-826 patients with PD-L1 expression (CPS ≥ 1)

16.15 Nonclinical performance evaluation: melanoma

Analytical sensitivity: melanoma

Analytical sensitivity of PD-L1 IHC 22C3 pharmDx was tested on 137 unique cases of melanoma FFPE specimens using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a dynamic range in tumor and immune cells, and exhibited 0–3 staining intensity.

Precision: melanoma

The precision of PD-L1 IHC 22C3 pharmDx on melanoma specimens was evaluated at Agilent. Average negative percent agreement (ANA), average positive percent agreement (APA), and overall percent agreement (OA) with two-sided 95% percentile bootstrap confidence intervals were determined for ≥ 1% PD-L1 expression (MEL Score ≥ 2) as shown in Table 58. For studies which resulted in 100.0% agreement, negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) with corresponding two-sided 95% Wilson score confidence intervals were computed.

Table 58. Precision of PD-L1 IHC 22C3 pharmDx tested at one site (MEL Score ≥ 2 (≥ 1%))

Precision Study	MEL Score	PD-L1 Expression	Study Design	% Agreement (95% CI)
Inter-operator	≥ 2	≥ 1%	All 16 melanoma specimens (7 PD-L1-negative and 9 PD-L1-positive) with a range of PD-L1 IHC expression were tested by 6 analysts using 1 Autostainer Link 48 instrument.	NPA 100.0% (91.6–100.0%) PPA 100.0% (93.4–100.0%) OA 100.0% (96.2–100.0%)
Inter-instrument	≥ 2	≥ 1%	All 16 melanoma specimens (7 PD-L1-negative and 9 PD-L1-positive) with a range of PD-L1 IHC expression were tested using 6 Autostainer Link 48 instruments.	NPA 100.0% (91.6–100.0%) PPA 100.0% (93.4–100.0%) OA 100.0% (96.2–100.0%)
Inter-lot	≥ 2	≥ 1%	All 16 melanoma specimens (7 PD-L1-negative and 9 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 3 replicates and 3 reagent lots using the Autostainer Link 48 instrument.	ANA 97.0% (92.3–100.0%) APA 97.4% (93.5–100.0%) OA 97.2% (93.1–100.0%)

Precision Study	MEL Score	PD-L1 Expression	Study Design	% Agreement (95% CI)
Inter-day	≥ 2	≥ 1%	All 16 melanoma specimens (7 PD-L1-negative and 9 PD-L1-positive) with a range of PD-L1 IHC expression were tested on 6 nonconsecutive days using the Autostainer Link 48 instrument.	NPA 100.0% (91.6–100.0%) PPA 100.0% (93.4–100.0%) OA 100.0% (96.2–100.0%)
Intra-run	≥ 2	≥ 1%	All 16 melanoma specimens (7 PD-L1-negative and 9 PD-L1-positive) with a range of PD-L1 IHC expression were tested with 6 replicates within a run using the Autostainer Link 48 instrument.	NPA 100.0% (91.6–100.0%) PPA 100.0% (93.4–100.0%) OA 100.0% (96.2–100.0%)
Inter-observer	≥ 2	≥ 1%	All 48 melanoma specimens (18 PD-L1-negative and 30 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, on 3 nonconsecutive days.	ANA 87.7% (79.3–94.7%) APA 91.8% (85.4–96.7%) OA 90.1% (83.1–95.8%)
Intra-observer	≥ 2	≥ 1%	All 48 melanoma specimens (22 PD-L1-negative and 26 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, were scored by 3 pathologists, on 3 nonconsecutive days.	ANA 89.9% (82.9–95.7%) APA 90.7% (83.3–96.2%) OA 90.3% (83.3–95.8%)

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall Percent Agreement
ANA=Average Negative Percent Agreement; APA=Average Positive Percent Agreement

17. Troubleshooting

Table 59. Troubleshooting

Problem	Probable Cause	Suggested Action
1. No staining of slides	1a. Programming error.	1a. Verify that the PD-L1 IHC 22C3 pharmDx protocol 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 EnVision FLEX Wash Buffer (20x) (Code K8007).
	1d. Degradation of Control Slide	1d. Check kit expiration date and kit storage conditions on outside of package.
2. Weak staining of specimen slides.	2a. Inappropriate fixation method used.	2a. Ensure that only neutral buffered formalin fixative and approved fixation methods are used.
	2b. Insufficient reagent volume applied.	2b. Check size of tissue section and reagent volume applied.
	2c. Inappropriate wash buffer used.	2c. Use only EnVision FLEX Wash Buffer (20x) (Code K8007).
3. Weak staining of specimen slides or of the positive cell line on the Agilent-provided Control Slide.	3a. Inadequate target retrieval.	3a. Verify that the 3-in-1 pretreatment procedure was correctly performed.
	3b. Inappropriate wash buffer used.	3b. Use only EnVision FLEX Wash Buffer (20x) (Code K8007).
4. Excessive nonspecific staining of slides.	4a. Paraffin incompletely removed.	4a. Verify that the 3-in-1 pretreatment procedure was correctly performed.
	4b. Slides dried after the 3-in-1 pretreatment procedure was performed.	4b. Ensure slides remain wet with 1x Envision FLEX Wash Buffer after the 3-in-1 pretreatment procedure and after loading on the Autostainer Link 48. Ensure that the Autostainer Link 48 lid is properly closed to prevent reagent evaporation during the staining procedure.
	4c. Nonspecific binding of reagents to tissue section.	4c. Check for proper fixation of the specimen and/or the presence of necrosis.
	4d. Inappropriate fixation method used.	4d. Ensure that only neutral buffered formalin fixative and recommended fixation methods are used.
	4e. Warped Autostainer Link 48 slide racks used.	4e. Ensure that only level Autostainer Link 48 slide racks are used.
5. Tissue detached from slides.	5a. Use of incorrect microscope slides.	5a. Use FLEX IHC Microscope Slides (Code K8020), or Superfrost Plus slides.
	5b. Inadequate preparation of specimens	5b. Cut sections should be placed in a 58 ± 2 ºC oven for 1 hour prior to staining.
6. Excessively strong specific staining.	6a. Inappropriate fixation method used.	6a. Ensure that only approved fixatives and fixation methods are used.
	6b. Inappropriate wash buffer used.	6b. Use only EnVision FLEX Wash Buffer (20x) (Code K8007).
7. 1x EnVision FLEX Target Retrieval Solution is cloudy in appearance when heated.	7. When heated the 1x EnVision FLEX Target Retrieval Solution turns cloudy in appearance.	7. This is normal and does not influence staining.
8. 1x EnVision FLEX Target Retrieval Solution does not meet pH specifications.	8a. pH meter is not calibrated correctly.	8a. Ensure pH meter is calibrated per manufacturer's recommendations. After re-calibration, re-test the pH of 1x EnVision FLEX Target Retrieval Solution. Do not modify the pH of 1x EnVision FLEX Target Retrieval Solution. If the pH is outside the acceptable

Problem	Probable Cause	Suggested Action
		range (6.1 ± 0.2), discard 1x EnVision FLEX Target Retrieval Solution. Prepare new 1x EnVision FLEX Target Retrieval Solution. Check the pH of the new 1x EnVision FLEX Target Retrieval Solution.
	8b. Inferior quality water is used to dilute the EnVision FLEX Target Retrieval Solution concentrate.	8b. Ensure that distilled or deionized water is used to prepare 1x EnVision FLEX Target Retrieval Solution.
	8c. Incorrect EnVision FLEX Target Retrieval Solution is used.	8c. Ensure that the correct EnVision FLEX Target Retrieval Solution specified in 'Materials Provided' Section 4 and 'Reagent Preparation' Section 9 is used.
9. Nonspecific > 1+ nuclear staining on PD-L1 and/or NCR slides	9a. Specimen slides dried prior to initiating the Autostainer Link 48 staining procedure.	9a. Ensure slides remain wet with 1x EnVision FLEX Wash Buffer after deparaffinization, rehydration, and target retrieval (3-in-1) procedure and after loading on the Autostainer Link 48. Ensure that the Autostainer Link 48 lid is properly closed to prevent reagent evaporation during the staining procedure.
	9b. Improper manual slide rinsing with 1x EnVision FLEX Wash Buffer before loading on the Autostainer Link 48.	9b. Slide racks should be placed one rack at a time on the Autostainer Link 48 and then 1x EnVision FLEX Wash Buffer should be manually applied to the slides using a wash bottle. Ensure slides remain wet prior to initiating the Autostainer Link 48 procedure.

NOTE: If the problem cannot be attributed to any of the causes in Table 59, or if the suggested corrective action fails to resolve the problem, please contact Agilent Pathology Support for further assistance. Additional information on staining techniques and specimen preparation can be found in the Education Guide: Immunohistochemical Staining Methods (available from Agilent Technologies).[20]

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Explanation of symbols

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

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