PD-L1 IHC 22C3 pharmDx
Rx Only

SK006
50 tests for use with Autostainer Link 48

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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), gastric or gastroesophageal junction (GEJ) adenocarcinoma, esophageal squamous cell carcinoma (ESCC), cervical cancer, urothelial carcinoma and head and neck squamous cell carcinoma (HNSCC) 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 gastric or GEJ adenocarcinoma, ESCC, cervical cancer, urothelial carcinoma and HNSCC 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.

Companion Diagnostic Indications

Tumor Indication	PD-L1 Expression Level	Intended Use
NSCLC	TPS ≥ 1%	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying NSCLC patients for treatment with KEYTRUDA® (pembrolizumab).**
Gastric or GEJ Adenocarcinoma	CPS ≥ 1	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying gastric or GEJ adenocarcinoma patients for treatment with KEYTRUDA® (pembrolizumab).
ESCC	CPS ≥ 10	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying ESCC patients for treatment with KEYTRUDA® (pembrolizumab).
Cervical Cancer	CPS ≥ 1	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying cervical cancer patients for treatment with KEYTRUDA® (pembrolizumab).
Urothelial Carcinoma	CPS ≥ 10	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying urothelial carcinoma patients for treatment with KEYTRUDA® (pembrolizumab). **
HNSCC	CPS ≥ 1	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying HNSCC patients for treatment with KEYTRUDA® (pembrolizumab). **

**See the KEYTRUDA® product label for specific clinical circumstances guiding PD-L1 testing.

2. Summary and Explanation

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

2.1 NSCLC

Merck Sharp & Dohme 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 stage III NSCLC, who are not candidates for surgical resection or definitive chemoradiation, or metastatic NSCLC patients that may respond to KEYTRUDA treatment. Refer to 'Clinical Performance Evaluation (NSCLC)' section below for KN042 study details.

Merck Sharp & Dohme 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. Refer to 'Clinical Performance Evaluation (NSCLC)' section below for KN024 study details.

Merck Sharp & Dohme 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 metastatic NSCLC patients that may respond to KEYTRUDA treatment. Refer to 'Clinical Performance Evaluation (NSCLC)' section below for KN010 study details.

2.2 Gastric or Gastroesophageal Junction (GEJ) Adenocarcinoma

Merck Sharp & Dohme sponsored clinical study, KEYNOTE-059 (KN059), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (CPS ≥ 1) gastric or GEJ adenocarcinoma patients with at least two prior systemic treatments for advanced disease that may respond to KEYTRUDA treatment. Refer to 'Clinical Performance Evaluation (gastric or GEJ adenocarcinoma)' section below for KN059 study details.

2.3 Esophageal Squamous Cell Carcinoma (ESCC)

Merck Sharpe & Dohme sponsored clinical study, KEYNOTE-181 (KN181), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (CPS ≥ 10) patients with recurrent locally advanced or metastatic esophageal cancer with disease progression on or after one prior line of systemic therapy, who may respond to KEYTRUDA treatment. Refer to 'Clinical Performance Evaluation (ESCC) section below for KN181 study details.

2.4 Cervical Cancer

Merck Sharp & Dohme sponsored clinical study, KEYNOTE-158 (KN158), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (CPS ≥ 1) cervical cancer patients, with disease progression on or after chemotherapy for recurrent or metastatic disease, that may respond to KEYTRUDA treatment. Refer to 'Clinical Performance Evaluation (cervical cancer)' Section below for KN158 Cohort E study details.

2.5 Urothelial Carcinoma

Merck Sharpe & Dohme sponsored clinical study, KEYNOTE-052 (KN052), investigated the clinical validity of PD-L1 IHC 22C3 pharmDx in identifying PD-L1 expressing (CPS ≥ 10) patients with advanced/unresectable or metastatic urothelial cancer who have not received prior systemic chemotherapy, and who are not eligible to receive cisplatin, who may respond to KEYTRUDA treatment. Refer to 'Clinical Performance Evaluation (UC)' section below for KN052 study details.

2.6 HNSCC

Merck Sharpe & Dohme 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 metastatic or recurrent HNSCC who had not previously received systemic therapy for metastatic disease or with recurrent disease who were considered incurable by local therapies, and who may respond to KEYTRUDA treatment. Refer to 'Clinical Performance Evaluation (HNSCC)' section below for KN048 study 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 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.

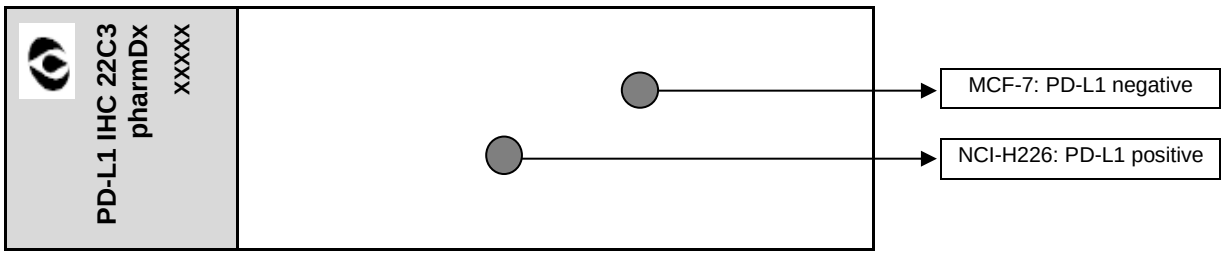
4. Materials Provided

Each kit includes 19.5 mL of PD-L1 primary antibody (approximately 3µg/mL protein concentration) and contains the reagents necessary to perform 50 tests in up to 15 individual runs. The materials listed below are sufficient for 50 tests (50 slides incubated with primary antibody to PD-L1 and 50 slides incubated with the corresponding NCR, 100 slides in total). For larger tissue sections three drop zones (3 x 150µL) per slide may be warranted. 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 antibody in a buffered solution, containing stabilizing protein, and 0.015 mol/L sodium azide.

Quantity	*Description*
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** **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) (2).

Note: All reagents included are formulated specifically for use with this kit. In order for the test to perform as specified, no substitutions, other than EnVision FLEX Target Retrieval Solution, Low pH (50x) (Code K8005) can be made. PD-L1 IHC 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)
Hematoxylin (Code K8008)
Distilled or deionized water (reagent-quality water)
Timer
Positive and negative tissues to use as process controls (see Quality Control section)
Microscope slides: Dako FLEX IHC Microscope Slides (Code K8020) or Superfrost Plus charged slides
Coverslips
Permanent mounting medium and ancillary reagents required for mounting coverslips
Light microscope (4x–40x objective magnification)

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 (3).
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 (4).
6. Incubation times, temperatures, or methods other than those specified may give erroneous results.
7. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.
8. The Visualization Reagent, Liquid DAB+ chromogen and prepared DAB+ Substrate-Chromogen solution may be affected adversely if exposed to excessive light levels. Do not store system components or perform staining in strong light, such as direct sunlight.
9. Paraffin residuals may lead to false negative results.
10. Use of reagent volumes other than recommended may result in loss of visible PD-L1 immunoreactivity.
11. Results from a small study showed a similar dynamic range of PD-L1 expression in primary and metastatic NSCLC specimen pairs. It is possible there may be differences in PD-L1 expression in primary tumors versus metastatic sites in the same patient.
12. Large tissue sections may require 3x150 µl of reagent.
13. As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper work procedures, the dangerous properties of the product and the necessary safety instructions. Please refer to Safety Data Sheet (SDS) for additional information.
14. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
15. Unused solution should be disposed of according to local, State and Federal regulations.
16. Safety Data Sheets are available on www.agilent.com or on request.
17. For countries outside of the United States, see the local KEYTRUDA product label for approved indications and expression cutoff values to guide therapy.

Danger

DAB+ Substrate Buffer: Contains Imidazole.

H360	May damage the unborn child.
P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
P280	Wear protective gloves. Wear eye or face protection. Wear protective clothing.
P308 + P313	IF exposed or concerned: Get medical attention.
P405	Store locked up.
P501	Dispose of contents and container in accordance with all local, regional, national and international regulations.

Danger

DAB+ Chromogen: Contains 3,3-Diaminobenzidine tetrahydrochloride.

H319	Causes serious eye irritation.
H350	May cause cancer.
H341	Suspected of causing genetic defects.
P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
P280	Wear protective gloves. Wear eye or face protection. Wear protective clothing.
P264	Wash hands thoroughly after handling.
P308 + P313	IF exposed or concerned: Get medical attention.
P305 + P351 + P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337 + P313	If eye irritation persists: Get medical attention.
P405	Store locked up.
P501	Dispose of contents and container in accordance with all local, regional, national and international regulations.

Warning

DAB Enhancer

H400	Very toxic to aquatic life.
H411	Toxic to aquatic life with long lasting effects.
P273	Avoid release to environment.
P391	Collect spillage.
P501	Dispose of contents and container in accordance with all local, regional, national and international regulations.

Warning

EnVision FLEX Target Retrieval Solution, Low pH (50x)

H319	Causes serious eye irritation.

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

7. Storage

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

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

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

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 Specimens

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 and dehydrated and cleared in a series of alcohols and xylene, followed by infiltration with melted paraffin. The paraffin temperature should not exceed 60 °C. NSCLC FFPE tissue blocks which are 5 years or older may result in a loss of PD-L1 immunoreactivity. Please refer to Section 15.2 (Product Specific Limitations) for gastric or GEJ adenocarcinoma specimens.

Tissue specimens should be cut into sections of 4-5 µm. After sectioning, tissues should be mounted on Dako FLEX IHC microscope slides (Code K8020) or Superfrost Plus slides and 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 Gastric or Gastroesophageal Junction (GEJ) Adenocarcinoma Cut Section Storage Recommendation

Cut sections must be stained within 5 months when stored at 2-8 ºC (preferred), or at 25 ºC.

8.2.3 ESCC 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 Cervical Cancer Cut Section Storage Recommendation

Cut sections must be stained within 5 months when stored at 2-8 ºC (preferred), or within 1 month when stored at 25 ºC.

8.2.5 Urothelial Carcinoma Cut Section Storage Recommendation

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

8.2.6 HNSCC Cut Section Storage Recommendation

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

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 Target Retrieval Solution, Low pH by diluting Target Retrieval Solution, Low pH (50x) 1:50 using distilled or deionized water (reagent-quality water); the pH of 1x Target Retrieval Solution must be 6.1 ± 0.2. 1x Target Retrieval Solution pH below 5.9 may give erroneous results. One 30 mL bottle of Target Retrieval Solution, Low pH (50x) diluted 1:50 will provide 1.5 L of 1x reagent, sufficient to fill one PT Link tank which will treat up to 24 slides per use. Discard 1x Target Retrieval Solution after three uses and do not use after 5 days following dilution. Please refer to Section 15.2 (Product Specific Limitations) for Target Retrieval Solution limitations in ESCC 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 Wash Buffer by diluting Wash Buffer (20x) 1:20 using distilled or deionized water (reagent-quality water) for the wash steps. Store unused 1x solution at 2-8 ºC for no more than one month. Discard buffer if cloudy in appearance. Refer to the User Guide for your Autostainer Link 48 for further information.

EnVision FLEX Wash Buffer (20x) is available as Code K8007.

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

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

Important Notes:

- ***If using an entire bottle of DAB+ Substrate Buffer, add 9 drops of DAB+ chromogen.** Although the label states 7.2 mL, this is the useable volume and does not account for the "dead volume" (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 above. 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 Section 6, Precautions).

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

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

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

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

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

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

Step 1: Deparaffinization, Rehydration and Target Retrieval (3-in-1) Procedure
For details, please refer to the PT Link User Guide.
Set PT Link (Code PT100/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 Target Retrieval Solution, Low pH, 1x working solution to cover the tissue sections.
►Preheat the Target Retrieval Solution to 65 °C.
►Immerse Autostainer racks containing mounted, FFPE tissue sections into the pre-heated Target Retrieval Solution, Low pH, (1x working solution) in PT Link tank. Incubate for 20 minutes at 97 °C.
►When target retrieval incubation has been completed and the temperature has cooled to 65 °C, remove each Autostainer slide rack with the slides from the PT Link tank and **immediately** place the Autostainer rack with slides into a tank (e.g., PT Link Rinse Station, Code PT109) containing diluted (1x), room temperature Wash Buffer (Code K8007).
►Incubate slides in diluted, room temperature Wash Buffer for five minutes.

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

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

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

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

11. Quality Control

Reagents in PD-L1 IHC 22C3 pharmDx have been quality controlled by immunohistochemistry using the target retrieval and staining procedures outlined above. Deviations in the recommended procedures for tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results. Quality controls should be included in each staining run. These quality controls are specified in Table 7 and include: a H&E stained patient tissue specimen; lab-supplied positive and negative control tissues; and a

Dako-supplied Control Cell Line Slide (5). In the USA, consult the quality control guidelines of the College of American Pathologists (CAP) Accreditation Program for Immunohistochemistry; see also CLSI Quality Assurance for Immunocytochemistry, Approved Guideline (5, 6, 7) for additional information.

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 the quality control section above. 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 39.

13. Staining and Scoring Interpretation

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.

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

Score partial or complete cell membrane staining (≥ 1+) that is perceived distinct from cytoplasmic staining. Cytoplasmic staining should be considered non-specific staining and is excluded in the assessment of staining intensity. Normal cells and tumor-associated immune cells such as infiltrating lymphocytes or macrophages ***should not*** be included in the scoring for the determination of PD-L1 expression level.

Table 1 below provides details about which tissue elements are included and/or excluded in determining the TPS.

Table 1. 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 7 (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 and ≤ 1+ non-specific staining.

The specimen should be considered to have PD-L1 expression if TPS ≥ 1% of the viable tumor cells exhibit membrane staining at any intensity. The specimen should be considered to have high PD-L1 expression if TPS ≥ 50% of the viable tumor cells exhibit membrane staining at any intensity.

Tumor Proportion Score			
PD-L1 Expression Levels	TPS < 1%	TPS ≥ 1%	TPS ≥ 50%
PD-L1 Expression Status	No PD-L1 Expression	PD-L1 Expression	High PD-L1 Expression

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

13.2 Gastric or GEJ Adenocarcinoma, Esophageal Squamous Cell Carcinoma (ESCC), Cervical Cancer, Urothelial Carcinoma, HNSCC – PD-L1 Expression Determined by Combined Positive Score

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

PD-L1 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:

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

Slide evaluation must be performed by a pathologist using a light microscope. For evaluation of the immunohistochemical staining, an objective of 10-20x magnification is 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 7 (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 and ≤ 1+ nonspecific staining.

Refer to sections 13.2.1, 13.2.2, 13.2.3 13.2.4, and 13.2.5 for tumor indication-specific information.

13.2.1 Gastric or Gastroesophageal Junction (GEJ) Adenocarcinoma – CPS Interpretation

A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide (biopsy and resection) for the specimen to be considered adequate for evaluation. If patient specimens include more than one biopsy (i.e. 3-5 endoscopic biopsies) on a slide, all tissues on the slide need to be evaluated to generate a single CPS for determining the PD-L1 expression level. Each biopsy should not be reported independently.

The CPS denominator includes all viable invasive tumor cells (PD-L1 staining and non-staining). Dysplasia, carcinoma in situ and all other cells are excluded.

Table 2 below provides details about which tissue elements are included in and excluded from the CPS numerator in gastric or GEJ adenocarcinoma

Table 2. CPS Numerator Inclusion/Exclusion Criteria for Gastric or GEJ Adenocarcinoma

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 gastric or GEJ adenocarcinoma tumor cells	• Non-staining tumor cells • Tumor cells with only cytoplasmic staining • Adenoma, dysplasia, and 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**: ○ Lymphocytes (including lymphocyte aggregates) ○ Macrophages*** • Only MICs directly associated with the response to the tumor are scored.	• Non-staining MICs • MICs associated with adenoma, dysplasia, and carcinoma in situ • MICs (including lymphoid aggregates) associated with ulcers, chronic gastritis, and other processes not associated with the tumor • MICs associated with normal structures • Neutrophils, eosinophils and plasma cells
Other Cells	• Not included	• Normal cells (including ganglion 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 CPS numerator.*
***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.*

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

Combined Positive Score		
PD-L1 Expression Level	CPS < 1	CPS ≥ 1
PD-L1 Expression Status	No PD-L1 Expression	PD-L1 Expression

Refer to PD-L1 IHC 22C3 pharmDx gastric or GEJ adenocarcinoma Interpretation Manual for additional guidance.

13.2.2 Esophageal Squamous Cell Carcinoma (ESCC) – CPS Interpretation

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

The CPS denominator includes all viable invasive tumor cells (PD-L1 staining and non-staining). Non-invasive neoplasia (including carcinoma in situ) and all other cells are excluded.

Table 3 below provides details about which tissue elements are included in and excluded from the CPS numerator in ESCC.

Table 3. CPS Numerator Inclusion/Exclusion Criteria for ESCC

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	• Non-staining tumor cells • Tumor cells with only cytoplasmic staining • Non-invasive neoplasia (including 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.	• Non-staining MICs • MICs associated with non-invasive neoplasia (including carcinoma in situ) • 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 are 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*.

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

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

13.2.3 Cervical Cancer – CPS Interpretation

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

The CPS denominator includes all viable invasive tumor cells (PD-L1 staining and non-staining). Dysplasia, carcinoma in situ and all other cells are excluded.

Table 4 below provides details about which tissue elements are included in and excluded from the CPS numerator in cervical cancer.

Table 4. 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 cervical tumor cells	• Non-staining tumor cells • Tumor cells with only cytoplasmic staining
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.	• Non-staining MICs • MICs associated with cervical intraepithelial neoplasia (CIN I-III) • 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 tumor such as cervicitis • Neutrophils, eosinophils and plasma cells
Other Cells	• Not included	• CIN I-III • 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 CPS numerator.*
***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.*

For cervical cancer, NSCLC tissue with its corresponding TPS scoring algorithm may be used as a positive and/or negative control if no cervical control tissue is available.

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

Combined Positive Score		
PD-L1 Expression Level	CPS < 1	CPS ≥ 1
PD-L1 Expression Status	No PD-L1 Expression	PD-L1 Expression

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

13.2.4 Urothelial Carcinoma – CPS Interpretation

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.

The CPS denominator includes all viable tumor cells (PD-L1 staining and non-staining). All immune cells, normal cells, necrotic cells, ulcers, chronic cystitis, and low-grade papillary carcinoma are excluded.

Table 5 below provides details about which tissue elements are included in and excluded from the CPS numerator in urothelial carcinoma.

Table 5. 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
		• Non-staining 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***
	Only MICs directly associated with the response to the tumor are scored.
		• Non-staining 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

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 are 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

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

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

13.2.5 HNSCC– CPS Interpretation

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.

The CPS denominator includes all viable invasive tumor cells (PD-L1 staining and non-staining). All immune cells, benign cells, non-viable tumor cells, carcinoma in situ, stromal cells (including fibroblasts), and necrotic cells and/or cellular debris are excluded.

Table 6 below provides details about which tissue elements are included in and excluded from the CPS numerator in HNSCC.

Table 6. 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	• Non-staining tumor cells • Tumor cells with only cytoplasmic staining
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.	• Non-staining 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	• Carcinoma in situ • 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*.

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

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

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

14. Slide Evaluation

Table 7. 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 (Supplied with kit)	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. • Non-specific staining < 1+ intensity. *MCF-7 (PD-L1-negative control cell line) acceptance criteria:* • No specific staining. • Non-specific staining < 1+ intensity. Note that staining of a few cells in the MCF-7 cell pellet may occasionally be observed. The following acceptance criteria are applicable: the presence of ≤ 10 total cells with distinct plasma membrane staining, or cytoplasmic 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.
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 non-specific 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 plasma membrane staining should be observed. Non-specific staining should be ≤1+.

Specimens	Rationale	Requirements
		Slide stained with Negative Control Reagent: No membrane staining. Non-specific 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 section 13.2.3 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. Non-specific staining should be ≤ 1+. **Slide stained with Negative Control Reagent**: No membrane staining. Non-specific 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 section 13.2.3 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 background staining of the assay.	Strong positive staining should be detected in portions of the crypt epithelium and weak to moderate staining of the follicular macrophages in the germinal centers. Negative staining should be observed in endothelium, fibroblasts as well as surface epithelium.
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. Non-specific 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. Refer to Summary and Explanation, Limitations, and Performance Characteristics for specific information regarding PD-L1 IHC 22C3 pharmDx immunoreactivity.	Positive staining intensity should be assessed within the context of any non-specific background staining observed on the patient's Negative Control Reagent slide in the same run. 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 Section 13 for scoring interpretation guidelines in PD-L1 expression.

15. Limitations

15.1 General Limitations

1. For prescription use only.
2. 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.
3. 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.
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 non-specific staining with horseradish peroxidase (7).
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 Technical Support with documented unexpected reactions.

8. False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (cytochrome C) (7).
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 Section 8.2).
2. For optimal and reproducible results, the PD-L1 protein requires target retrieval pre-treatment when tissues are routinely fixed (neutral buffered formalin) and paraffin embedded.
3. Do not substitute reagents from different lot numbers of this product, or from kits of other manufacturers. The only exception is the EnVision FLEX Target Retrieval Solution, Low pH (50x), which, if required, is available as Code K8005.
4. Stained control cell lines should be used only for validation of the staining run and should not be used to score the staining reaction in tissue sections.
5. Use of PD-L1 IHC 22C3 pharmDx on tissues with fixatives other than 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.
8. The clinical study in gastric or GEJ adenocarcinoma was conducted with the guidance to provide a minimum of 3 and up to 5 core needle tissue biopsies per patient. The reliability of determining patients' PD-L1 expression level based on testing fewer passes is unknown. Refer to Section 13.2 for guidance on scoring interpretation.
9. The clinical study in urothelial carcinoma was conducted with guidance to provide a minimum of 3 and up to 5 core needle tissue biopsies per patient. The reliability of determining patients' PD-L1 expression level based on testing fewer passes is unknown. Refer to Section 13.2 for guidance on scoring interpretation.
10. If PD-L1 expression is not detected in an archival* gastric or GEJ adenocarcinoma specimen, evaluate the feasibility of obtaining an additional tumor biopsy for PD-L1 testing. (See Table 21 in Clinical Section 16.5)
11. 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.
12. Laboratories should pay particular attention to the pH of the Target Retrieval Solution for pre-treatment of ESCC specimens as pH 5.9 may affect PD-L1 staining performance.
13. The studies carried out to assess TRS use up to three times in esophageal cancer did not meet acceptance criteria for qualitative evaluation of PD-L1 expression status, therefore TRS reuse is not recommended for ESCC specimens.

*In the context of clinical trial KN059, a newly obtained biopsy was defined as a specimen obtained up to 6 weeks (42 days) prior to initiation of treatment on Day 1 (Cycle 1) with KEYTRUDA and with no additional anti-cancer treatment having been given after the specimen was obtained. Specimens that were >42 days were classified as archival.

16. Performance Evaluation

16.1 Non-Clinical Performance Evaluation: Normal and Neoplastic Tissues

Normal tissues: Table 8 summarizes monoclonal mouse anti-PD-L1, Clone 22C3 immunoreactivity on the recommended panel of normal tissues. Plasma membrane staining was observed on immune cells and cells of epithelial origin. Cytoplasmic staining was noted in some cell types but was not recorded as positive staining. 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 (8, 9).

Table 8: Summary of PD-L1 IHC 22C3 pharmDx Normal Tissue Reactivity

Tissue Type (# tested)	Positive Plasma Membrane Staining: Tissue Elements	Positive Cytoplasmic Staining: Tissue Elements	Non-specific Staining
Adrenal (3)	0/3	1/3 Medullary cells	0/3
Bone marrow (3)	3/3 Megakaryocytes	3/3 Megakaryocytes	0/3
Breast (3)	0/3	0/3	0/3
Cerebellum (3)	0/3	0/3	0/3
Cerebrum (3)	0/3	0/3	0/3
Cervix (3)	1/3 Epithelium	0/3	0/3
Colon (3)	2/3 Macrophages	0/3	0/3
Esophagus (3)	0/3	0/3	0/3
Kidney (3)	1/3 Tubular epithelium	0/3	0/3
Liver (3)	1/3 Macrophages 1/3 Hepatocytes	0/3	0/3
Lung (3)	3/3 Alveolar macrophages	0/3	0/3
Mesothelial cells (2)	0/2	0/2	0/2
Muscle, cardiac (3)	0/3	0/3	0/3
Muscle, skeletal (3)	0/3	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 (2)	2/2 Epithelium	0/2	0/2
Salivary gland (3)	0/3	0/3	0/3
Skin (3)	0/3	0/3	0/3
Small intestine (3)	0/3	0/3	0/3
Spleen (3)	2/3 Macrophages	0/3	0/3

Tissue Type (# tested)	Positive Plasma Membrane Staining: Tissue Elements	Positive Cytoplasmic Staining: Tissue Elements	Non-specific Staining
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 9 summarizes monoclonal mouse anti-PD-L1, Clone 22C3 immunoreactivity on a panel of neoplastic tissues. Plasma membrane staining was observed on immune cells and cells of epithelial origin. Cytoplasmic staining was noted in some cell types but was not recorded as positive staining. 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 (8-11).

Table 9: Summary of PD-L1 IHC 22C3 pharmDx Neoplastic Tissue Reactivity

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

Tumor Type	Location	PD-L1 positive/total N=159
	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 carcinoma	Metastatic colon signet ring cell carcinoma to ovary	0/1
	Colon	0/1
Small cell carcinoma	Lung	0/1
Spermatocytoma	Testis	0/2
Squamous cell carcinoma	Metastatic esophageal squamous cell carcinoma to lymph node	0/1
	Cervix	2/5
	Esophagus	0/7
	Head & neck	0/2
	Lung	1/2
	Skin	0/2
	Uterus	0/1
Synovial sarcoma	Pelvic cavity	0/1
Thymoma	Mediastinum	1/1
Transitional cell carcinoma	Bladder	0/6
	Kidney	0/1

16.2 Non-Clinical Performance Evaluation: NSCLC

Analytical Sensitivity/Specificity
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 Dako. 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 studies which resulted in 100% agreement, negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with two-sided 95% Wilson score confidence intervals for the TPS ≥ 1% cutoff and TPS ≥ 50% cutoff.

Table 10: Precision of PD-L1 IHC 22C3 pharmDx tested at one site (TPS ≥ 1%)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-instrument	TPS ≥ 1%	Each of 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression was tested on each of six Autostainer Link 48 instruments.	NPA 100% (94.0-100%) PPA 100% (94.0-100%) OA 100% (96.9-100%)
Inter-operator	TPS ≥ 1%	Each of 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression was tested using six analysts on one Autostainer Link 48 instrument.	NPA 100% (93.9-100%) PPA 100% (94.0-100%) OA 100% (96.9-100%)
Inter-day	TPS ≥ 1%	Each of 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression was tested on six non-consecutive days on the Autostainer Link 48 instrument.	NPA 100% (94.0-100%) PPA 100% (94.0-100%) OA 100% (96.9-100%)
Inter-lot	TPS ≥ 1%	Each of 24 NSCLC specimens (13 PD-L1-negative and 11 PD-L1-positive) with a range of PD-L1 IHC expression was tested with three replicates and each of three reagent lots on the Autostainer Link 48 instrument.	ANA 98.3% (95.9-100%) APA 97.9% (94.6-100%) OA 98.1% (95.3-100%)
Intra-run (Repeatability)	TPS ≥ 1%	Each of 24 NSCLC specimens (12 PD-L1-negative and 12 PD-L1-positive) with a range of PD-L1 IHC expression was tested with six replicates within a run on the Autostainer Link 48 instrument.	NPA 100% (94.0-100%) PPA 100% (93.8-100%) OA 100% (96.8-100%)

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 11: Precision of PD-L1 IHC 22C3 pharmDx tested at one site (TPS ≥ 50%)

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

NPA=Negative Percent Agreement; PPA=Positive Percent Agreement; OA=Overall 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 agreements were calculated since no natural reference exists in reproducibility parameters such as site and observer. 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.

Table 12: 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%	Each of 36 NSCLC specimens (16 PD-L1-negative and 20 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 2700 pair-wise comparisons.	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%	Each of 36 NSCLC specimens (16 PD-L1-negative and 20 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days at each of three study sites. Intra-site analysis was performed for three sites on a total of 1080 pair-wise comparisons.	ANA 96.2% (94.1-97.5%) APA 96.7% (95.0-97.9%) OA 96.5% (95.2-97.4%)
Inter-observer	TPS ≥ 1%	Scoring of 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, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 1674 pair-wise comparisons.	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%	Scoring of 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, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 558 pair-wise comparisons.	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 13: 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%	Each of 36 NSCLC specimens (21 PD-L1-negative and 15 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 2700 pair-wise comparisons.	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%	Each of 36 NSCLC specimens (21 PD-L1-negative and 15 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days at each of three study sites. Intra-site analysis was performed for three sites on a total of 1080 pair-wise comparisons.	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%	Scoring of 62 NSCLC specimens (30 PD-L1-negative and 32 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 1674 pair-wise comparisons.	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%	Scoring of 62 NSCLC specimens (30 PD-L1-negative and 32 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 558 pair-wise comparisons.	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

KN042: First-line treatment of metastatic NSCLC as a single agent

The efficacy of KEYTRUDA was investigated in KEYNOTE-042 (NCT02220894), a randomized, multicenter, open-label, active-controlled trial conducted in 1274 patients with stage III NSCLC, who were not candidates for surgical resection or definitive chemoradiation, or metastatic NSCLC, whose tumors expressed PD-L1 (TPS ≥ 1%) by an immunohistochemistry assay using the PD-L1 IHC 22C3 pharmDx Kit, and who had not received prior systemic treatment for metastatic NSCLC. 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 radiation in the thoracic region within the prior 26 weeks of initiation of study were ineligible. Randomization was stratified by ECOG performance status (0 vs. 1), histology (squamous vs. nonsquamous), geographic region (East Asia vs. non-East Asia), and PD-L1 expression (TPS ≥ 50% vs. TPS 1 to 49%). Patients were randomized (1:1) to receive KEYTRUDA 200 mg intravenously every 3 weeks or investigator's choice of either of the following platinum-containing chemotherapy regimens:

- Pemetrexed 500 mg/m^2 every 3 weeks and carboplatin AUC 5 to 6 mg/mL/min every 3 weeks on Day 1 for a maximum of 6 cycles followed by optional pemetrexed 500 mg/m^2 every 3 weeks for patients with nonsquamous histologies;
- Paclitaxel 200 mg/m^2 every 3 weeks and carboplatin AUC 5 to 6 mg/mL/min every 3 weeks on Day 1 for a maximum of 6 cycles followed by optional pemetrexed 500 mg/m^2 every 3 weeks for patients with nonsquamous histologies.

Treatment with KEYTRUDA continued until RECIST v1.1 (modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ)-defined progression of disease, unacceptable toxicity, or a maximum of 24 months. Administration of KEYTRUDA was permitted beyond RECIST-defined disease progression if the patient was clinically stable and deriving clinical benefit as determined by the investigator. Treatment with KEYTRUDA could be reinitiated at the time of subsequent disease progression and administered for up to 12 months. Assessment of tumor status was performed every 9 weeks. The main efficacy outcome measure was OS. Additional efficacy outcome measures were PFS and ORR as assessed by a BICR review according to RECIST v1.1, modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ.

The study population characteristics were: median age of 63 years (range: 25 to 90), 45% age 65 or older; 71% male; 64% White, 30% Asian, and 2% Black. Nineteen percent were Hispanic or Latino. Sixty-nine percent had ECOG performance status of 1; 39% with squamous and 61% with nonsquamous histology; 87% with M1 disease and 13% with Stage IIIA (2%) or Stage IIIB (11%) who were not candidates for surgical resection or definitive chemoradiation per investigator assessment; and 5% with treated brain metastases at baseline. Forty-seven percent of patients had TPS ≥ 50% NSCLC and 53% had TPS 1 to 49% NSCLC.

The trial demonstrated a statistically significant improvement in OS for patients randomized to KEYTRUDA as compared with chemotherapy. Table 14 and Figure 1 summarize the efficacy results in the subgroup of patients with TPS ≥ 50% and in all randomized patients with TPS ≥ 1%.

Table 14: Efficacy Results of All Randomized Patients (TPS ≥ 1% and TPS ≥ 50%) in KEYNOTE-042

	TPS ≥ 1%		TPS ≥ 50%	
Endpoint	**KEYTRUDA 200 mg every 3 weeks n=637**	**Chemotherapy n=637**	**KEYTRUDA 200 mg every 3 weeks n=299**	**Chemotherapy n=300**
OS				
Number of events (%)	371 (58%)	438 (69%)	157 (53%)	199 (66%)
Median in months (95% CI)	16.7 (13.9, 19.7)	12.1 (11.3, 13.3)	20.0 (15.4, 24.9)	12.2 (10.4, 14.2)
Hazard ratio* (95% CI)	0.81 (0.71, 0.93)		0.69 (0.56, 0.85)	
p-Value†	0.0036		0.0006	
PFS				
Number of events (%)	507 (80%)	506 (79%)	221 (74%)	233 (78%)
Median in months (95% CI)	5.4 (4.3, 6.2)	6.5 (6.3, 7.0)	7.1 (5.9, 9.0)	6.4 (6.1, 6.9)
Hazard ratio*,‡ (95% CI)	1.07 (0.94, 1.21)		0.81 (0.67, 0.99)	
p-Value†	- ‡		NS§	
Objective Response Rate				
ORR‡ (95% CI)	27% (24, 31)	27% (23, 30)	39% (33.9, 45.3)	32% (26.8, 37.6)
Complete response rate	0.5%	0.5%	0.7%	0.3%
Partial response rate	27%	26%	39%	32%
Duration of Response				
% with duration ≥ 12 months¶	47%	16%	42%	17%
% with duration ≥ 18 months¶	26%	6%	25%	5%

* Based on the stratified Cox proportional hazard model

† Based on a stratified log-rank test; compared to a p-Value boundary of 0.0291

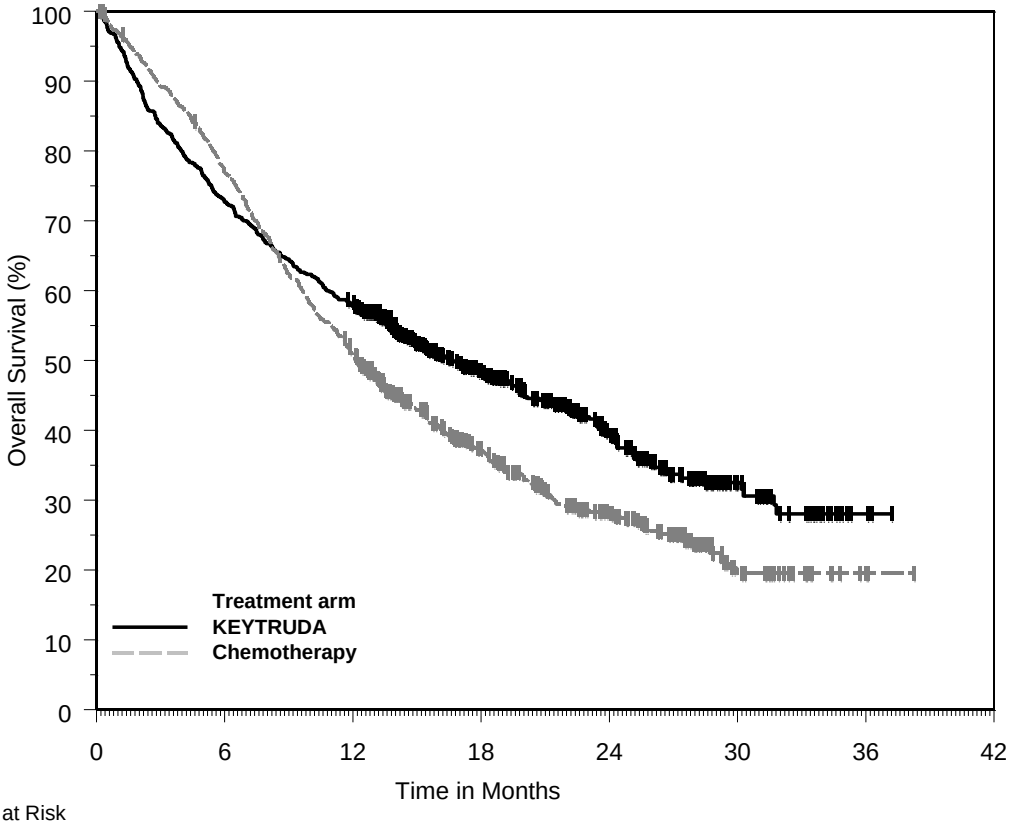
‡ Not evaluated for statistical significance as a result of the sequential testing procedure for the secondary endpoints

§ Not significant compared to a p-Value boundary of 0.0291

¶ Based on observed duration of response

In a pre-specified exploratory subgroup analysis for patients with TPS 1-49% NSCLC, the median OS was 13.4 months (95% CI: 10.7, 18.2) for the pembrolizumab group and 12.1 months (95% CI: 11.0, 14.0) in the chemotherapy group, with an HR of 0.92 (95% CI: 0.77, 1.11).

Figure 1: Kaplan-Meier Curve for Overall Survival in all Randomized Patients in KEYNOTE-042 (TPS ≥ 1%)



Number at Risk								
KEYTRUDA:	637	463	365	214	112	35	2	0
Chemotherapy:	637	485	316	166	88	24	1	0

KEYNOTE 024: Controlled trial of first-line treatment of patients with NSCLC

The efficacy of KEYTRUDA was investigated in Trial 24, a randomized (1:1), open-label, multicenter, controlled trial (12). Key eligibility criteria were metastatic NSCLC, PD-L1 expression tumor proportion score (TPS) of 50% or greater by an immunohistochemistry assay using PD-L1 IHC 22C3 pharmDx, and no prior systemic treatment for metastatic NSCLC. 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 were ineligible. Patients were randomized to receive KEYTRUDA 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. Non-squamous patients could receive pemetrexed maintenance). Patients were treated with KEYTRUDA until unacceptable toxicity or disease progression, or up to 35 administrations. Subsequent disease progression could be retreated for up to 1 additional year. Treatment could continue beyond disease progression if the patient was clinically stable and was considered to be deriving clinical benefit by the investigator. Assessment of tumor status was performed every 9 weeks. Patients on chemotherapy who experienced progression of disease were offered KEYTRUDA.

Among the 305 patients in Trial 24, baseline characteristics were: median age 65 years (54% age 65 or older); 61% male; 82% White and 15% Asian; and 35% and 65% with an ECOG performance status 0 and 1, respectively. Disease characteristics were squamous (18%) and non-squamous (82%); M1 (99%); and brain metastases (9%).

The major efficacy outcome measure was progression-free survival (PFS) as assessed by blinded independent central review (BICR) using Response Evaluation Criteria on Solid Tumors Version 1.1 (RECIST 1.1). Additional efficacy outcome measures were overall survival (OS) and objective response rate (ORR) as assessed by BICR using RECIST 1.1. Table 15 summarizes key efficacy measures for the entire intent to treat (ITT) population.

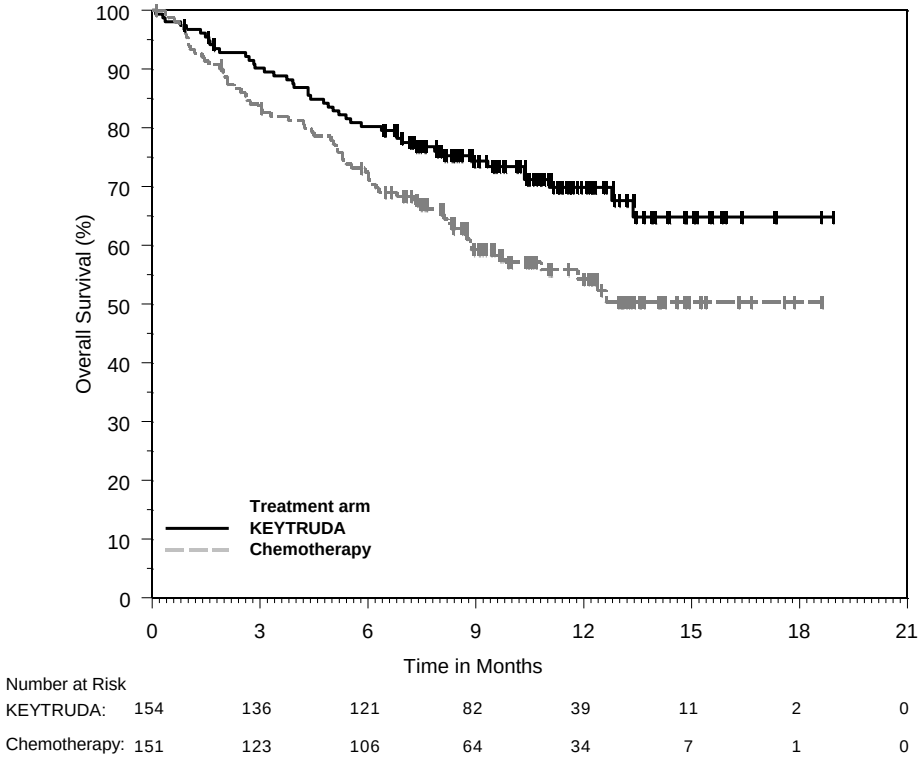
Table 15: Efficacy Results in Trial 24

Endpoint	KEYTRUDA 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	44 (29%)	64 (42%)
Hazard ratio† (95% CI)	0.60 (0.41, 0.89)	---
p-Value‡	0.005	---
Median in months (95% CI)	Not reached (NA, NA)	Not reached (9.4, NA)
Objective Response Rate*		
ORR % (95% CI)	45% (37, 53)	28% (21, 36)
Complete response %	4%	1%
Partial response %	41%	27%

* Assessed by BICR using RECIST 1.1
† Hazard ratio (KEYTRUDA compared to chemotherapy) based on the stratified Cox proportional hazard model
‡ Based on stratified Log rank test
NA = not available

Among the 69 patients randomized to KEYTRUDA 200 mg with an objective response, response durations ranged from 1.9+ to 14.5+ months. Eighty-eight percent of these responders had a response duration of 6 months or longer (based on Kaplan-Meier estimation; Figure 2).

Figure 2: Kaplan-Meier Curve for Overall Survival in Trial 24



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

The efficacy of KEYTRUDA was investigated in Trial 10, a randomized (1:1), open-label, multicenter, controlled trial (13). Key eligibility criteria were advanced NSCLC that had progressed following platinum-containing chemotherapy, and if appropriate, targeted therapy for ALK or EGFR mutations, and PD-L1 expression tumor proportion score (TPS) of 1% or greater by a clinical trial assay (CTA) version of PD-L1 IHC 22C3 pharmDx. Forty-four and 56 percent of patients were enrolled based on testing of an archival tumor sample or a new tumor sample, respectively. 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 were ineligible. Patients were randomized (1:1:1) to receive 2 mg/kg (n=344) or 10 mg/kg (n=346) of KEYTRUDA every 3 weeks or 75 mg/m^2 of docetaxel every 3 weeks (n=343). Patients were treated with KEYTRUDA until unacceptable toxicity or disease progression that was symptomatic, was rapidly progressive, required urgent intervention, occurred with a decline in performance status, or was confirmed at 4 to 6 weeks with repeat imaging. Patients without disease progression were treated for up to 24 months or 35 administrations, whichever was longer. Subsequent disease progression could be retreated for up to 1 additional year. 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 (≥ 1% of viable tumor cells exhibiting membrane staining at any intensity) and samples from 94 patients did not have PD-L1 expression (< 1% of viable tumor cells exhibiting membrane staining at any intensity). Within these 413 patients with PD-L1 expression, specimens from 163 patients had high PD-L1 expression (≥ 50% of viable tumor cells exhibiting membrane staining at any intensity).

The level of agreement achieved between the CTA and PD-L1 IHC 22C3 pharmDx is shown in Table 16.

Table 16: 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 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 17 and 18. KEYTRUDA demonstrated durable clinical benefit in NSCLC patients with PD-L1 expression (TPS ≥ 1%), which was enhanced in patients with high 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. The tables below summarize key efficacy measures in the overall population with PD-L1 expression (TPS ≥ 1%) and in the high PD-L1 expression (TPS ≥ 50%) subset for the overall clinical study (TPS ≥ 1% by CTA) and in the population with PD-L1 expression 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 3. Efficacy results were similar for the 2 mg/kg and 10 mg/kg KEYTRUDA arms.

Table 17: Response to KEYTRUDA in Previously Treated NSCLC Patients: Overall Clinical Trial 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 (63%)	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)

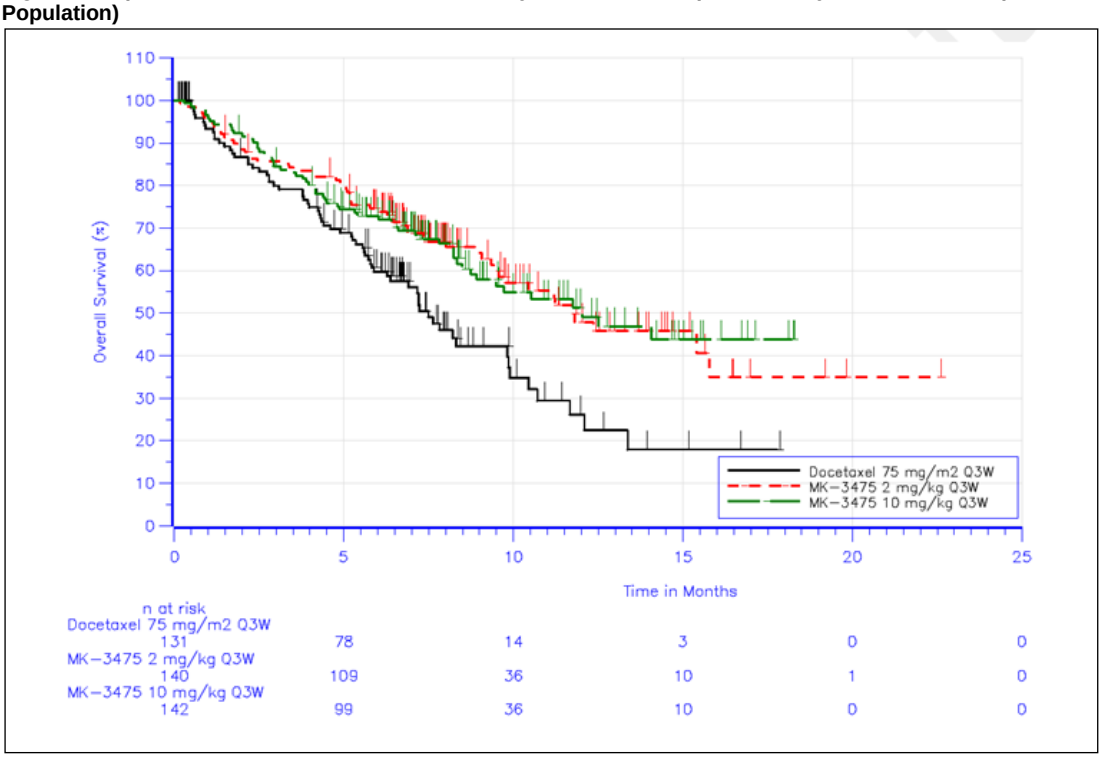
* Hazard ratio (KEYTRUDA compared to docetaxel) based on the stratified Cox proportional hazard model
† Based on stratified Log rank test
‡ Assessed by BICR using RECIST 1.1
§ All responses were partial responses

Table 18: Response to KEYTRUDA in Previously Treated NSCLC Patients: Overall Clinical Trial and Patients with PD-L1 High 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 3: Kaplan-Meier Curve for Overall Survival by Treatment Arm (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 Non-Clinical Performance Evaluation: Gastric or Gastroesophageal Junction (GEJ) Adenocarcinoma

The following histologies were tested in the non-clinical performance evaluation of gastric or GEJ adenocarcinoma: intestinal, diffuse including signet ring cell carcinoma, and mucinous types.

Analytical Sensitivity/Specificity: Gastric or GEJ Adenocarcinoma
Analytical sensitivity of PD-L1 IHC 22C3 pharmDx was tested on 100 FFPE gastric or GEJ adenocarcinoma specimens (stage I to IV) using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a range of CPS 0-100. 60% of the specimens had PD-L1 expression, with expression defined by CPS ≥ 1.

Precision: Gastric or GEJ Adenocarcinoma
The precision of PD-L1 IHC 22C3 pharmDx in gastric or GEJ adenocarcinoma was evaluated at Dako. Inter-instrument, inter-operator, inter-day, and inter-lot were tested in combined precision. Repeatability was tested in intra-run precision. Intra-observer and inter-observer precision were also assessed. Negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with two-sided 95% Wilson score confidence intervals for the CPS ≥ 1 cutoff.

Table 19: Precision of PD-L1 IHC 22C3 pharmDx in gastric or GEJ adenocarcinoma, tested at one site (CPS ≥ 1)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI):
Combined Precision (inter-instrument, inter-operator, inter-lot, inter-day,)	CPS ≥ 1	Each of 24 gastric or GEJ adenocarcinoma specimens (12 PD-L1-positive and 12 PD-L1-negative) with a range of PD-L1 expression was tested using three Autostainer Link 48 instruments, four operators, three kit lots, over three non-consecutive days.	NPA 100% (94.9-100%) PPA 95.8% (88.5-98.6%) OA 97.9% (94.1-99.3%
Intra-run precision (Repeatability)	CPS ≥ 1	Each of 24 gastric or GEJ adenocarcinoma specimens (13 PD-L1-negative and 11 PD-L1-positive) with a range of PD-L1 IHC expression was tested with five replicates within a run on the Autostainer Link 48 instrument.	NPA 96.9% (89.5-99.2%) PPA 100% (93.5-100%) OA 98.3% (94.1-99.5%)
Inter-observer precision	CPS ≥ 1	60 gastric or GEJ adenocarcinoma 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 three pathologists over three non-consecutive days	NPA 91.5% (87.2-94.4%) PPA 96.1% (93.3-97.7%) OA 94.1% (91.8-95.8%)
Intra-observer precision	CPS ≥ 1	60 gastric or GEJ adenocarcinoma 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 three pathologists over three non-consecutive days	NPA 96.0% (92.6-97.9%) PPA 96.8% (94.3-98.3%) OA 96.5% (94.6-97.7%)

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

External Reproducibility: Gastric or GEJ Adenocarcinoma
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% percentile bootstrap confidence intervals (CI) for the CPS ≥ 1 cutoff. In an initial study the acceptance criteria for the CI lower bound of OA and NPA for inter-observer reproducibility and CI lower bound of NPA for intra-observer reproducibility were not met. A root cause assessment indicated that one of the three observers in the study did not pass post-study proficiency testing. A second inter- and intra-observer study was conducted with three naïve observers, and the results met the acceptance criteria. Results are shown in Table 20 below. Proficiency assessment is recommended to ensure correct observer scoring interpretation.

Table 20: Reproducibility of PD-L1 IHC 22C3 pharmDx in gastric or GEJ adenocarcinoma, tested at three external sites (CPS ≥ 1)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	CPS ≥ 1	Each of 36 gastric or GEJ adenocarcinoma specimens (16 PD-L1 negative and 20 PD-L1 positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 540 comparisons to majority call.	NPA 92.5% (86.2-97.5%) PPA 91.7% (84.7-97.7%) OA 92.0% (87.4-96.3%)
Intra-site	CPS ≥ 1	Each of 36 gastric or GEJ adenocarcinoma specimens (16 PD-L1 negative and 20 PD-L1 positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days at each of three study sites. Intra-site analysis was performed for three sites on a total of 540 comparisons to majority call.	NPA 93.1% (89.2-96.5%) PPA 98.2% (96.4-99.6%) OA 95.7% (93.7-97.6%)
Inter-observer	CPS ≥ 1	Scoring of 68 gastric or GEJ adenocarcinoma specimens (36 PD-L1- negative and 32 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 612 comparisons to majority call.	NPA 96.6% (92.9-99.4%) PPA 96.5% (93.1-99.3%) OA 96.6% (94.0-98.7%)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Intra-observer	CPS ≥ 1	Scoring of 68 gastric or GEJ adenocarcinoma specimens (36 PD-L1-negative and 32 PD-L1-positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 612 comparisons to majority call.	NPA 97.2% (94.8-99.1%) PPA 97.2% (94.8-99.3%) OA 97.2% (95.3-98.9%)

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

16.5 Clinical Performance Evaluation: Gastric or Gastroesophageal (GEJ) Adenocarcinoma

The efficacy of KEYTRUDA was investigated in KEYNOTE 059 (KN059), a multicenter, non-randomized, open-label multi-cohort trial that enrolled 259 patients with gastric or GEJ adenocarcinoma who progressed on at least two prior systemic treatments for advanced disease. Previous treatment must have included a fluoropyrimidine and platinum doublet. HER2/neu positive patients must have previously received treatment with approved HER2/neu targeted therapy. Patients with active autoimmune disease or a medical condition that required immunosuppression or with clinical evidence of ascites by physical exam were ineligible.

Patients received KEYTRUDA 200 mg every 3 weeks until unacceptable toxicity or disease progression that was symptomatic, rapidly progressive, required urgent intervention, occurred with a decline in performance status, or was confirmed at least 4 weeks later with repeat imaging. Patients without disease progression were treated for up to 24 months. Assessment of tumor status was performed every 6 to 9 weeks. The major efficacy outcome measures were ORR according to RECIST 1.1, as assessed by blinded independent central review, and duration of response.

PD-L1 expression level for 259 patient tumor biopsy or resection tissue (167 archival and 90 newly obtained (refer to definition in Table 21) was determined using PD-L1 IHC 22C3 pharmDx. PD-L1 expression level for 2 samples was not evaluable. Overall, 58% (148/257) of the patients had tumors that expressed PD-L1 with a combined positive score (CPS) ≥ 1. Seventy-three percent (66/90) of patients whose tumors were newly obtained for PD-L1 testing and 49% (82/167) of patients whose archival tumors were tested expressed PD-L1 at CPS ≥ 1 (Table 21).

Table 21: Tumor PD-L1 Expression by Specimen Type

Tumor Tissue	PD-L1 Expression (CPS ≥ 1) n (%)	No PD-L1 Expression (CPS < 1) n (%)
Overall study n=257 (%)	148 (58)	109 (42)
Archival Tissue* n=167	82 (49)	85 (51)
Newly Obtained Tissue* n= 90	66 (73)	24 (27)

*In the context of clinical trial KN059, a newly obtained biopsy was defined as a specimen obtained up to 6 weeks (42 days) prior to initiation of treatment on Day 1 (Cycle 1) with KEYTRUDA and with no additional anti-cancer treatment having been given after the specimen was obtained. Specimens that were >42 days were classified as archival.

Of 148 patients with PD-L1 expression at CPS ≥ 1, 143 were assessed to be either microsatellite stable (MSS) tumor status or had undetermined MSI or MMR status. The baseline characteristics of these 143 patients were: median age 64 years (47% age 65 or older); 77% male; 82% White, 11% Asian; and ECOG PS of 0 (43%) and 1 (57%). Eighty-five percent had M1 disease and 7% had M0 disease. Fifty-one percent had two and 49% had three or more prior lines of therapy in the recurrent or metastatic setting.

For the 143 patients that have PD-L1 expression (CPS ≥ 1), the ORR was 13.3% (95% CI: 8.2, 20.0); 1.4% had a complete response and 11.9% had a partial response. Among the 19 responding patients, the duration of response ranged from 2.8+ to 19.4+ months, with 11 patients (58%) having responses of 6 months or longer and 5 patients (26%) having responses of 12 months or longer.

16.6 Non-Clinical Performance Evaluation: Esophageal Cancer

The non-clinical 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 Dako. Inter-instrument, inter-operator, inter-day, and inter-lot 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 22.

Table 22: 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-Day, and Inter-Lot as combined variables)	CPS ≥ 10	Each of 32 esophageal cancer specimens (15 PD-L1-negative and 17 PD-L1-positive) with a range of PD-L1 IHC expression was tested using three operators, on three Autostainer Link 48 instruments, over three non-consecutive days, using three reagent lots.	NPA 97.8% (93.3-100%) PPA 98.0% (94.1-100%) OA 97.9% (94.8-100%)
Intra-run precision	CPS ≥ 10	Each of 32 esophageal cancer specimens (21	NPA 98.1% (95.2-100%)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
(Repeatability)		PD-L1-negative and 11 PD-L1-positive) with a range of PD-L1 IHC expression was tested with five replicates within a run on the Autostainer Link 48 instrument.	PPA 92.7% (83.6-100%) OA 96.2% (93.1-98.8%)
Inter-observer precision	CPS ≥ 10	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 three pathologists over three non-consecutive 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	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 three pathologists over three non-consecutive days.	NPA 96.2% (93.4-98.8%) PPA 98.5% (96.5-100%) 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. 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.

Table 23: Reproducibility of the 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	Each of 36 esophageal cancer specimens (23 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 540 comparisons to majority call.	NPA 99.7% (99.1-100%) PPA 99.0% (96.9-100%) OA 99.4% (98.5-100%)
Intra-Site	CPS ≥ 10	Each of 36 esophageal cancer specimens (23 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Intra-site analysis was performed for three sites on a total of 540 comparisons to majority call.	NPA 99.7% (99.1-100%) PPA 99.0% (96.9-100%) OA 99.4% (98.5-100%)
Inter-observer	CPS ≥ 10	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, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 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	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 three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 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

16.7 Clinical Performance Evaluation: Esophageal Squamous Cell Carcinoma

The efficacy of KEYTRUDA was investigated in KEYNOTE 181 (NCT02564263), a multicenter, randomized, open-label, active-controlled trial that enrolled 628 patients with recurrent locally advanced or metastatic esophageal cancer who progressed on or after one prior line of systemic treatment for advanced disease. Patients with HER2/neu positive esophageal cancer were required to have received treatment with approved HER2/neu targeted therapy. All patients were required to have tumor specimens for PD-L1 testing at a central laboratory; PD-L1 status was determined using the PD-L1 IHC 22C3 pharmDx kit. Patients with a history of non-infectious pneumonitis that required steroids or current pneumonitis, active autoimmune disease, or a medical condition that required immunosuppression were ineligible.

Patients were randomized (1:1) to receive either KEYTRUDA 200 mg every 3 weeks or investigator's choice of any of the following chemotherapy regimens, all given intravenously: paclitaxel 80-100 mg/m2 on Days 1, 8, and 15 of every 4-week cycle, docetaxel 75 mg/m2 every 3 weeks, or irinotecan 180 mg/m2 every 2 weeks. Randomization was stratified by tumor histology (esophageal squamous cell carcinoma [ESCC] vs. esophageal adenocarcinoma [EAC]/Siewert type I EAC of the gastroesophageal junction [GEJ]), and geographic region (Asia vs. ex-Asia). Treatment with KEYTRUDA or chemotherapy continued until unacceptable toxicity or disease progression. Patients randomized to KEYTRUDA were permitted to continue beyond the first RECIST v1.1 (modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ)-defined disease progression if clinically stable until the first radiographic evidence of disease progression was confirmed at least 4 weeks later with repeat imaging. Patients treated with KEYTRUDA without disease progression could be treated for up to 24 months. Assessment of tumor status was performed every 9 weeks. The major efficacy outcome measure was OS evaluated in the following co-primary populations: patients with ESCC, patients with tumors expressing PD-L1 CPS ≥10, and all randomized patients. Additional efficacy outcome measures were PFS, ORR, and DoR, according to RECIST v1.1, modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ, as assessed by BICR.

A total of 628 patients were enrolled and randomized to KEYTRUDA (n=314) or investigator's treatment of choice (n=314). Of these 628 patients, 167 (27%) had ESCC that expressed PD L1 with a CPS ≥10. Of these 167 patients, 85 patients were randomized to KEYTRUDA

and 82 patients to investigator's treatment of choice [paclitaxel (n=50), docetaxel (n=19), or irinotecan (n=13)]. The baseline characteristics of these 167 patients were: median age of 65 years (range: 33 to 80), 51% age 65 or older; 84% male; 32% White and 68% Asian; 38% had an ECOG PS of 0 and 62% had an ECOG PS of 1. Ninety percent had M1 disease and 10% had M0 disease. Prior to enrollment, 99% of patients had received platinum-based treatment, and 84% had also received treatment with a fluoropyrimidine. Thirty-three percent of patients received prior treatment with a taxane.

The observed OS hazard ratio was 0.77 (95% CI: 0.63, 0.96) in patients with ESCC, 0.70 (95% CI: 0.52, 0.94) in patients with tumors expressing PD-L1 CPS ≥10, and 0.89 (95% CI: 0.75, 1.05) in all randomized patients. On further examination, patients whose ESCC tumors expressed PD-L1 (CPS ≥10), an improvement in OS was observed among patients randomized to KEYTRUDA as compared with chemotherapy (hazard ratio of 0.64, 95% CI: 0.46, 0.90).

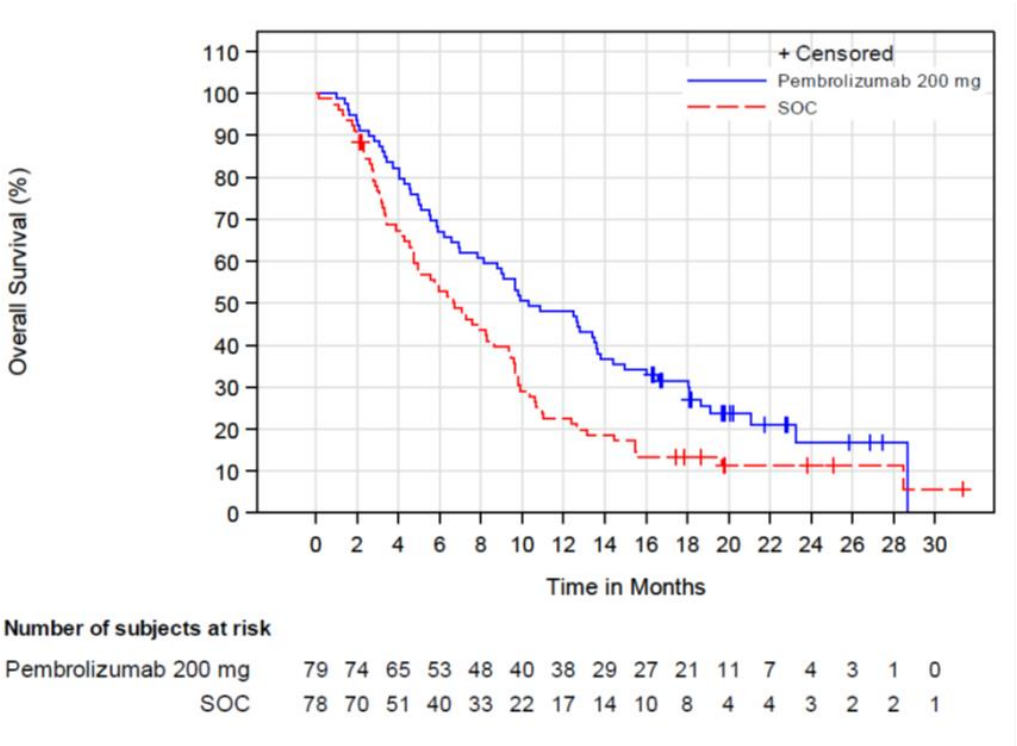
Ten (10) of the 167 ESCC participants with tumors expressing PD-L1 CPS ≥10 had specimens stained outside the stability window. These 10 participants have been excluded from the efficacy results summarized in Table 24 and the Kaplan-Meier curve for OS shown in Figure 4. The efficacy results for the population excluding the 10 participants with specimens outside the stability window are consistent with the efficacy conclusions detailed above.

Table 24: Efficacy Results in Patients with Recurrent or Metastatic ESCC (CPS ≥10) in KEYNOTE-181 (Excluding 10 Participants with Specimens Outside the Stability Window)

Endpoint	KEYTRUDA 200 mg every 3 weeks n=79	Chemotherapy n=78
OS		
Number (%) of patients with event	62 (78.5)	68 (87.2)
Median in months (95% CI)	10.3 (7.8, 13.6)	6.7 (4.8, 9.3)
Hazard ratio* (95% CI)	0.63 (0.45, 0.90)	
PFS (BCS per RECIST 1.1)		
Number (%) of patients with event	70 (88.6)	72 (92.3)
Median in months (95% CI)	3.2 (2.1, 4.3)	2.6 (2.1, 3.7)
Median follow-up (95% CI), months	3.2 (2.1, 4.4)	2.8 (2.1, 4.0)
Hazard ratio* (95% CI)	0.67 (0.48, 0.95)	
Objective Response Rate		
ORR (95% CI)	21.5 (13.1, 32.2)	7.7 (2.9, 16.0)
Number (%) of complete responses	3 (3.8)	1 (1.3)
Number (%) of partial responses	14 (17.7)	5 (6.4)
Median duration of response in months (range)	10.3 (2.8, 18.8+)	7.7 (4.3, 16.8+)

Cox proportional hazards model stratified by geographic region (Asia vs ex-Asia)
"+" indicates there is no progressive disease by the time of last disease assessment.

Figure 4: Kaplan-Meier Curve for Overall Survival in KEYNOTE-181 (ESCC CPS ≥10) (Excluding 10 Participants with Specimens Outside the Stability Window)



16.8 Non-Clinical Performance Evaluation: Cervical Cancer including Combined Squamous Cancers

Non-clinical studies were performed on PD-L1 IHC 22C3 pharmDx on FFPE human cervical cancer tissue specimens. Squamous cell (SQ) cancers from vulva, anal and salivary gland were also included in these non-clinical study sample sets to supplement the SQ cervical

cancer. These supplemental cancer types were also included in the Merck study KEYNOTE 158. The non-clinical studies comprised of analytical validation, stability and external reproducibility studies.

Analytical Sensitivity/Specificity: Cervical Cancer
Sensitivity of PD-L1 IHC 22C3 pharmDx was analyzed on 370 FFPE cervical cancer specimens (stage I to IV). Assessment of PD-L1 expression demonstrated staining across a range of CPS 0-100. Approximately 85% of cervical cancer specimens had PD-L1 expression, with expression defined by CPS ≥ 1.

Precision: Cervical Cancer Including Squamous Cell Cancers
The precision of PD-L1 IHC 22C3 pharmDx in cervical cancers were evaluated at Dako using squamous cell (SQ) cancers from cervical, vulva, anal and salivary gland. Inter-instrument, inter-operator, inter-day, and inter-lot were tested in combined precision. Repeatability was tested in intra-run precision. Inter- and intra-observer precision were also assessed. Negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were calculated with two-sided 95% Wilson score confidence intervals for the CPS ≥ 1 cutoff.

Table 25: Precision of PD-L1 IHC 22C3 pharmDx in cervical cancer including squamous cell cancers, tested at one site (CPS ≥ 1)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Squamous Cell Histological Subgroup Combined Precision (inter-instrument, inter-operator, inter-lot, inter-day,)	CPS ≥ 1	Each of 18 specimens of the squamous subgroup (7 PD-L1 negative and 11 PD-L1-positive; 6 Cervical Cancer (3 PD-L1 positive/3 PD-L1 negative)) with a range of PD-L1 expression were tested using three Autostainer Link 48 instruments, four operators, three kit lots, over three non-consecutive days.	NPA 100% (91.4-100%) PPA 100% (94.5-100%) OA 100% (96.5-100%)
Squamous Cell Histological Subgroup Intra-run precision (Repeatability)	CPS ≥ 1	Each of 8 specimens (0 PD-L1-negative and 8 PD-L1-positive; 2 Cervical Cancer (2 PD-L1 positive)) with a range of PD-L1 IHC expression was tested with five replicates within a run on the Autostainer Link 48 instrument.	NPA NA PPA 97.5% (87.1-99.6%) OA 97.5% (87.1-99.6%)
Squamous Cell Histological Subgroup Inter-observer precision	CPS ≥ 1	52 squamous specimens (22 PD-L1 negative/30 PD-L1 positive) with a range of PD-L1 IHC expression, were stained with PD-L1 IHC 22C3 pharmDx and then scored by three pathologists over three non-consecutive days.	NPA 98.4% (95.5-99.5%) PPA 98.9% (96.8-99.6%) OA 98.7% (97.2-99.4%)
Squamous Cell Histological Subgroup Intra-observer precision	CPS ≥ 1	52 squamous specimens (22 PD-L1 negative/30 PD-L1 positive) with a range of PD-L1 IHC expression, were stained with PD-L1 IHC 22C3 pharmDx and then scored by three pathologists over three non-consecutive days.	NPA 97.9% (94.8-99.2%) PPA 99.6% (97.9-99.9%) OA 98.9% (97.5-99.5%)

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

Precision: Cervical Cancer
The precision of PD-L1 IHC 22C3 pharmDx in cervical cancer was evaluated at Dako. Inter-instrument, inter-operator, inter-day, and inter-lot were tested in combined precision. Repeatability was tested in intra-run precision. Inter- and intra-observer precision were also assessed. Negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were calculated with two-sided 95% Wilson Score confidence intervals for the CPS ≥ 1 cutoff.

Table 26: 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)
Cervical Cancer Combined Precision (inter-instrument, inter-operator, inter-lot, inter-day,)	CPS ≥ 1	6 Cervical Cancer specimens (3 PD-L1 negative and 3 PD-L1-positive) with a range of PD-L1 expression were tested using three Autostainer Link 48 instruments, four operators, three kit lots, over three non-consecutive days.	NPA 100% (81.6-100%) PPA 100% (82.4-100%) OA 100% (90.1-100%)
Cervical Cancer Intra-run precision (Repeatability)	CPS ≥ 1	2 Cervical Cancer specimens (0 PD-L1-negative and 2 PD-L1-positive) with a range of PD-L1 IHC expression were tested with five replicates within a run on the Autostainer Link 48 instrument.	NPA NA PPA 100% (72.2-100%) OA 100% (72.2-100%)
Cervical Cancer Inter-observer precision	CPS ≥ 1	21 Cervical Cancer specimens (8 PD-L1 negative/13 PD-L1 positive) with a range of PD-L1 IHC expression, were stained with PD-L1 IHC 22C3 pharmDx and then scored by three pathologists over three non-consecutive days.	NPA 100% (94.9-100%) PPA 99.1% (95.3-99.8%) OA 99.5% (97.1-99.9%)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Cervical Cancer Intra-observer precision	CPS ≥ 1	21 Cervical Cancer specimens (8 PD-L1 negative/13 PD-L1 positive) with a range of PD-L1 IHC expression, were stained with PD-L1 IHC 22C3 pharmDx and then scored by three pathologists over three non-consecutive days.	NPA 100% (94.9-100%) PPA 99.1% (95.3-99.8%) OA 99.5% (97.1-99.9%)

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

External Reproducibility: Cervical Cancer Including Squamous Cell Cancers
The reproducibility of PD-L1 IHC 22C3 pharmDx was evaluated at three external sites using squamous cell cancers (SQ) from cervical, vulva, anal and salivary gland. Negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with two-sided 95% Wilson score confidence intervals for the CPS ≥ 1 cutoff.

Table 27: Reproducibility of PD-L1 IHC 22C3 pharmDx in cervical cancer including squamous cell cancers, tested at three external sites (CPS ≥ 1)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	CPS ≥ 1	Each of 22 specimens of the squamous subgroup (9 PD-L1 negative and 13 PD-L1 positive; n=6 Cervical Cancer) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 329 comparisons to majority call.	NPA 94.8% (89.7-97.5%) PPA 97.4% (94.1-98.9%) OA 96.4% (93.7-97.9%)
Intra-site	CPS ≥ 1	Each of 22 specimens of the squamous subgroup (9 PD-L1 negative and 13 PD-L1 positive; n=6 Cervical Cancer) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Intra-site analysis was performed for three sites on a total of 329 comparisons to majority call.	NPA 98.5% (94.6-99.6%) PPA 97.5% (94.3-98.9%) OA 97.9% (95.7-99.0%)
Inter-observer	CPS ≥ 1	Each of 22 specimens of the squamous subgroup (9 PD-L1 negative and 13 PD-L1 positive; n=6 Cervical Cancer) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 194 comparisons to majority call.	NPA 95.1% (88.0-98.1%) PPA 99.1% (95.2-99.8%) OA 97.4% (94.1-98.9%)
Intra-observer	CPS ≥ 1	Each of 22 specimens of the squamous subgroup (9 PD-L1 negative and 13 PD-L1 positive; n=6 Cervical Cancer) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 194 comparisons to majority call.	NPA 97.4% (91.1-99.3%) PPA 98.3% (93.9-99.5%) OA 97.9% (94.8-99.2%)

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 using cervical cancer. Negative percent agreement (NPA), positive percent agreement (PPA), and overall percent agreement (OA) were computed with two-sided 95% Wilson score confidence intervals for the CPS ≥ 1 cutoff.

Table 28: 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	6 Cervical Cancer specimens (2 PD-L1 negative and 4 PD-L1 positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 90 comparisons to majority call.	NPA 100% (88.6-100%) PPA 95.0% (86.3-98.3%) OA 96.7% (90.7-98.9%)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Intra-site	CPS ≥ 1	6 Cervical Cancer specimens (2 PD-L1 negative and 4 PD-L1 positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Intra-site analysis was performed for three sites on a total of 90 comparisons to majority call.	NPA 100% (88.6-100%) PPA 95.0% (86.3-98.3%) OA 96.7% (90.7-98.9%)
Inter-observer	CPS ≥ 1	6 Cervical Cancer specimens (2 PD-L1 negative and 4 PD-L1 positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 54 comparisons to majority call.	NPA 100% (82.4-100%) PPA 100% (90.4-100%) OA 100% (93.4-100%)
Intra-observer	CPS ≥ 1	6 Cervical Cancer specimens (2 PD-L1 negative and 4 PD-L1 positive) with a range of PD-L1 IHC expression, stained with PD-L1 IHC 22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 54 comparisons to majority call.	NPA 100% (82.4-100%) PPA 100% (90.4-100%) OA 100% (93.4-100%)

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

16.9 Clinical Performance Evaluation: Cervical Cancer

The efficacy of KEYTRUDA was investigated in 98 patients with recurrent or metastatic cervical cancer enrolled in a single cohort (Cohort E) in Study KEYNOTE 158 (NCT02628067), a multicenter, non-randomized, open-label, multi-cohort trial. The trial excluded patients with autoimmune disease or a medical condition that required immunosuppression.

Patients were treated with KEYTRUDA intravenously at a dose of 200 mg every 3 weeks until unacceptable toxicity or documented disease progression. Patients with initial radiographic disease progression could receive additional doses of treatment during confirmation of progression unless disease progression was symptomatic, was rapidly progressive, required urgent intervention, or occurred with a decline in performance status. Patients without disease progression could be treated for up to 24 months. Assessment of tumor status was performed every 9 weeks for the first 12 months, and every 12 weeks thereafter. The major efficacy outcome measures were ORR according to RECIST 1.1, as assessed by blinded independent central review, and duration of response.

Among the 98 patients in Cohort E, 77 (79%) had tumors that expressed PD-L1 with a CPS ≥ 1 and received at least one line of chemotherapy in the metastatic setting. PD-L1 status was determined using PD-L1 IHC 22C3 pharmDx. The baseline characteristics of these 77 patients were: median age was 45 years (range: 27 to 75 years); 81% were White, 14% Asian, 3% Black; ECOG PS was 0 (32%) or 1 (68%); 92% had squamous cell carcinoma, 6% adenocarcinoma, and 1% adenosquamous histology; 95% had M1 disease and 5% had recurrent disease; 35% had one and 65% had two or more prior lines of therapy in the recurrent or metastatic setting.

No responses were observed in patients whose tumors did not have PD-L1 expression (CPS < 1).

Efficacy results are summarized in Table 29.

Table 29: Efficacy Results in Cohort E of KEYNOTE-158 (CPS ≥ 1)

Endpoint	n=77*
Objective response rate	
ORR (95% CI)	14.3% (7.4, 24.1)
Complete response rate	2.6%
Partial response rate	11.7%
Response duration	
Median in months (range)	NR (4.1, 18.6+)†
% with duration ≥ 6 months	91%

*Median follow-up time of 11.7 months (range 0.6 to 22.7 months)
†Based on patients (n=11) with a response by independent review
+Denotes ongoing
NR = not reached

16.10 Non-Clinical Performance Evaluation: Urothelial Carcinoma

The non-clinical studies were performed on FFPE urothelial carcinoma specimens.

Analytical Sensitivity/Specificity: Urothelial Carcinoma
Analytical sensitivity of PD-L1 IHC 22C3 pharmDx was tested on 104 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. One specimen was not evaluable due to high background staining.

Precision: Urothelial Carcinoma
The precision of PD-L1 IHC 22C3 pharmDx was evaluated at Dako. Inter-instrument, inter-operator, inter-day, and inter-lot 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 30.

Table 30: Precision of PD-L1 IHC 22C3 pharmDx in urothelial carcinoma, tested at one site (CPS ≥ 10)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Combined Precision (Inter-Operator, Inter-Instrument, Inter-Day, and Inter-Lot as combined variables)	CPS ≥ 10	Each of 46 urothelial carcinoma specimens (26 PD-L1-negative and 20 PD-L1-positive) with a range of PD-L1 IHC expression was tested using three operators, on three Autostainer Link 48 instruments, over three non-consecutive days, using three reagent lots.	NPA 96.2% (92.3-100%) PPA 98.3% (95.0-100%) OA 97.1% (94.2-99.3%)
Intra-run precision* (Repeatability)	CPS ≥ 10	Each of 32 urothelial carcinoma specimens (17 PD-L1-negative and 15 PD-L1-positive) with a range of PD-L1 IHC expression was tested with five replicates within a run on the Autostainer Link 48 instrument.	NPA 100% (95.7-100%)* PPA 96.0% (92.0-100%) OA 98.1% (96.2-100%)
Inter-observer precision	CPS ≥ 10	60 urothelial carcinoma specimens (28 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 three pathologists over three non-consecutive days.	NPA 95.2% (90.3-99.2%) PPA 94.1% (89.9-97.6%) OA 94.6% (91.4-97.4%)
Intra-observer precision	CPS ≥ 10	60 urothelial carcinoma specimens (28 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 three pathologists over three non-consecutive days.	NPA 96.8% (94.3-99.2%) PPA 96.5% (94.1-98.6%) OA 96.7% (94.8-98.3%)

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

*The percentile bootstrap method cannot compute confidence intervals if 100% agreement is observed, therefore the Wilson score method was used to compute confidence intervals for intra-run precision NPA agreement. Note that the Wilson score method has limitations as it assumes independence of data. Since one specimen contributes more than one comparison to majority call, the data are not independent.

External Reproducibility: Urothelial Carcinoma

The reproducibility of PD-L1 IHC 22C3 pharmDx was evaluated at three external sites using urothelial carcinoma specimens. 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.

Table 31: Reproducibility of the PD-L1 IHC 22C3 pharmDx in urothelial carcinoma, tested at three external sites (CPS ≥ 10)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
Inter-site	CPS ≥ 10	Each of 36 urothelial carcinoma specimens (20 PD-L1-negative and 16 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 539 comparisons to majority call.	NPA 94.0% (87.7-99.3%) PPA 84.6% (77.1-91.7%) OA 89.8% (85.0-94.1%)
Intra-site	CPS ≥ 10	Each of 36 urothelial carcinoma specimens (20 PD-L1-negative and 16 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days at each of three study sites. Intra-site analysis was performed for three sites on a total of 539 comparisons to majority call.	NPA 96.2% (92.9-98.8%) PPA 95.0% (92.4-97.4%) OA 95.7% (93.5-97.6%)
Inter-observer	CPS ≥ 10	Scoring of 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, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 540 comparisons to majority call.	NPA 97.3% (94.3-99.6%) PPA 90.7% (86.4-94.6%) OA 93.9% (91.3-96.3%)
Intra-observer	CPS ≥ 10	Scoring of 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, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 540 comparisons to majority call.	NPA 95.7% (93.8-97.8%) PPA 96.1% (93.6-98.3%) OA 95.9% (94.3-97.4%)

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

16.11 Clinical Performance Evaluation: Urothelial Carcinoma

The efficacy of KEYTRUDA was investigated in Study KEYNOTE 052 (NCT02335424), a multicenter, open-label, single-arm trial in 370 patients with locally advanced or metastatic urothelial carcinoma who were not eligible for cisplatin-containing chemotherapy. The trial excluded patients with autoimmune disease or a medical condition that required immunosuppression.

Patients received KEYTRUDA 200 mg every 3 weeks until unacceptable toxicity or disease progression. Patients with initial radiographic disease progression could receive additional doses of treatment during confirmation of progression unless disease progression was symptomatic, was rapidly progressive, required urgent intervention, or occurred with a decline in performance status. Patients without disease progression could be treated for up to 24 months. Tumor response assessments were performed at 9 weeks after the first dose,

then every 6 weeks for the first year, and then every 12 weeks thereafter. The major efficacy outcome measures were ORR according to RECIST 1.1 as assessed by independent radiology review and duration of response.

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 and PD-L1 status was unknown for 9 patients. Baseline characteristics of these patients were: median age 73 years, 68% male, and 88% 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-one percent had M1 disease, and 26% 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.

Efficacy results are summarized in Table 32.

Table 32: Efficacy Results in KN052

Endpoint	CPS < 10 in Validation Set (N=185)	CPS ≥ 10 in Validation Set (N=80)
Objective Response Rate*		
ORR (95% CI)	22% (16, 28)	51% (40, 63)
Complete response rate	2%	16%
Partial response rate	20%	35%
Duration of Response		
Median in months (range)	9.7 (1.4+ - 11.0+)	NR (1.4+ - 11.1+)

+ Denotes ongoing
NR = not reached; *excludes patients with unknown PD-L1 status

KEYNOTE-361 (NCT02853305) is an ongoing, multicenter, randomized study in previously untreated patients with metastatic urothelial carcinoma who are eligible for platinum-containing chemotherapy. The study compares KEYTRUDA with or without platinum-based chemotherapy (i.e., cisplatin or carboplatin with gemcitabine) to platinum-based chemotherapy alone. The trial also enrolled a third arm of monotherapy with KEYTRUDA to compare to platinum-based chemotherapy alone. The independent Data Monitoring Committee (iDMC) for the study conducted a review of early data and found that in patients classified as having PD-L1 expression of CPS < 10, those treated with KEYTRUDA monotherapy had decreased survival compared to those who received platinum-based chemotherapy. The iDMC recommended to stop further accrual of patients with PD-L1 expression of CPS < 10 in the monotherapy arm, however, no other changes were recommended, including any change of therapy for patients who had already been randomized to and were receiving treatment in the monotherapy arm.

16.12 Non-Clinical Performance Evaluation: HNSCC

The non-clinical 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.

Precision: HNSCC
The precision of PD-L1 IHC 22C3 pharmDx was evaluated at Dako. Inter-instrument, inter-operator, inter-day, and inter-lot 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% bootstrap confidence intervals for the CPS ≥ 1 cutoff and the CPS ≥ 20 cutoff as shown in Tables 33 and 34 respectively.

Table 33: 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-Day, and Inter-Lot as combined variables)	CPS ≥ 1	Each of 34 HNSCC specimens (12 PD-L1-negative and 22 PD-L1-positive) with a range of PD-L1 IHC expression was tested using five operators, on five Autostainer Link 48 instruments, over five days, using five reagent lots.	NPA 100% (94.0-100%)* PPA 99.1% (97.3-100%) OA 99.4% (98.2-100%)
Intra-run precision (Repeatability)	CPS ≥ 1	Each of 34 HNSCC specimens (16 PD-L1-negative and 18 PD-L1-positive) with a range of PD-L1 IHC expression was tested with five replicates within a run on the Autostainer Link 48 instrument.	NPA 98.8% (96.2-100%) PPA 97.8% (94.4-100%) OA 98.2% (95.9-100%)
Inter-observer precision	CPS ≥ 1	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	NPA 88.9% (78.8-98.0%) PPA 99.1% (97.4-100%) OA 94.4% (89.8-98.6%)

Precision Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
		by three pathologists over three non-consecutive days with a minimum two-week washout period in between each read.	
Intra-observer precision	CPS ≥ 1	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 three pathologists over three non-consecutive days with a minimum two-week washout period in between each read.	NPA 98.8% (96.0-100%) 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

*The percentile bootstrap method cannot compute confidence intervals if 100% agreement is observed, therefore the Wilson score method was used to compute confidence intervals for combined precision NPA agreement. Note that the Wilson score method has limitations as it assumes independence of data. Since one specimen contributes more than one comparison to majority call, the data are not independent.

Table 34: 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-Day, and Inter-Lot as combined variables)	CPS ≥ 20	Each of 34 HNSCC specimens (17 PD-L1-negative and 17 PD-L1-positive) with a range of PD-L1 IHC expression was tested using five operators, on five Autostainer Link 48 instruments, over five days, using five reagent lots.	NPA 100% (95.7-100%)* PPA 96.5% (90.6-100%) OA 98.2% (95.3-100%)
Intra-run precision (Repeatability)	CPS ≥ 20	Each of 34 HNSCC specimens (18 PD-L1-negative and 16 PD-L1-positive) with a range of PD-L1 IHC expression was tested with five replicates within a run on the Autostainer Link 48 instrument.	NPA 97.7% (92.9-100%) PPA 98.7% (96.2-100%) OA 98.2% (95.2-100%)
Inter-observer precision	CPS ≥ 20	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 three pathologists over three non-consecutive days with a minimum two-week washout period in between each read.	NPA 96.3% (91.8-100%) PPA 93.1% (87.3-97.9%) OA 94.9% (91.4-97.9%)
Intra-observer precision	CPS ≥ 20	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 three pathologists over three non-consecutive days with a minimum two-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

*The percentile bootstrap method cannot compute confidence intervals if 100% agreement is observed, therefore the Wilson score method was used to compute confidence intervals for combined precision NPA agreement. Note that the Wilson score method has limitations as it assumes independence of data. Since one specimen contributes more than one comparison to majority call, the data are not independent.

External Reproducibility: HNSCC

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 two-sided 95% confidence intervals using the bootstrap method.

Table 35: 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	Each of 38 HNSCC specimens (19 PD-L1-negative and 19 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed for three sites on a total of 570 comparisons to majority call.	NPA 96.8% (92.6-100%) PPA 93.3% (86.7-98.6%) OA 95.1% (91.2-98.2%)
Intra-site	CPS ≥ 1	Each of 38 HNSCC specimens (19 PD-L1-negative and 19 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days at each of three study sites. Intra-site analysis was performed for three sites on a total of 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	Scoring of 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, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 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	Scoring of 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	NPA 97.3% (95.4-98.9%) PPA 98.3% (96.8-99.7%) OA 97.8% (96.8-98.9%)

Reproducibility Study	Diagnostic Cutoff	Study Design	% Agreement (95% CI)
		22C3 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 555 comparisons to majority call.	

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

Table 36: 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	Each of 38 HNSCC specimens (25 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 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	Each of 38 HNSCC specimens (25 PD-L1-negative and 13 PD-L1-positive) with a range of PD-L1 IHC expression was tested on five non-consecutive days at each of three study sites. Intra-site analysis was performed for three sites on a total of 570 comparisons to majority call.	NPA 96.9% (94.6-98.8%) PPA 90.6% (86.3-94.9%) OA 94.9% (92.8-96.8%)
Inter-observer	CPS ≥ 20	Scoring of 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, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 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	Scoring of 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, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 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.

16.13 Clinical Performance Evaluation: HNSCC

The efficacy of KEYTRUDA was investigated in KEYNOTE-048 (NCT02358031), a randomized, multicenter, open label, active controlled trial conducted in 882 patients with metastatic or recurrent HNSCC who had not previously received systemic therapy for metastatic disease or with recurrent disease who were considered incurable by local therapies. Patients with active autoimmune disease that required systemic therapy within two years of treatment or a medical condition that required immunosuppression were ineligible. Randomization was stratified by tumor PD-L1 expression (TPS ≥50% or <50%) according to the PD-L1 IHC 22C3 pharmDx Kit, HPV status according to p16 IHC (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 intravenously every 3 weeks
- KEYTRUDA 200 mg intravenously every 3 weeks, carboplatin AUC 5 mg/mL/min intravenously every 3 weeks or cisplatin 100 mg/m^2 intravenously every 3 weeks, and FU 1000 mg/m^2/day as a continuous intravenous infusion over 96 hours every 3 weeks (maximum of 6 cycles of platinum and FU)
- Cetuximab 400 mg/m^2 intravenously as the initial dose then 250 mg/m^2 intravenously once weekly, carboplatin AUC 5 mg/mL/min intravenously every 3 weeks or cisplatin 100 mg/m^2 intravenously every 3 weeks, and FU 1000 mg/m^2/day as a continuous intravenous infusion over 96 hours every 3 weeks (maximum of 6 cycles of platinum and FU)

Treatment with KEYTRUDA continued until RECIST v1.1-defined progression of disease as determined by the investigator, unacceptable toxicity, or a maximum of 24 months. Administration of KEYTRUDA 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. A retrospective re-classification of patients' tumor PD-L1 status according to CPS according to the PD-L1 IHC 22C3 pharmDx Kit was conducted using the tumor specimens used for randomization.

The main efficacy outcome measures were OS and PFS as assessed by BICR according to RECIST v1.1 (modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ) sequentially tested in the subgroup of patients with CPS ≥20, the subgroup of patients with CPS ≥ 1, and the overall population.

A total of 601 patients were randomized to the KEYTRUDA as a single agent and cetuximab in combination with chemotherapy arms; 301 patients to the KEYTRUDA as a single agent arm and 300 patients to the cetuximab in combination with chemotherapy arm. The study population characteristics were: median age of 61 years (range: 22 to 94); 36% age 65 or older; 85% male; 74% White and 19% Asian, and 1.7% Black; 61% ECOG PS of 1; and 79% were former/current smokers. Twenty-two percent of patients' tumors were HPV-positive, and 96% had Stage IV disease (Stage IVA 20%, Stage IVB 6%, and Stage IVC 70%).

For the subgroup of patients randomized to KEYTRUDA as a single agent or to cetuximab in combination with chemotherapy, PD-L1 expression level for 601 patient tumor biopsy or resection tissue (159 archival and 442 newly obtained; refer to definition in Table 37) was determined using PD-L1 IHC 22C3 pharmDx. Overall, 85% (512/601) of the patients had tumors that expressed PD-L1 with CPS ≥ 1. Eighty-six percent (380/442) of patients whose tumors were newly obtained for PD-L1 testing and 83% (132/159) of patients whose archival tumors were tested expressed PD-L1 at CPS ≥ 1. Forty-three percent (255/597) of the patients had tumors that expressed PD-L1 with CPS ≥ 20; four patients had unknown PD-L1 expression status (one specimen was archival tissue and three specimens were newly obtained tissue). Forty-two percent (186/439) of patients whose tumors were newly obtained for PD-L1 testing and 44% (69/158) of patients whose archival tumors were tested expressed PD-L1 at CPS ≥ 20 (Table 37).

Table 37: Tumor PD-L1 Expression by Specimen Type

Tumor Tissue	Number (%) with CPS < 1	Number (%) with CPS ≥ 1	Number (%) with CPS ≥ 20
Overall study n=601	89 (15)	512 (85)	255 (43)**
Archival Tissue* n=159	27 (17)	132 (83)	69 (44)**
Newly Obtained Tissue* n= 442	62 (14)	380 (86)	186 (42)**

*In the context of clinical trial KEYNOTE-048, newly obtained tissue biopsy was defined as the biopsy collected within 90 days of initiation of treatment with pembrolizumab. Specimens that were > 90 days were classified as archival.
**Based on patients with known PD-L1 expression; 4 patients had unknown PD-L1 expression status (one specimen was archival tissue and three specimens were newly obtained tissue).

The trial demonstrated a statistically significant improvement in OS for the subgroup of patients with PD-L1 CPS ≥ 1 randomized to KEYTRUDA as a single agent compared to those randomized to cetuximab in combination with chemotherapy. At the time of the interim analysis, there was no significant difference in OS between the KEYTRUDA single agent arm and the control arm for the overall population.

Table 38 summarizes efficacy results for KEYTRUDA as a single agent in the subgroup of patients with CPS ≥ 1 HNSCC and CPS ≥ 20 HNSCC. Figure 5 summarizes the OS results in the subgroup of patients with CPS ≥ 1 HNSCC.

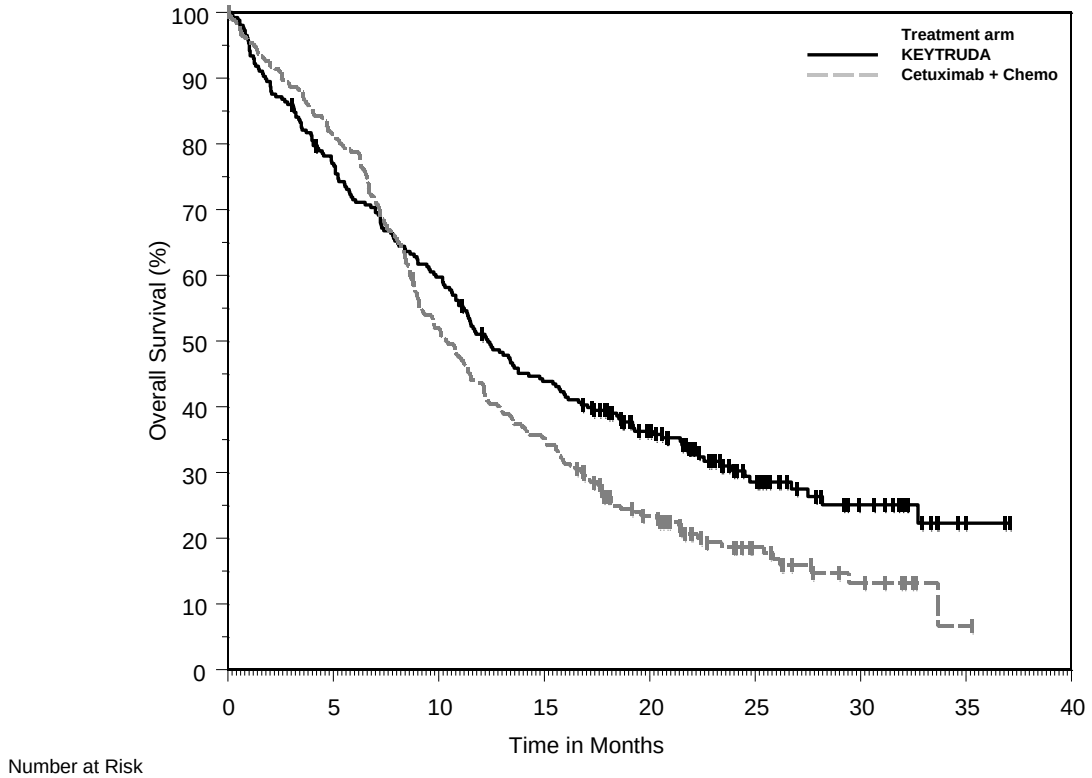
Table 38: Efficacy Results for KEYTRUDA as a Single Agent in KEYNOTE-048 (CPS ≥ 1 and CPS ≥ 20)

Endpoint	CPS ≥ 1		CPS ≥ 20	
	KEYTRUDA 200 mg every 3 weeks n=257	Cetuximab Platinum FU n=255	KEYTRUDA 200 mg every 3 weeks n=133	Cetuximab Platinum FU n=122
OS				
Number of events (%)	177 (69%)	206 (81%)	82 (62%)	95 (78%)
Median in months (95% CI)	12.3 (10.8, 14.9)	10.3 (9.0,11.5)	14.9 (11.6, 21.5)	10.7 (8.8, 12.8)
Hazard ratio* (95% CI)	0.78 (0.64, 0.96)		0.61 (0.45, 0.83)	
p-Value†	0.0171		0.0015	
PFS				
Number of events (%)	225 (88%)	231 (91%)	113 (85%)	111 (91%)
Median in months (95% CI)	3.2 (2.2, 3.4)	5.0 (4.8, 5.8)	3.4 (3.2, 3.8)	5.0 (4.8, 6.2)
Hazard ratio‡ (95% CI)	1.15(0.95, 1.38)		0.99 (0.75, 1.29)	
Objective Response Rate				
ORR‡ (95% CI)	19% (14.5, 24.4)	35% (29.1, 41.1)	23% (16.4, 31.4)	36% (27.6, 45.3)
Complete response rate	5%	3%	8%	3%
Partial response rate	14%	32%	16%	33%
Duration of Response				
Median in months (range)	20.9 (1.5+, 34.8+)	4.5 (1.2+, 28.6+)	20.9 (2.7, 34.8+)	4.2 (1.2+, 22.3+)

* Based on the stratified Cox proportional hazard model
† Based on a stratified log-rank test
‡ Response: Best objective response as confirmed complete response or partial response

In an exploratory subgroup analysis for patients with CPS 1-19 HNSCC, the median OS was 10.8 months (95% CI: 9.0, 12.6) for KEYTRUDA as a single agent and 10.1 months (95% CI: 8.7, 12.1) for cetuximab in combination with chemotherapy, with an HR of 0.90 (95% CI: 0.68, 1.18).

Figure 5: Kaplan-Meier Curve for Overall Survival for KEYTRUDA as a Single Agent in KEYNOTE-048 (CPS ≥ 1)



17. Troubleshooting

Table 39: Troubleshooting

Problem	Probable Cause	Suggested Action
1. No staining of slides	1a. Programming error.	1a. Verify that the PD-L1 IHC 22C3 pharmDx program was selected for programming of slides.
	1b. Lack of reaction with DAB+ Substrate-Chromogen Solution (DAB)	1b. Verify that DAB+ Substrate-Chromogen Solution was prepared properly.
	1c. Sodium azide in wash buffer.	1c. Use only Dako Wash Buffer (Code K8007).
	1d. Degradation of Control Slide	1d. Check kit expiration date and kit storage conditions on outside of package.
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 Dako Wash Buffer (Code K8007).
3. Weak staining of specimen slides or of the positive cell line on the Control Slide provided with the kit	3a. Inadequate target retrieval.	3a. Verify that the 3-in-1 pre-treatment procedure was correctly performed.
	3b. Inappropriate wash buffer used.	3b. Use only Dako Wash Buffer (Code K8007).
4. Excessive background staining of slides.	4a. Paraffin incompletely removed.	4a. Verify that the 3-in-1 pre-treatment procedure was correctly performed.
	4b. Slides dried while loading onto the Autostainer Link 48.	4b. Ensure slides remain wet with buffer while loading and prior to initiating run.
	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.
5. Tissue detached from slides.	5a. Use of incorrect microscope slides.	5a. Use Dako 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 °C 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 Dako Wash Buffer (Code K8007).
7. Target Retrieval Solution is cloudy in appearance when heated.	7. When heated the Target Retrieval Solution turns cloudy in appearance.	7. This is normal and does not influence staining.

NOTE: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call Agilent Technical Support for further assistance. Additional information on staining techniques and specimen preparation can be found in Dako Education Guide: Immunohistochemical Staining Methods (5) (available from Agilent).

18. References

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Explanation of symbols

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

Dako North America, Inc.
6392 Via Real
Carpinteria, California 93013 USA

Tel 805 566 6655
Fax 805 566 6688
Technical Support 800 424 0021
Customer Service 800 235 5763

EC REP Dako Denmark A/S
Produktionsvej 42
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www.agilent.com

PT0020/Rev D

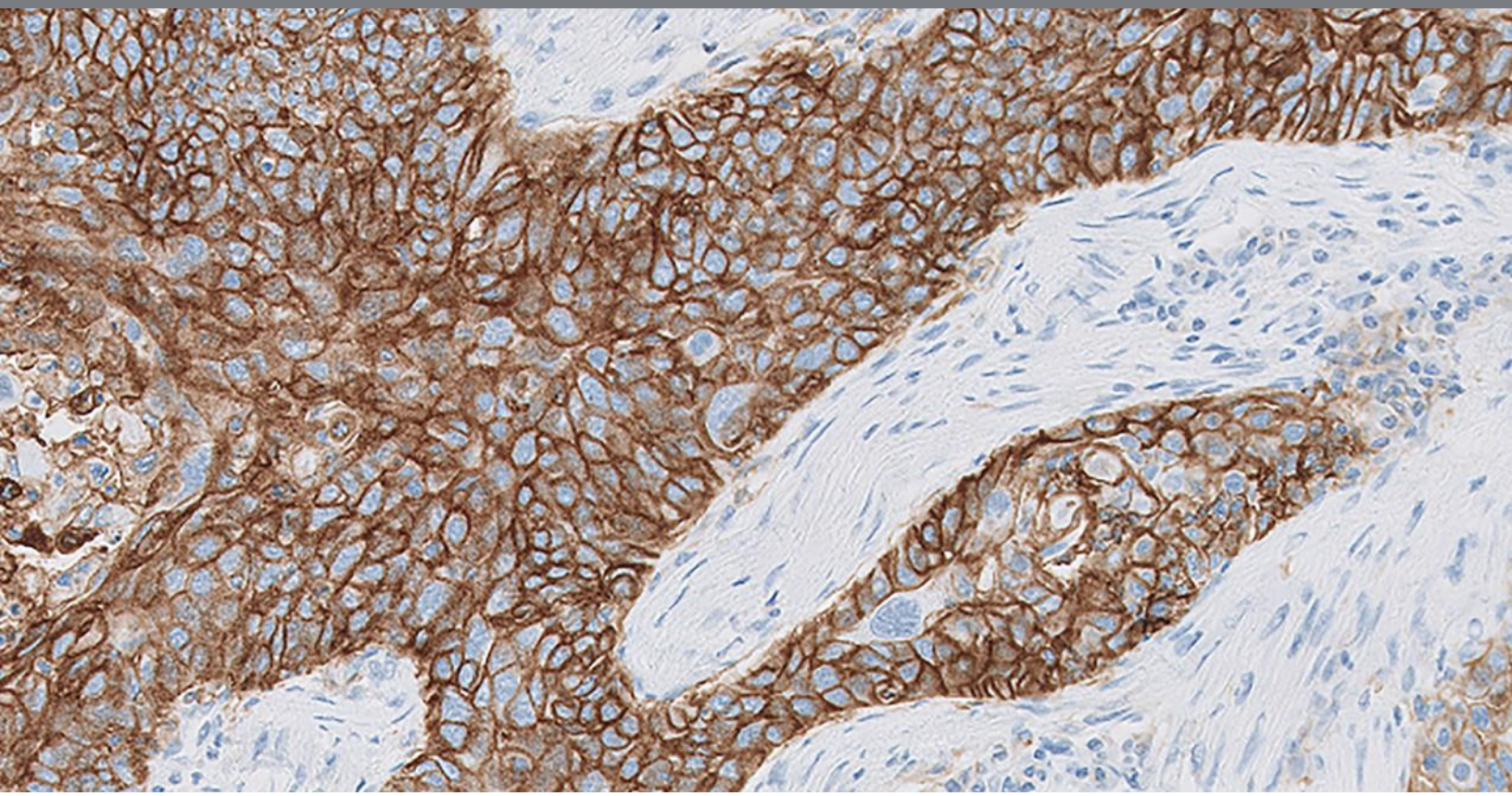
Revision 2019.07

PD-L1 IHC 22C3 pharmDx Interpretation Manual – Esophageal Squamous Cell Carcinoma (ESCC)

Agilent Dako

FDA-approved for in vitro diagnostic use

Rx only



Agilent Trusted Answers

For countries outside of the United States, see the local KEYTRUDA product label for approved indications and expression cutoff values to guide therapy.

Table of Contents

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), gastric or gastroesophageal junction (GEJ) adenocarcinoma, esophageal squamous cell carcinoma (ESCC), cervical cancer, urothelial carcinoma and head and neck squamous cell carcinoma (HNSCC) 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 gastric or GEJ adenocarcinoma, ESCC, cervical cancer, urothelial carcinoma and HNSCC 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.

Companion Diagnostic Indications

Tumor Indication	PD-L1 Expression Level	Intended Use
NSCLC	TPS ≥ 1%	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying NSCLC patients for treatment with KEYTRUDA® (pembrolizumab).**
Gastric or GEJ Adenocarcinoma	CPS ≥ 1	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying gastric or GEJ adenocarcinoma patients for treatment with KEYTRUDA® (pembrolizumab).
ESCC	CPS ≥ 10	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying ESCC patients for treatment with KEYTRUDA®
Cervical Cancer	CPS ≥ 1	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying cervical cancer patients for treatment with KEYTRUDA® (pembrolizumab)
Urothelial Carcinoma	CPS ≥ 10	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying urothelial carcinoma patients for treatment with KEYTRUDA® (pembrolizumab) **
HNSCC	CPS ≥ 1	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying HNSCC patients for treatment with KEYTRUDA® (pembrolizumab).**

*** See the KEYTRUDA® product label for specific clinical circumstances guiding PD-L1 testing.*

Introduction

PD-L1 IHC 22C3 pharmDx is the only companion diagnostic FDA-approved as an aid in identifying patients with esophageal squamous cell carcinoma (ESCC) for treatment with KEYTRUDA® (pembrolizumab). This Interpretation
Manual is provided as a tool to help guide pathologists and laboratory personnel in achieving correct and reproducible results in assessing PD-L1 expression in FFPE ESCC specimens. PD-L1 expression evaluation may be used to identify patients for treatment with KEYTRUDA.

The manual provides detailed scoring guidelines and technical information from the PD-L1 IHC 22C3 pharmDx Instructions for Use (IFU) to ensure high-quality staining and diagnostic assessment. To help familiarize you with the requirements for scoring ESCC stains with PD-L1 IHC 22C3 pharmDx, example cases of various PD-L1 expression levels are provided as references. These example cases and in-depth recommendations for interpretation of ESCC specimens stained with PD-L1 IHC 22C3 pharmDx can help individual labs achieve reproducible and reliable results.

PD-L1 IHC 22C3 pharmDx is considered a qualitative immunohistochemical assay. PD-L1 expression in ESCC 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.

ESCC tissue specimens that are tested for PD-L1 expression are scored and divided into PD-L1 expression levels based on a Combined Positive Score (CPS):

– CPS < 10

– CPS ≥ 10

PD-L1 expression levels are used to inform patient eligibility for treatment with KEYTRUDA. For more details on staining and interpretation, please refer to the current version of the IFU provided with PD-L1 IHC 22C3 pharmDx, Code SK006 or visit www.agilent.com.

Assay Interpretation

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

Reporting Results

To help understand what information should be reported to the treating physician, please refer to the Reporting Results section of this manual on page 32.

Photomicrographs

The included photomicrographs are of ESCC, except for Figure 36 which is squamous cell carcinoma from the cervix, and Figures 34, 35b, and 37 which are esophageal adenocarcinoma.

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

Tissue samples supplied by BioIVT.

The data and biospecimens used in this project were provided by US Biolab, Rockville, MD and by SageBio LLC, Sharon, MA, USA with appropriate ethics approval and through Trans-Hit Biomarkers Inc.

PD-L1 Overview

The PD-1/PD-L1 Pathway Controls the Immune Response in Normal Tissue

Programmed death-ligand 1 (PD-L1) is a transmembrane protein that binds to the programmed death-1 receptor (PD-1) during immune system modulation. The PD-1 receptor is typically expressed on cytotoxic T-cells and other immune cells, while the PD-L1 ligand is typically expressed on normal cells. Normal cells use the PD-1/PD-L1 interaction as a mechanism of protection against immune recognition by inhibiting the action of T-cells (Figure 1). Inactivation of cytotoxic T-cells downregulates the immune response such that the inactive T-cell is exhausted, ceases to divide, and might eventually die by programmed cell death, or apoptosis.

The Tumor Escapes Detection by Utilizing the PD-1/PD-L1 Pathway

Many tumor cells are able to upregulate the expression of PD-L1 as a mechanism to evade the body's natural immune response. Activated T-cells recognize the PD-L1 marker on the tumor cell, similar to that of a normal cell, and PD-L1 signaling renders the T-cell inactive (Figure 2). The tumor cell escapes the immune cycle, continues to avoid detection for elimination, and is able
to proliferate.

Anti-PD-1 Therapy Enables the Immune Response Against Tumors

PD-1/PD-L1 interaction between tumor cells and activated T-cells (Figure 3) is a mechanistic pathway used by immunotherapeutic agents. When the tumor cell is unable to interact with the activated T-cell, the immune system remains active, helping to prevent immunosuppression.

PD-L1 IHC 22C3 pharmDx Detects PD-L1 in ESCC Specimens

PD-L1 upregulation in ESCC is a biomarker for response to anti-PD-1 therapy. PD-L1 IHC 22C3 pharmDx was the only PD-L1 assay used in the KEYTRUDA® (pembrolizumab) clinical trial (KEYNOTE-181) to evaluate the relationship between PD-L1 expression and clinical efficacy. KEYTRUDA is a humanized monoclonal PD-1-blocking antibody.

The PD-1/PD-L1 Pathway

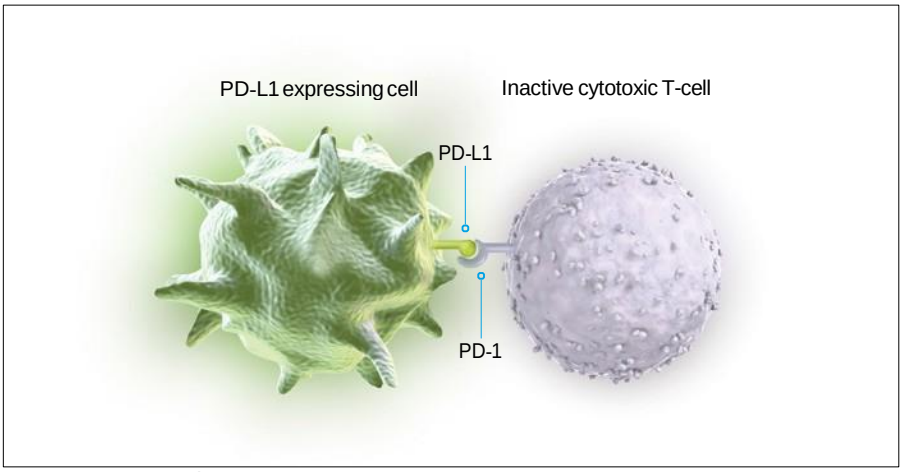


Figure 1: Inactivation of T-cells limits damage to normal tissue.

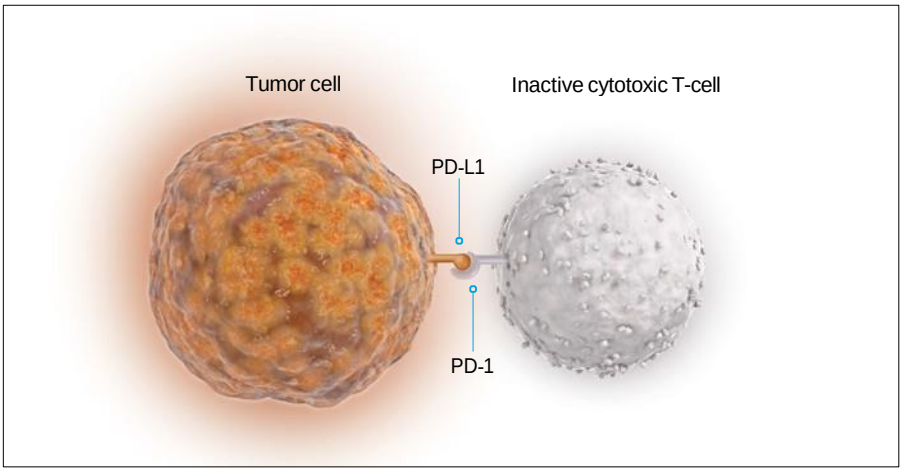


Figure 2: Inactivation of T-cells reduces tumor cell death and elimination.

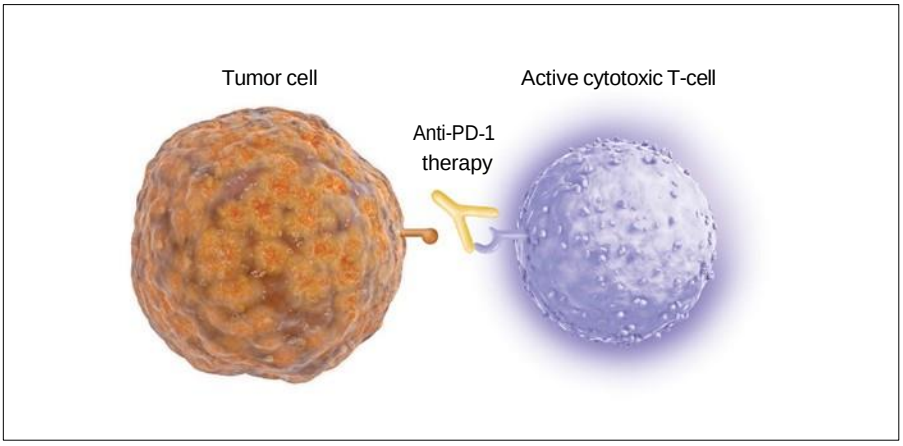


Figure 3: Blocking the PD-1/PD-L1 interaction helps to enable active T-cells and tumor cell death and elimination.

PD-L1 IHC 22C3 pharmDx Overview

What is PD-L1 IHC 22C3 pharmDx?

PD-L1 IHC 22C3 pharmDx is the only companion diagnostic indicated as an aid in identifying patients with ESCC for treatment with KEYTRUDA® (pembrolizumab). PD-L1 IHC 22C3 pharmDx is a qualitative immunohistochemical (IHC) assay intended for use in the detection of PD-L1 protein in FFPE ESCC tissue samples using EnVision FLEX visualization system on Autostainer Link 48.

Components of PD-L1 IHC 22C3 pharmDx

PD-L1 IHC 22C3 pharmDx contains optimized reagents to perform an IHC staining procedure using a linker and a chromogen enhancement reagent (Figure 4). Deparaffinization, rehydration, and target retrieval is performed using a 3-in-1 procedure on PT Link. Following peroxidase block, specimens are
incubated with the monoclonal mouse primary antibody to PD-L1 or the Negative Control Reagent. Specimens are then incubated with a Mouse LINKER, followed by incubation with a ready-to-use Visualization Reagent consisting of secondary antibody molecules and horseradish peroxidase molecules coupled to a dextran polymer backbone.

The enzymatic conversion of the subsequently added chromogen results in precipitation of a visible reaction product at the site of the antigen. The color of the chromogenic reaction is modified by a chromogen enhancement reagent. The specimen may then be counterstained and coverslipped. Results are interpreted using a light microscope.

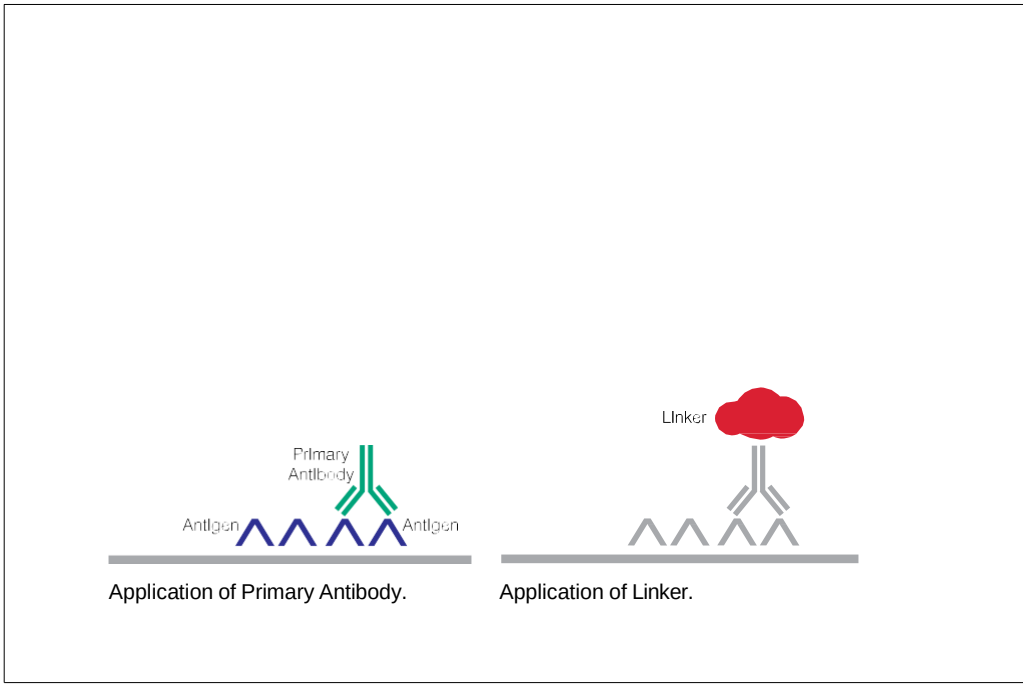


Figure 4: PD-L1 IHC 22C3 pharmDx staining procedure.

Kit Configuration (SK006)

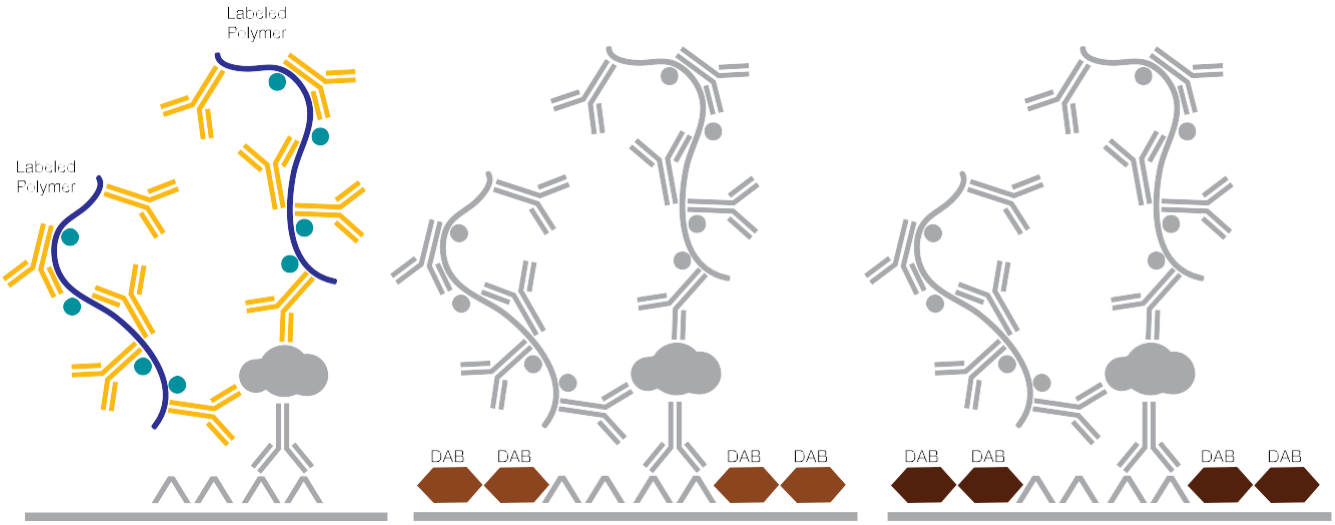
Figure 5: PD-L1 IHC 22C3 pharmDx components.

PD-L1 IHC 22C3 pharmDx (Code SK006) contains reagents to perform 50 tests in up to 15 individual runs (Figure 5):

1. EnVision FLEX Target Retrieval Solution, Low pH, (50×)
2. Peroxidase-blocking Reagent
3. Primary Antibody: Monoclonal Mouse Anti-PD-L1, Clone 22C3
4. Negative Control Reagent
5. Mouse LINKER
6. Visualization Reagent-
7. HRP DAB+ Substrate
8. Buffer DAB+ Chromogen
9. DAB Enhancer
10. PD-L1 IHC 22C3 pharmDx Control Cell Line Slides*

EnVision FLEX Wash Buffer, (20×) (Code K8007) and EnVision FLEX Hematoxylin (Code K8008) are required but not included in the kit.

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



Application of Visualization Reagent. Substrate

Application of DAB+ Chromogen Solution.

Application of DAB Enhancer.

Technical Considerations

Technical problems related to PD-L1 IHC 22C3 pharmDx may arise and can be attributed to two factors: specimen collection and preparation prior to performing the test, and the actual performance of the test itself. Technical problems are generally related to procedural deviations and can be controlled and minimized through training and, where necessary, clarification of the product instructions.

Specimen Preparation

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

In-house Control Tissue

Differences in processing and embedding in the user's laboratory may produce significant variability in results. Include positive and negative in-house control tissue in each staining run, in addition to the PD-L1 IHC 22C3 pharmDx Control Cell Line Slide.

Select positive and negative control tissue from fresh specimens of the same tumor indication as the patient specimen. Fix, process, and embed the control tissue in the same manner. Control tissues processed differently from the patient specimen validate reagent performance only and do not verify tissue preparation.

The ideal positive control tissue provides a complete dynamic representation of weak-to-moderate staining of tumor cells and tumor-associated mononuclear inflammatory cells (MICs: lymphocytes and macrophages). The ideal negative control tissue should demonstrate no staining on tumor cells and immune cells. However, because prevalence of PD-L1 expression on immune cells is high, a few staining immune cells are acceptable.

Optional Additional In-house Control: Tonsil Tissue

Tonsil stained with PD-L1 should be pre-screened to exhibit strong staining in portions of the crypt epithelium and weak-to-moderate staining of the follicular macrophages in the germinal centers. PD-L1 expression of the endothelium, fibroblasts, and the surface epithelium should be absent.

Tissue Processing

FFPE tissues have been validated for use. Block specimens into a thickness of 3 mm or 4 mm, fix in formalin and dehydrate and clear in a series of alcohols
and xylene, followed by infiltration with melted paraffin. The paraffin temperature should not exceed 60 °C. Feasibility studies on NSCLC tissue samples were performed with fixation in 10% neutral buffered formalin for 12–72 hours. Fixation times of 3 hours or less should not be used for PD-L1 assessment. The use of PD-L1 IHC 22C3 pharmDx on decalcified tissues or tissues processed with other fixatives has not been validated and is not recommended.

Cut tissue specimens into sections of 4–5 µm. After sectioning, tissues should be mounted on Dako FLEX IHC Microscope Slides (Code K8020) or Superfrost Plus slides, and then placed in a 58 ± 2 °C oven for 1 hour. To preserve antigenicity, store tissue sections in the dark at 2–8 °C (preferred) and stain within 4.5 months of sectioning, or at room temperature up to 25 °C and stain within 1 month of sectioning.

PD-L1 IHC 22C3 pharmDx Staining Procedure

The PD-L1 IHC 22C3 pharmDx reagents and instructions have been designed for optimal performance. Further dilution of the reagents, alteration of incubation times, temperatures, or materials may give erroneous results. All of the required steps and incubation times for staining are pre-programmed in the DakoLink software.

Reagent Storage

Store all components of PD-L1 IHC 22C3 pharmDx, including Control Cell Line Slides, in the dark at 2–8 °C when not in use.

Reagent Preparation

Equilibrate all components to room temperature (20–25 °C) prior to immunostaining. Do not use after the expiration date printed on the outside of the package.

EnVision FLEX Target Retrieval Solution, Low pH

Dilute EnVision FLEX Target Retrieval Solution, Low pH, (50×) 1:50 using distilled or deionized water (reagent-quality water). One 30 mL bottle of concentrate provides 1.5 L of working solution, which is sufficient to fill one PT Link tank. Discard 1× EnVision FLEX Target Retrieval Solution, Low pH after 3 uses or 5 days after dilution. Please refer to the Product-specific Limitations
Section on page 16 for Target Retrieval Solution limitations in ESCC specimens.

EnVision FLEX Wash Buffer

Dilute EnVision FLEX Wash Buffer (20×) 1:20 using distilled or deionized water (reagent-quality water). Store unused 1× buffer at 2–8 °C for no more than
1 month. Discard if cloudy in appearance.

DAB+ Substrate-Chromogen Solution

Add 1 drop of DAB+ Chromogen per mL of DAB+ Substrate Buffer and mix. Prepared DAB+ Substrate-Chromogen is stable for 5 days if stored in the dark at 2–8 °C. Mix the DAB+ Substrate-Chromogen Solution thoroughly prior to use. Any precipitate developing in the solution will not affect staining quality.

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

Controls to Assess Staining Quality

The following quality controls should be included in each staining run:

- One PD-L1 IHC 22C3 pharmDx Control Cell Line Slide stained with the primary antibody
- Positive and negative in-house control tissues stained with the primary antibody
- Subsequent sections of each patient specimen stained with the Negative Control Reagent

Deparaffinization, Rehydration, and Target Retrieval

Use PT Link to perform a Deparaffinization, Rehydration, and Target Retrieval 3-in-1 procedure:

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

Staining and Counterstaining

- Place the Autostainer rack with slides on the Autostainer Link 48
- Ensure slides remain wet with buffer while loading and prior to initiating the run. Dried tissue sections may display increased non-specific staining
- Select the PD-L1 IHC 22C3 pharmDx protocol. The instrument performs the staining and counterstaining procedures by applying the appropriate reagent, monitoring the incubation time, and rinsing slides between reagents
- Counterstain slides using EnVision FLEX Hematoxylin, Code K8008

Mounting

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

Product-specific Limitations

- Laboratories should pay particular attention to the pH of the Target Retrieval Solution for pre-treatment of ESCC specimens as pH 5.9 may affect PD-L1 staining performance
- The studies carried out to assess TRS use up to three times in esophageal cancer did not meet acceptance criteria for qualitative evaluation of PD-L1 expression status, therefore TRS reuse is not recommended for ESCC specimens

Technical Checklist

Use the checklist below to ensure correct usage of PD-L1 IHC 22C3 pharmDx:

Customer Name/Institution _____

Name and Title _____

Autostainer Link 48 Serial Number_____Software Version _____

	Yes	No
Regular preventive maintenance is performed on the Autostainer Link 48 and PT Link?	☐	☐
PD-L1 IHC 22C3 pharmDx is used before the expiration date printed on the outside of the box?	☐	☐
All PD-L1 IHC 22C3 pharmDx components, including Control Cell Line Slides, are stored in the dark at 2–8 °C?	☐	☐
All PD-L1 IHC 22C3 pharmDx components, including Control Cell Line Slides, are equilibrated to room temperature (20–25 °C) prior to immunostaining?	☐	☐
Appropriate positive and negative control tissues from ESCC are identified?	☐	☐
Tissues are fixed in neutral buffered formalin?	☐	☐
Tissues are infiltrated with melted paraffin, at or below 60 °C?	☐	☐
Tissue sections of 4–5 µm are mounted on Dako FLEX IHC Microscope Slides or Superfrost Plus slides?	☐	☐
Specimens are oven-dried at 58 ± 2 °C for 1 hour?	☐	☐
Specimens are stained within 4.5 months of sectioning when stored in the dark at 2–8 °C (preferred) or within 1 month when stored in the dark at room temperature up to 25 °C?	☐	☐
EnVision FLEX Target Retrieval Solution, Low pH is prepared properly? pH of 1× Target Retrieval Solution must be 6.1 ± 0.2.	☐	☐
EnVision FLEX Wash Buffer is prepared properly?	☐	☐
DAB+ Substrate-Chromogen Solution is prepared properly?	☐	☐
Slides are counterstained with EnVision FLEX Hematoxylin?	☐	☐
The Deparaffinization, Rehydration, and Target Retrieval 3-in-1 procedure is followed using PT Link?	☐	☐
Slides remain wet with buffer while loading and prior to initiating run on Autostainer Link 48?	☐	☐
The PD-L1 IHC 22C3 pharmDx protocol is selected on Autostainer Link 48?	☐	☐
Do you have all the necessary equipment to perform the PD-L1 IHC 22C3 pharmDx according to protocol? If not, specify what is missing in comments below.	☐	☐

Additional observations or comments:

_____

_____

Slide Evaluation

General Considerations

PD-L1 IHC 22C3 pharmDx evaluation should be performed by a qualified pathologist using a light microscope. Details of the PD-L1 IHC 22C3 pharmDx interpretation guidelines are reviewed on page 30. Before examining the patient specimen for PD-L1 staining, it is important to examine the controls to assess staining quality.

PD-L1 interpretation is best assessed by requesting 3 serial tissue sections (H&E, PD-L1 stain, and NCR stain) so that if the H&E is first assessed and is acceptable, the 2 remaining serial sections are likely to be acceptable for use in IHC staining.

Each PD-L1 IHC 22C3 pharmDx is configured with Control Cell Line Slides that should be included in each IHC run. Guidelines on interpreting the Control Cell Line Slide are reviewed to the right. In-house control tissue slides should also be assessed with every IHC run.

Specimen Adequacy

Confirm the Presence of at Least 100 Viable Tumor Cells

A hematoxylin and eosin (H&E) stain of the tissue specimen is evaluated first to assess tissue histology and preservation quality. PD-L1 IHC 22C3 pharmDx and the H&E staining should be performed on serial sections from the same paraffin block of the specimen. Tissue specimens should be intact, well preserved, and should confirm tumor indication.

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.

Instructions for Patient Specimens with Less Than 100 Viable Tumor Cells

Tissue from a deeper level of the block, or potentially another block, could have a sufficient number of viable tumor cells for PD-L1 IHC 22C3 pharmDx testing.

Evaluating Controls

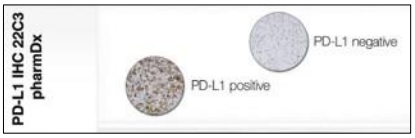


Figure 6: Each Control Cell Line Slide contains sections of cell pellets with positive and negative PD-L1 expression.

PD-L1 IHC 22C3 pharmDx Control Cell Line Slide

Examine the PD-L1 IHC 22C3 pharmDx Control Cell Line Slide to determine that reagents are functioning properly. Each slide contains sections of cell pellets with positive and negative PD-L1 expression (Figure 6). Assess the percentage of
positive cells, staining intensity, and non-specific staining in both cell pellets. If any staining of the Control Cell Line Slide is not satisfactory, all results with the patient specimens should be considered invalid. Do not use the Control Cell Line Slide as an aid in interpretation of patient results.

Evaluate the overall staining intensity using the following guide:

0	Negative
1+	Weak intensity
2+	Moderate intensity
3+	Strong intensity

Positive Control Cell Pellet

The following staining is acceptable for the PD-L1 positive cell pellet (Figure 7):

- Cell membrane staining of ≥ 70% of cells
- ≥ 2+ average staining intensity
- Non-specific staining < 1+ intensity

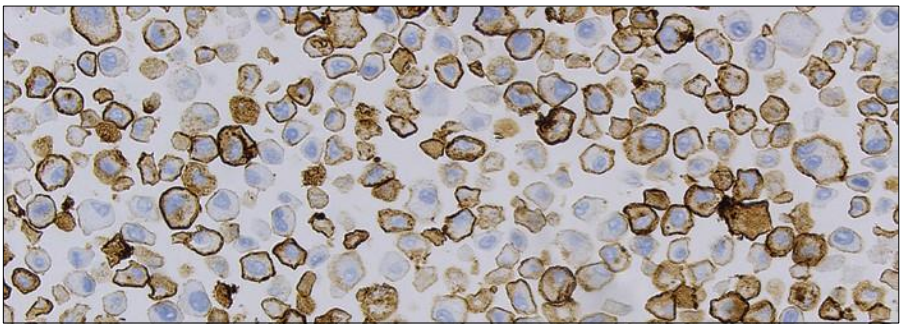


Figure 7: Positive cell pellet with acceptable staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20× magnification).

Negative Control Cell Pellet

For the PD-L1 negative cell pellet, the following staining is acceptable (Figure 8):

- The majority of cells should demonstrate no staining. **Note:** The presence of 10 or fewer cells with distinct cell membrane staining is acceptable
- Non-specific staining < 1+ intensity

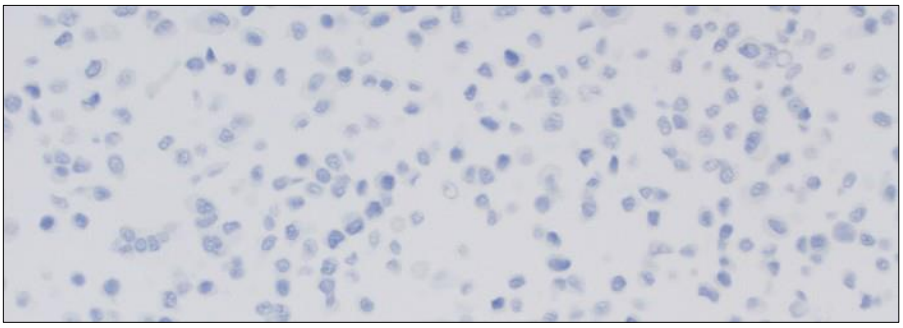


Figure 8: Negative cell pellet with no staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20× magnification).

Positive and Negative In-house Control Tissue (ESCC)

Examine the positive in-house ESCC control tissue to determine that the tissues are correctly prepared and reagents are functioning properly. The ideal positive control tissue provides a complete dynamic representation of weak-to-moderate staining of tumor cells and tumor-associated mononuclear inflammatory cells (MICs) (Figure 9). If staining of positive in-house control tissue is not satisfactory, all results with the patient specimen should be considered invalid.

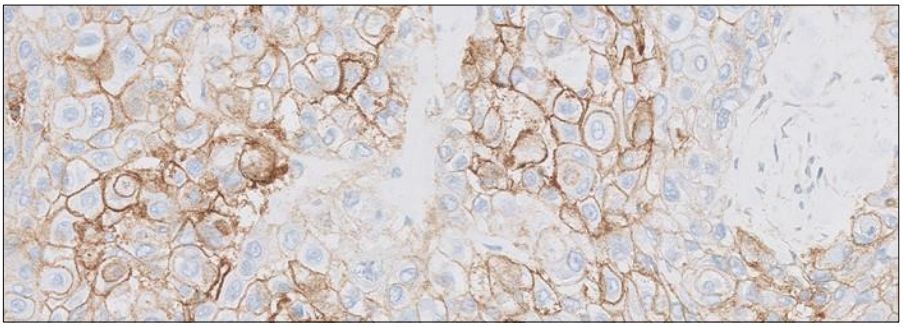


Figure 9: Positive in-house control tissue (20× magnification).

The ideal ESCC negative control tissue should demonstrate no staining of tumor cells and immune cells (Figure 10). However, because prevalence of PD-L1 expression on immune cells is high, a few staining immune cells are acceptable. Examine the negative in-house control tissue to determine the expected staining. The variety of different cell types present in most tissue sections offers internal negative control sites; this should be verified by the user.

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

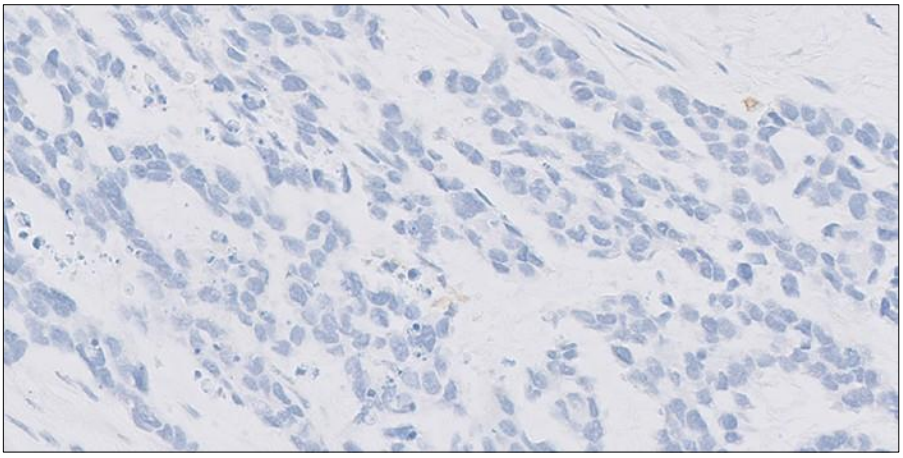


Figure 10: Negative in-house control tissue demonstrating lack of staining of tumor cells and MICs (20× magnification).

Optional Control Tissue

In addition to the Control Cell Line Slide and in-house control tissues, FFPE tonsil may also be used as an optional control specimen. Tonsil stained with PD-L1 should exhibit strong membrane staining in portions of the crypt epithelium and weak-to-moderate membrane staining of the follicular macrophages in the germinal centers (Figure 11).

PD-L1 expression of the endothelium, fibroblasts, and the surface epithelium should be absent.

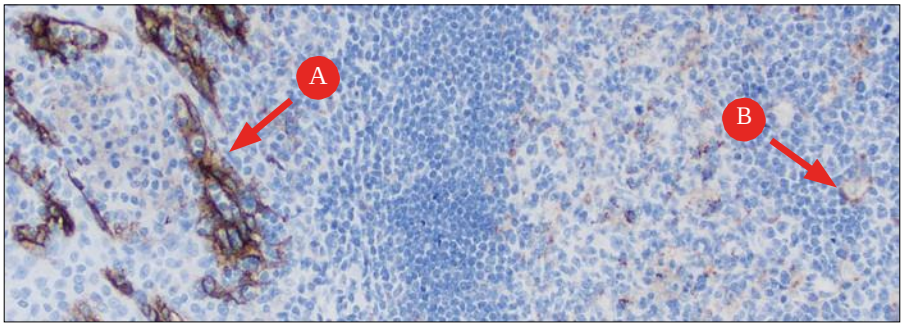


Figure 11: Tonsil stained with PD-L1 primary antibody exhibiting strong membrane staining in portions of the crypt epithelium (A) and weak-to-moderate membrane staining of follicular macrophages in the germinal centers (B) (10× magnification).

Do not use in-house control tissue as an aid in interpretation of patient results.

Negative Control Reagent (NCR)

Examine the slides stained with the NCR to identify non-specific background staining that may interfere with PD-L1 staining interpretation, making the specimen non-evaluable. Satisfactory performance is indicated by the absence of staining (Figure 12).

Examine the patient specimens stained with the NCR to determine if there is any non-specific staining that may interfere with interpreting the PD-L1 stained slide.

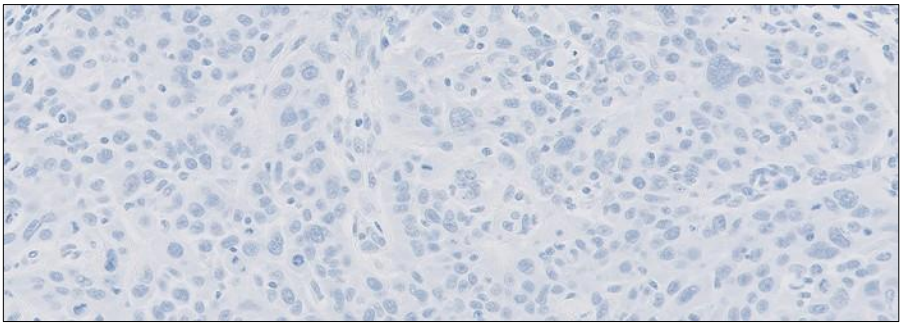


Figure 12: ESCC tissue specimen stained with NCR (20× magnification).

NCR-stained slides indicate non-specific background staining and allow for better interpretation of patient specimens stained with the primary antibody.

Slide Evaluation Flowchart

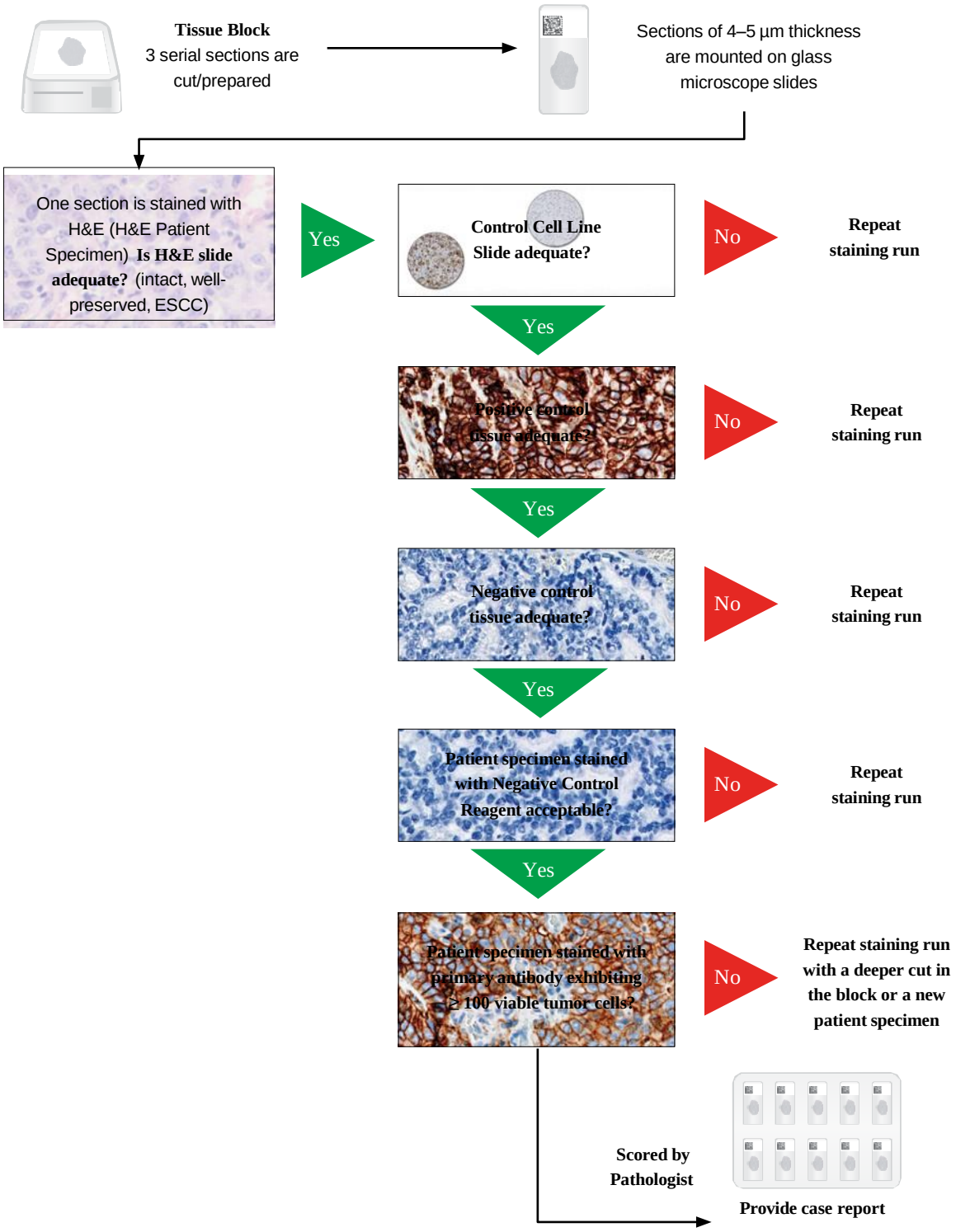


Figure 13: Recommended order of slide evaluation.

Combined Positive Score

Definition of Combined Positive Score (CPS)

PD-L1 expression in ESCC 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. Although the result of the calculation can exceed 100, the maximum score is defined as CPS 100.

CPS is defined accordingly:

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

** Macrophages and histiocytes are considered the same cells*

CPS Numerator Inclusion and Exclusion Criteria

Any perceptible and convincing partial or complete linear membrane staining (≥ 1+) of viable tumor cells that is perceived as distinct from cytoplasmic staining is considered PD-L1 staining and should be included in the scoring.

Any membrane and/or cytoplasmic staining (≥ 1+) of lymphocytes and macrophages (mononuclear inflammatory cells, MICs) within tumor nests and/or adjacent supporting stroma is considered PD-L1 staining and should be included in the CPS numerator. Only MICs directly associated with the response against the tumor are scored.

See Tables 1 and 2 on page 26 for additional CPS inclusion/exclusion criteria.

Determining Combined Positive Score

- At lower magnifications, examine all well-preserved tumor areas. Evaluate overall areas of PD-L1 staining and non-staining tumor cells, keeping in mind that partial membrane staining or 1+ membrane staining may be difficult to see at low magnifications. Ensure there are at least 100 viable tumor cells in the sample
  - A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide (biopsy and resection) for the specimen to be considered adequate for evaluation
- For specimens with less than 100 viable tumor cells, tissue from a deeper level of the block or potentially another block could have a sufficient number of tumor cells for evaluation of PD-L1 expression
- At higher magnification (20×), evaluate PD-L1 expression and calculate CPS:
  - Determine the total number of viable tumor cells, both PD-L1 staining and non-staining (CPS denominator)
  - Determine the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) (CPS numerator; see Tables 1 and 2 on page 26 for additional CPS inclusion/exclusion criteria)
  - Calculate CPS
- Evaluation of membrane staining should be performed at no higher than 20× magnification. Slide reviewer should not perform the CPS calculation at 40× magnification

Table 1: CPS Numerator Inclusion/Exclusion Criteria

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	– Non-staining tumor cells – Tumor cells with only cytoplasmic staining – Non-invasive neoplasia (including 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†: – Lymphocytes (including lymphocyte aggregates) – Macrophages‡ Only MICs directly associated with the response to the tumor are scored	– Non-staining MICs – MICs associated with non-invasive neoplasia (including carcinoma in situ) – 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 are included in the score*

† Adjacent MICs are defined as being within the same 20× 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 2: CPS Denominator Inclusion/Exclusion Criteria

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	All viable invasive tumor cells	– Non-viable tumor cells – Non-invasive neoplasia (including carcinoma in situ)
Immune Cells	Not included	All immune cells
Other Cells	Not included	– Benign cells – Stromal cells (including fibroblasts) – Necrotic cells and/or cellular debris

Suggested Methods

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

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

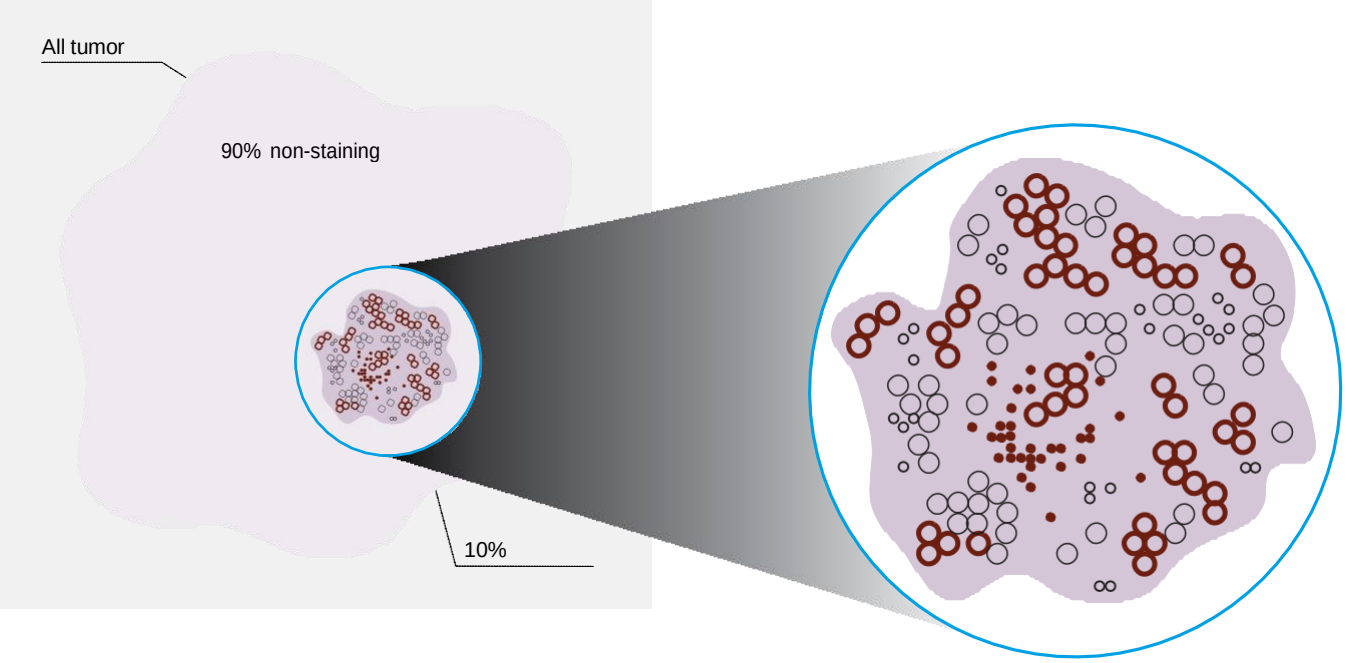
Example 1: Calculation of Combined Positive Score Based on a Small PD-L1 Staining Area

First: Evaluate the tumor area for perceptible and convincing staining as described in "Determining Combined Positive Score" on page 25.

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

Second: Evaluate the area of staining to estimate the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages).

Assessment: *There are approximately 100 viable tumor cells and about 80 PD-L1 staining cells (per the CPS numerator)*



Calculate the Combined Positive Score of the entire tumor area:

Assessment:

CPS of area with staining:

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells}^{\S}}{\text{Total \# of viable tumor cells}} \times 100 = \frac{\text{~80 PD-L1 staining cells}}{\text{100 tumor cells}} \times 100 = 80$$

CPS of entire tumor area: 10% × 80 ≈ CPS 8

Clinical Interpretation: CPS < 10

§ *Including tumor cells, lymphocytes, macrophages*

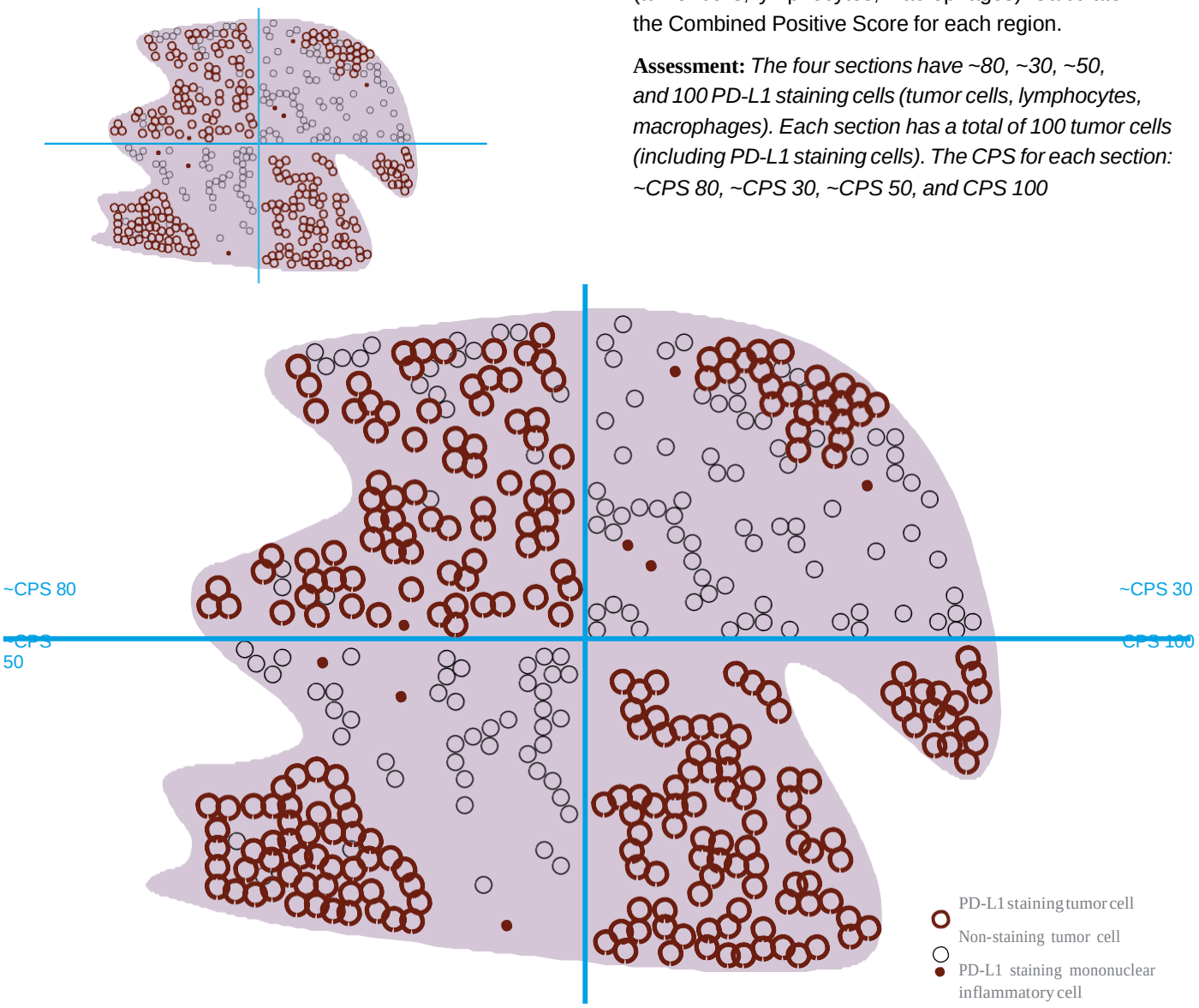
Figure 14: Example of tumor with small PD-L1 staining area.

Example 2: Calculation of Combined Positive Score Based on a Heterogeneous PD-L1 Staining Area

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

Second: Observe each region and estimate the total number of viable tumor cells and PD-L1 staining cells (tumor cells, lymphocytes, macrophages). Calculate the Combined Positive Score for each region.

Assessment: *The four sections have ~80, ~30, ~50, and 100 PD-L1 staining cells (tumor cells, lymphocytes, macrophages). Each section has a total of 100 tumor cells (including PD-L1 staining cells). The CPS for each section: ~CPS 80, ~CPS 30, ~CPS 50, and CPS 100*



Calculate the Combined Positive Score of the entire tumor area:

Assessment:

Combined Positive Score:

(80 + 30 + 50 + 100) / 4 ≈ CPS 65

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

Clinical Interpretation: CPS ≥ 10

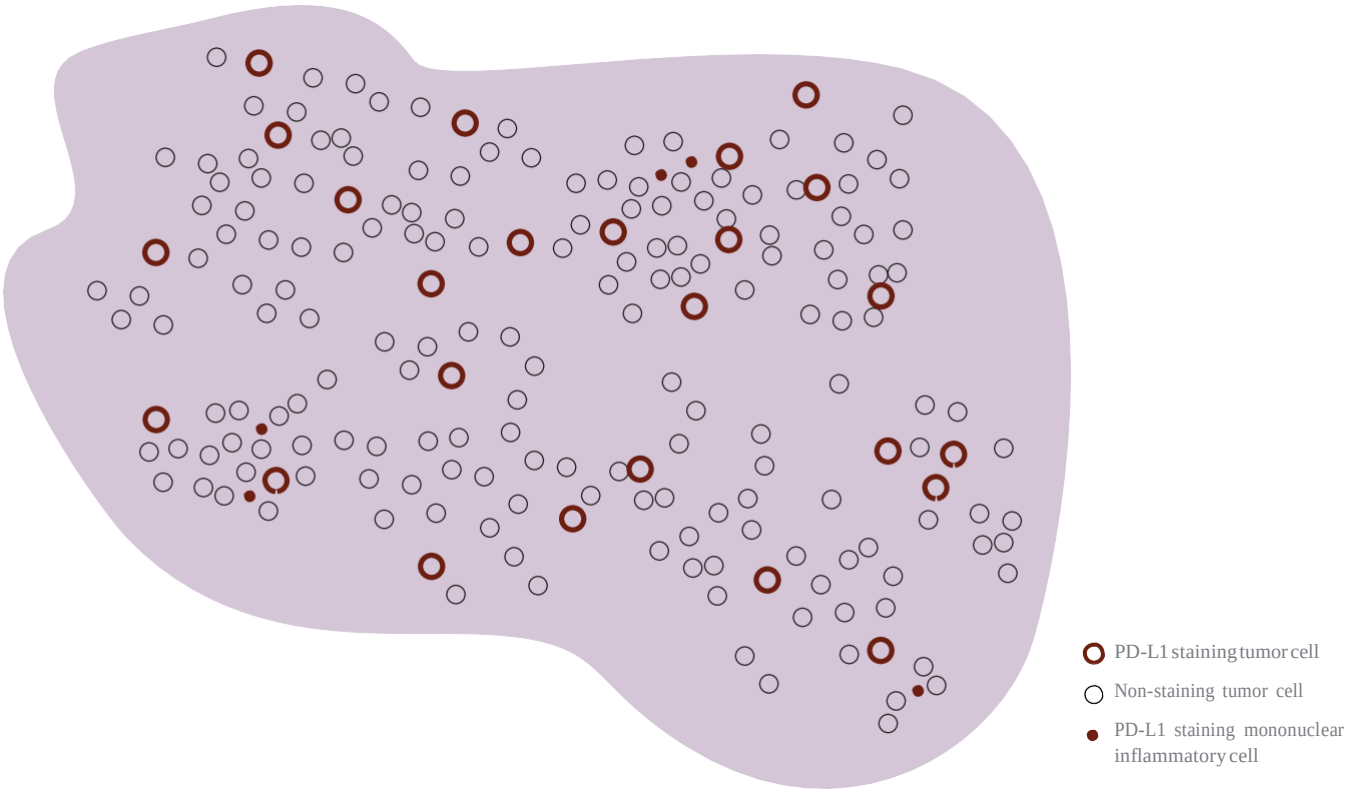
Figure 15: Example with heterogeneous PD-L1 staining area.

Example 3: Calculation of Combined Positive Score for a Near Cut-off Specimen

First: Evaluate the specimen for perceptible and convincing staining as described in "Determining Combined Positive Score" on page 25.

Second: Confirm that there is no staining in areas that appeared void of staining at lower magnifications. Evaluate all staining areas and estimate the total number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages).
Then re-evaluate the entire specimen (staining and non-staining areas) and estimate the total number of viable tumor cells (PD-L1 staining and non-staining tumor cells). Calculate the Combined Positive Score.

Assessment: *Tumor specimen has perceptible and convincing staining. There are 30 PD-L1 staining cells (tumor cells, lymphocytes, macrophages). There are approximately 200 viable tumor cells present in the entire specimen*



Calculate the Combined Positive Score of the entire tumor area:

Assessment:

Combined Positive Score:

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells*}}{\text{Total \# of viable tumor cells}} \times 100 \quad \frac{\text{30 PD-L1 staining cells}}{\text{200 tumor cells}} \times 100 = \text{CPS 15}$$

Clinical Interpretation: CPS ≥ 10

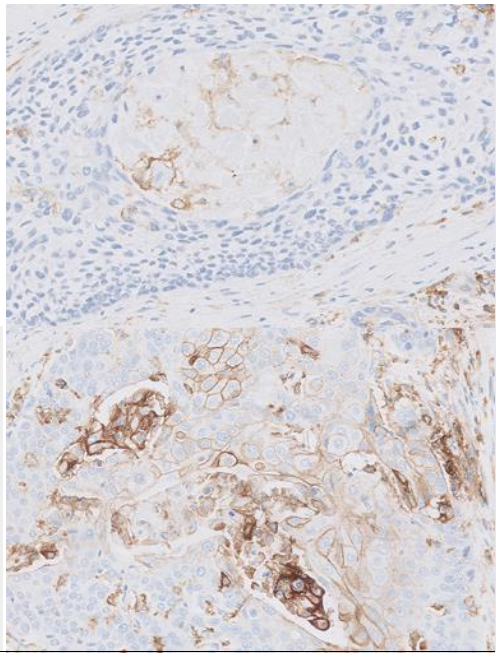
** Including tumor cells, lymphocytes, macrophages*

Figure 16: Example of near cut-off specimen.

Interpretation of CPS

The Combined Positive Score (CPS) determines the PD-L1 expression levels of the specimen. See the table below for scoring interpretation examples.

Table 3: CPS and Corresponding PD-L1 Expression Levels

CPS	PD-L1 Expression Level	Image (20× magnification)
< 10	CPS is less than 10	
≥ 10	CPS is greater than or equal to 10	

Identifying Patients With ESCC for Treatment

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

Clinical Validation of PD-L1 IHC 22C3 pharmDx in Previously Treated Patients With ESCC

The clinical validity of PD-L1 IHC 22C3 pharmDx in evaluating PD-L1 expression in previously treated patients with ESCC is based on the KEYTRUDA KEYNOTE-181 study sponsored by Merck & Co. Specimens from patients with esophageal cancer who progressed on or after one prior line of standard therapy for advanced disease (advanced/metastatic adenocarcinoma or squamous cell carcinoma) were tested for PD-L1 expression using PD-L1 IHC 22C3 pharmDx. In the KEYNOTE-181 clinical trial, 42.8% of enrolled patients had ESCC that expressed PD-L1 with a Combined Positive Score (CPS) of greater than or equal to 10 (CPS ≥ 10) (Table 4). Clinical efficacy of KEYTRUDA treatment is presented in the Clinical Performance Evaluation section on pages 63–65.

Table 4: PD-L1 Prevalence in Patients with Recurrent or Metastatic ESCC Enrolled in KEYNOTE-181*

PD-L1 Expression	CPS < 10	CPS ≥ 10
Prevalence % (n)	57.2% (210)	42.8% (157)

** Prevalence calculation based on patients with known PD-L1 expression (patients with unknown PD-L1 expression, n=6) and excludes patients with specimens outside the stability window (n=28)*

PD-L1 IHC 22C3 pharmDx Testing Scheme

Use the following flowchart to help you understand which patients are indicated for treatment with KEYTRUDA based on their CPS.

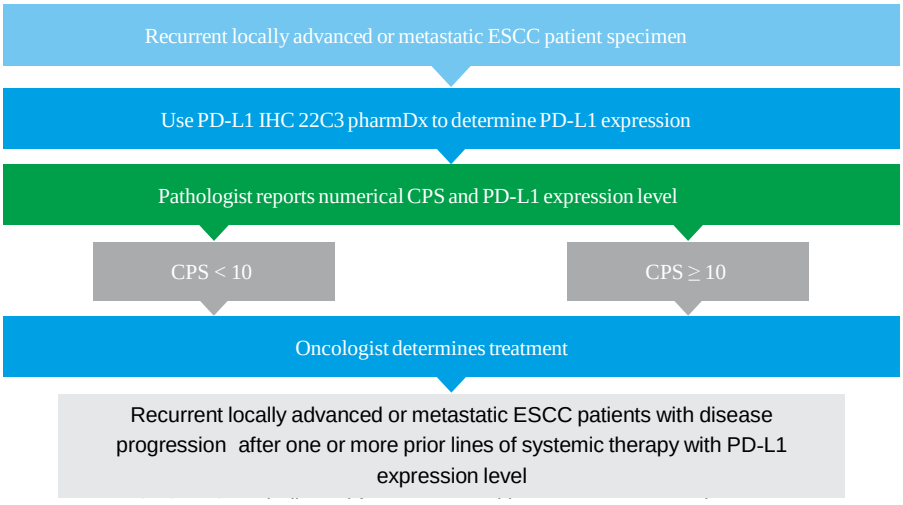


Figure 17: Testing scheme for PD-L1 IHC 22C3 pharmDx.

Reporting Results

Suggested information to include when reporting results with PD-L1 IHC 22C3 pharmDx.

PD-L1 IHC 22C3 pharmDx Summary of Sample Tested

Date of Run: _____

PD-L1 IHC 22C3 pharmDx Lot: _____

Staining Run Log ID: _____

Specimen ID: _____

Patient Identifiers: _____

Type of Service: IHC Stain with Manual Interpretation _____

Other: _____

PD-L1 Testing Results

Control Cell Line Slide Results: Pass: ☐ Fail: ☐

Adequate Tumor Cells Present (≥ 100 cells): Yes ☐ No: ☐

PD-L1 IHC 22C3 pharmDx Result to Treating Physician

Combined Positive Score: _____

CPS ≥ 10: ☐ CPS < 10: ☐

Comments to Treating Physician:

– KEYTRUDA® (pembrolizumab) is indicated for the treatment of patients with recurrent locally advanced or metastatic ESCC with disease progression after one or more prior lines of systemic therapy whose tumors exhibit PD-L1 expression level CPS ≥ 10 as determined by an FDA-approved test. See KEYTRUDA prescribing information for details.

Combined Positive Score Summary and Examples

Key Considerations in Scoring PD-L1 IHC 22C3 pharmDx Stained Specimens

By definition, PD-L1 staining cells in ESCC are:

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

PD-L1 expression status in ESCC is determined by 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.

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

This section will define and illustrate scoring inclusions and exclusions for accurate determination of Combined Positive Score. All images are ESCC, except for Figure 36, which is squamous cell carcinoma of the cervix, and Figures 34, 35b, and 37, which are esophageal adenocarcinoma.

Image Guide for Interpretation of PD-L1 IHC 22C3 pharmDx Staining in ESCC

PD-L1 Staining Cells Included in the Combined Positive Score (CPS)

Tumor cells, lymphocytes, and macrophages exhibiting appropriate PD-L1 expression are defined as PD-L1 staining cells. All PD-L1 staining cells are included in the CPS numerator for determination of the Combined Positive Score (see Tables 1 and 2 on page 26 for additional CPS inclusion/exclusion criteria). All viable tumor cells should be included in the denominator. Below are common staining characteristics of PD-L1 staining cells that must be included in the CPS numerator. All images are ESCC unless otherwise noted in the figure caption.

Tumor Cells

Linear Membrane Staining

Tumor cells exhibiting perceptible and convincing partial and/or complete smooth or granular linear membrane staining are considered PD-L1 staining cells. Linear membrane staining can be present at any intensity and must be perceptible and convincing at no higher than 20× magnification.

Perceptible and convincing staining of tumor cells (linear membrane staining) is often heterogeneous, with various staining intensities present.

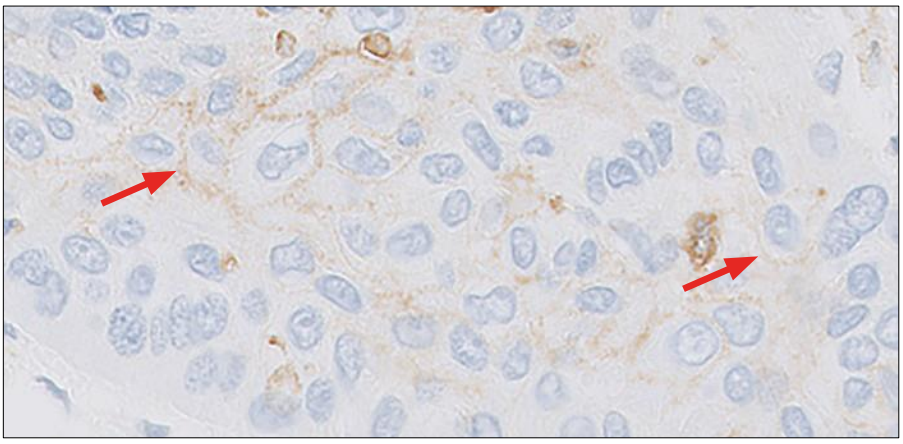


Figure 18a: ESCC specimen stained with PD-L1 primary antibody exhibiting 1+ linear membrane staining of tumor cells (arrows) (20× magnification).

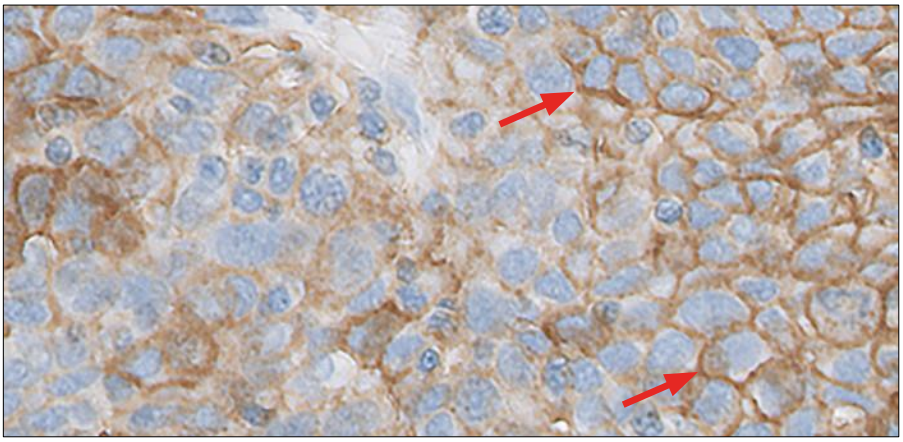


Figure 18b: ESCC specimen stained with PD-L1 primary antibody exhibiting 2+ linear membrane staining of tumor cells (arrows) (20× magnification).

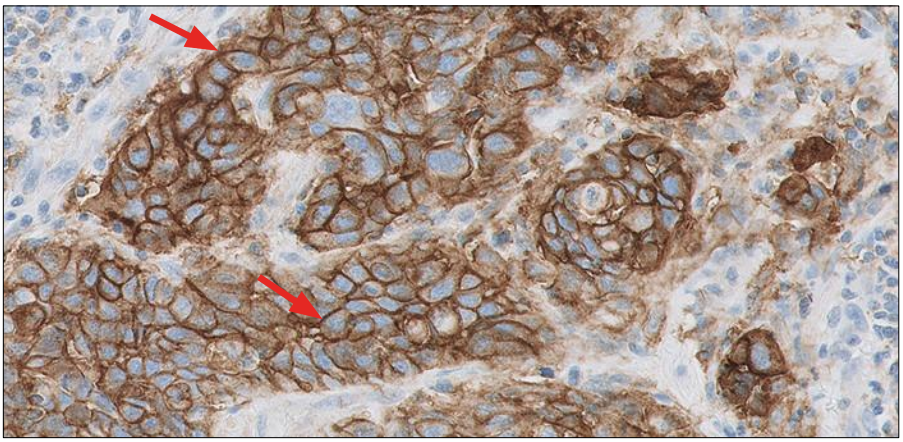


Figure 18c: ESCC specimen stained with PD-L1 primary antibody exhibiting 3+ linear membrane staining of tumor cells (arrows) (20× magnification).

Key Point

Perceptible and convincing linear membrane staining of tumor cells at any intensity should be included in the CPS numerator

Partial Linear Membrane Staining

Tumor cells can exhibit partial linear membrane staining. At a 20× magnification, any partial linear membrane staining observed at any intensity must be included in the CPS numerator.

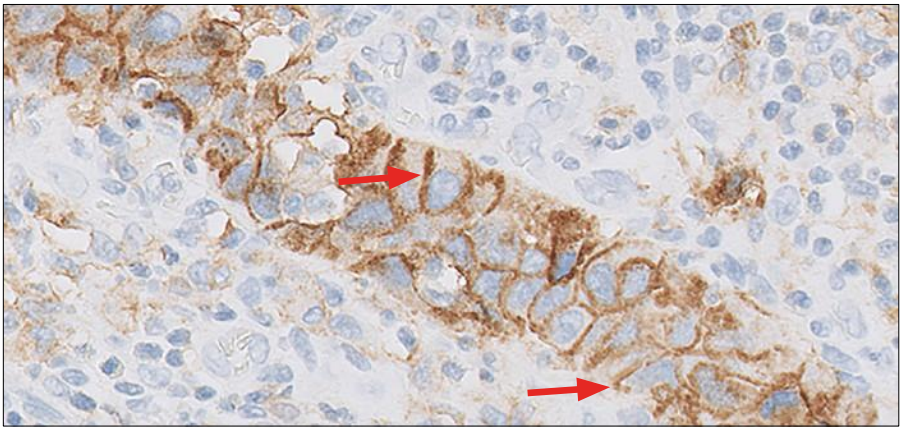


Figure 19: ESCC specimen stained with PD-L1 primary antibody exhibiting partial linear membrane staining of tumor cells (arrows) (20× magnification).

Key Point

Perceptible and convincing partial linear membrane staining of tumor cells should be included in the CPS numerator

Linear Membrane and Cytoplasmic Staining

Tumor cells with both perceptible and convincing linear membrane staining (≥ 1+ intensity) and cytoplasmic staining at 20× magnification should be included in the CPS numerator. Tumor cells exhibiting only cytoplasmic staining are excluded from the CPS numerator, as this is considered non-specific staining. Additionally, linear PD-L1 staining of tumor cells can be smooth or granular. If partial or complete linear membrane staining is distinct from cytoplasmic staining, then the cell should be included in the CPS numerator.

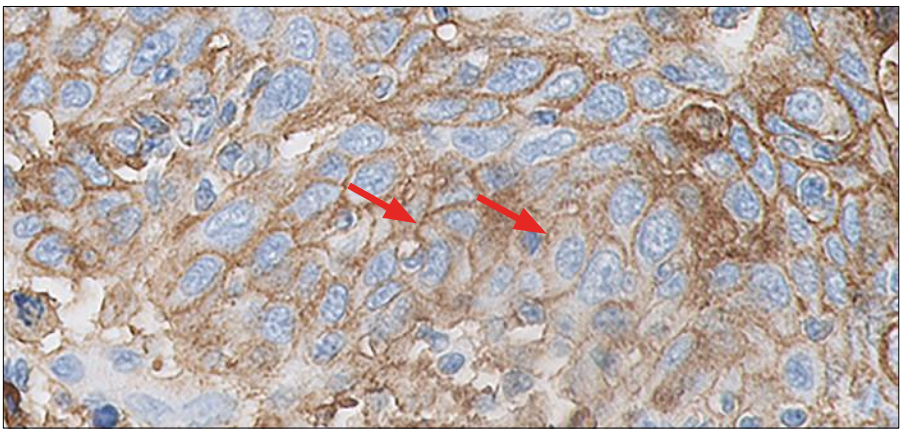


Figure 20: ESCC specimen stained with PD-L1 primary antibody exhibiting linear membrane staining distinct from cytoplasmic staining (arrows) (20× magnification).

Key Point

Tumor cells exhibiting perceptible and convincing linear membrane staining that is distinct from cytoplasmic staining are included in the CPS numerator

Granular Staining

Tumor cells can exhibit a granular membrane staining pattern where membrane and cytoplasmic staining are indistinguishable. Only perceptible and convincing membrane staining of tumor cells (≥ 1+ intensity) observed at no higher than 20× magnification should be included in the CPS numerator.

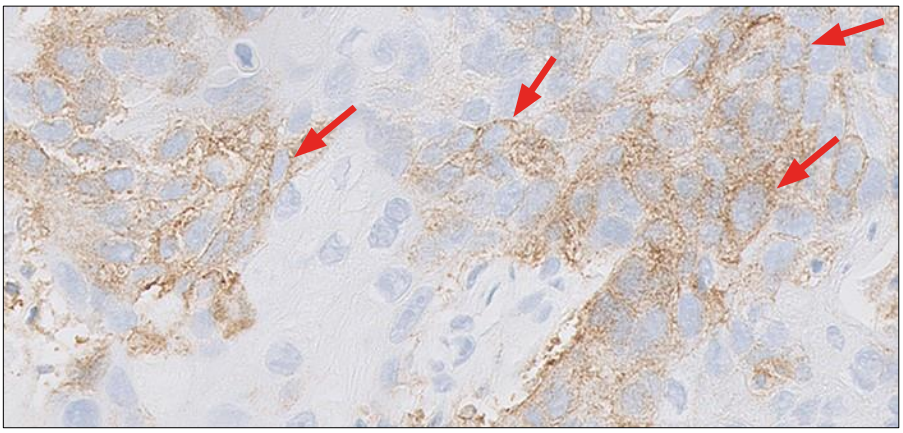


Figure 21: ESCC specimen stained with PD-L1 primary antibody exhibiting granular linear membrane staining pattern (arrows) (20× magnification).

Key Point

Granular staining of tumor cells must exhibit a perceptible and convincing linear membrane pattern to be included in the CPS numerator

Multinucleate Tumor Cells

Some tumor cells in ESCC may be multinucleate and each multinucleate tumor cell should be counted as one cell. The same rules should apply for inclusion in the numerator and denominator: all viable tumor cells should be included in the denominator and all tumor cells with partial or complete linear membrane staining should be included in the numerator.

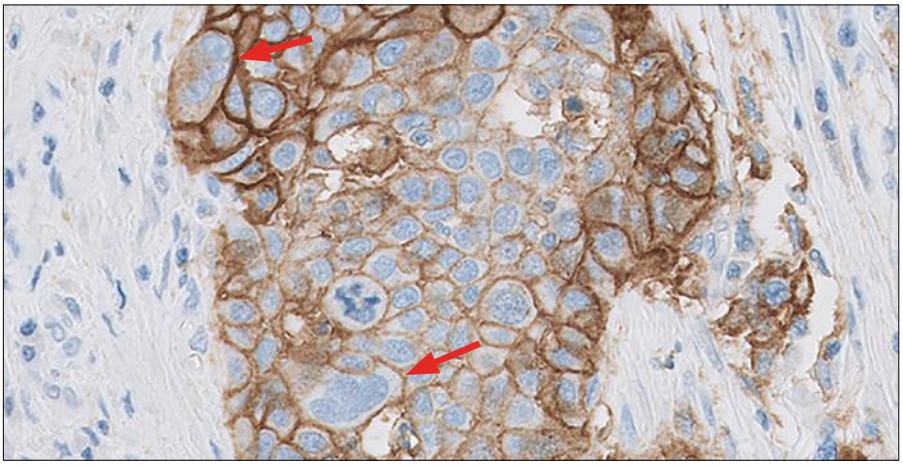


Figure 22: Multinucleate tumor cells (arrows) (20× magnification).

Key Point

Multinucleate tumor cells can be seen in ESCC and follow the same criteria for inclusion/exclusion as mononucleate tumor cells

Immune Cells

Tumor-associated Mononuclear Inflammatory Cells (MICs)

Tumor-associated lymphocytes and macrophages (mononuclear inflammatory cells, MICs) exhibiting membrane and/or cytoplasmic staining at a 20× magnification (≥ 1+ intensity) are considered PD-L1 staining cells and should be included in the CPS numerator. Tumor-associated MICs are present within the tumor nests and/or adjacent supporting stroma and are directly associated with the response against the tumor.

Staining of tumor-associated lymphocytes and macrophages (membrane and/or cytoplasmic) is often heterogeneous, with various staining intensities present.

Note: PD-L1 staining lymphocytes often have indistinguishable membrane and cytoplasmic staining due to a high nuclear to cytoplasmic ratio; PD-L1 staining macrophages often have distinct membrane staining and low cytoplasmic staining. All PD-L1 staining tumor-associated MICs should be included in the CPS numerator.

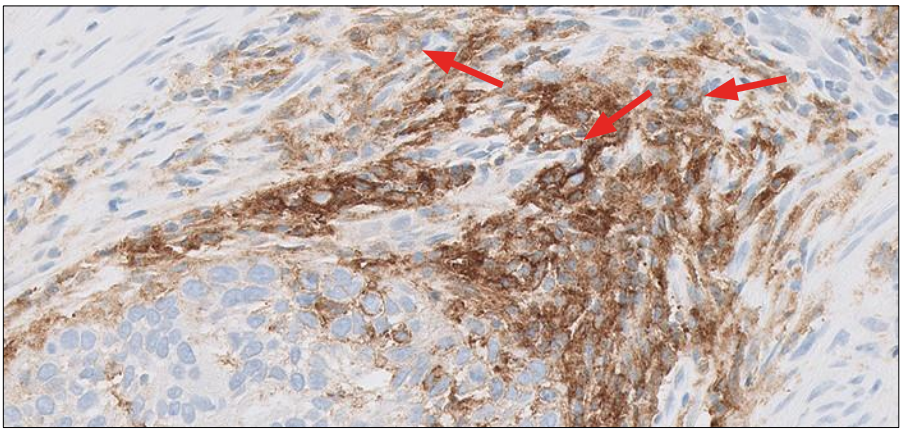


Figure 23a: ESCC specimen stained with PD-L1 primary antibody exhibiting staining of tumor-associated lymphocytes (arrows) (20× magnification).

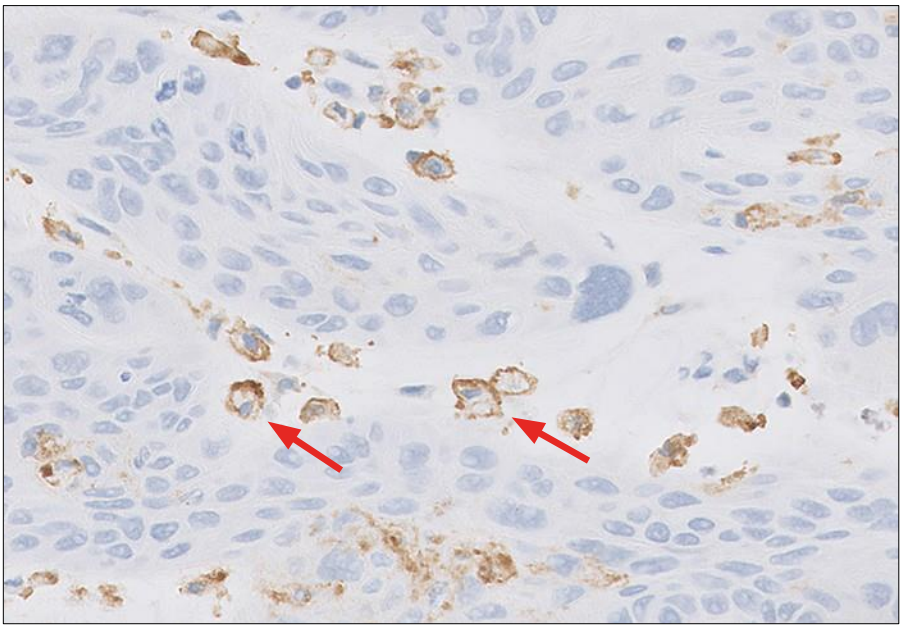


Figure 23b: ESCC specimen stained with PD-L1 primary antibody exhibiting staining of tumor-associated macrophages (arrows) (20× magnification).

Multinucleate Giant Cells

Multinucleate giant cells can be seen in ESCC and, if PD-L1 staining is present on these cells, each multinucleate giant cell should be counted as one cell and included in the numerator.

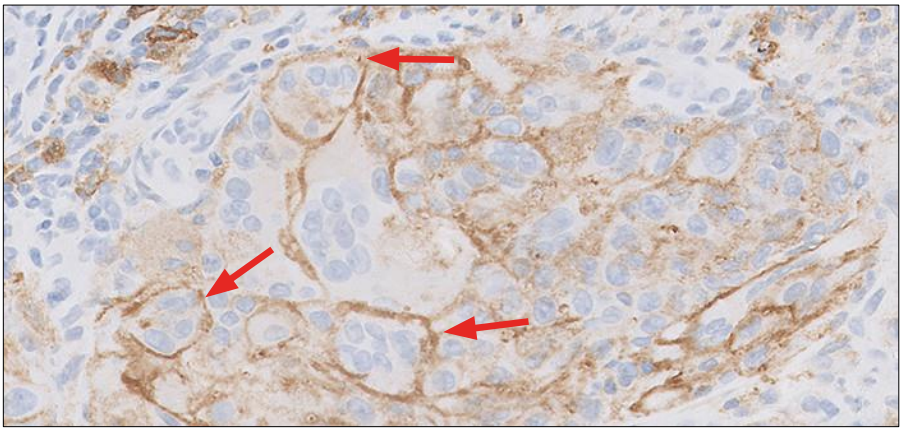


Figure 24: ESCC specimen stained with PD-L1 primary antibody exhibiting staining of multinucleate giant cells (arrows) (20× magnification).

Key Point

Tumor-associated lymphocytes and macrophages with membrane and/or cytoplasmic staining should be included in the CPS numerator

Indistinguishable Tumor and Immune Cells

Tumor cells and tumor-associated lymphocytes and macrophages may be indistinguishable from each other when examining the slide with PD-L1 antibody staining due to small tumor cell size and staining characteristics. It is recommended to use the corresponding H&E slide to distinguish cell morphology. This is especially important when determining the denominator.

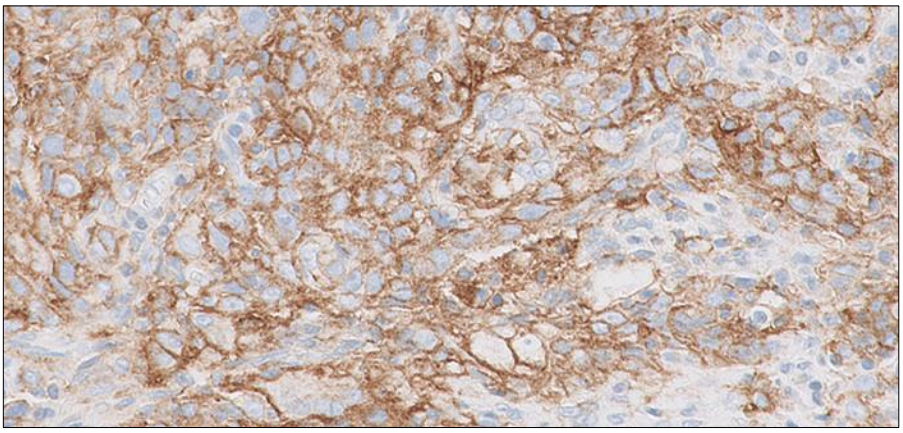


Figure 25a: Tumor and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other and exhibit PD-L1 primary antibody staining (20× magnification).

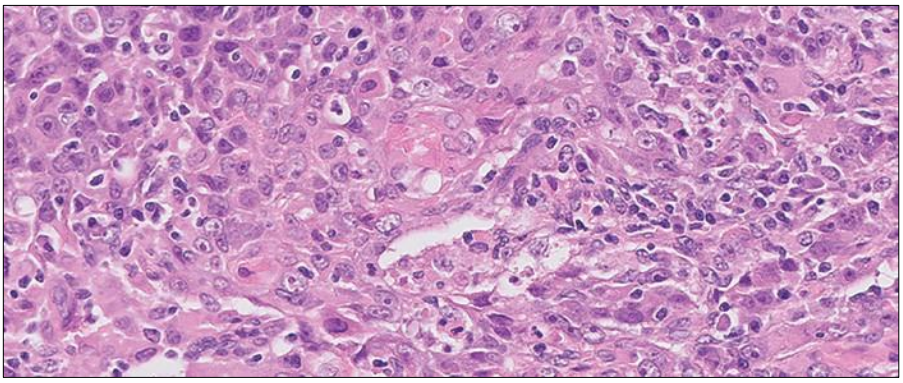


Figure 25b: Corresponding H&E to reference when tumor and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other (20× magnification).

Key Point

Utilize the H&E slide when it is challenging to distinguish tumor cells from immune cells

Immune Cell Inclusion/Exclusion: 20× Rule

PD-L1 staining mononuclear inflammatory cells (MICs) must be directly associated with the response against the tumor to be included in the CPS numerator. MICs are considered tumor-associated if they are present within the tumor nests and/or adjacent supporting stroma within a 20× magnification field of view. In cases where it is difficult to tell if MICs are tumor-associated, the following is suggested as a guideline:

Move the slide so that the tumor is in the approximate center of a 20× field. Immune cells surrounding the tumor in this field should be included in scoring. Immune cells outside of this field should be excluded from scoring as long as they do not surround neighboring tumor cells. In general, include PD-L1 staining MICs that are within 0.5 mm of the tumor cells. This rule may be applied to tumors within lymph nodes that contain PD-L1 staining MICs. See Figures 26a–26c for an example of determining which MICs are included in the CPS numerator.

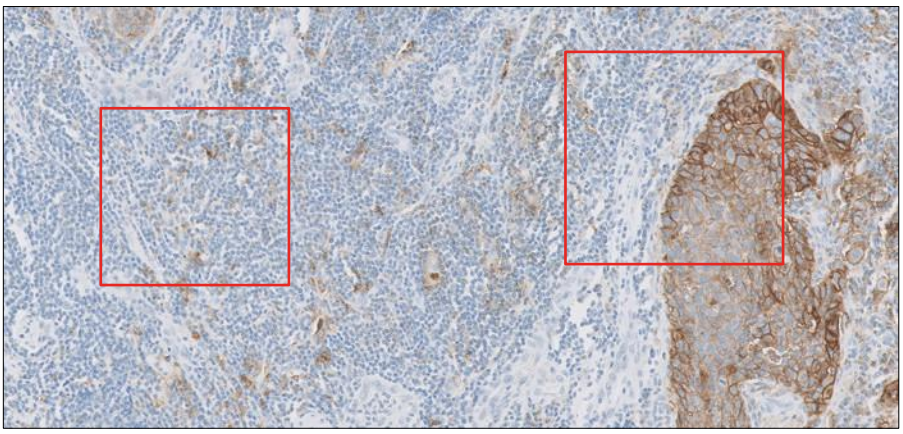


Figure 26a: At 5× magnification, several areas of PD-L1 staining mononuclear inflammatory cells are visible. To demonstrate which immune cells to include in the numerator, zoom in to 20× magnification on the boxed fields (5× magnification).

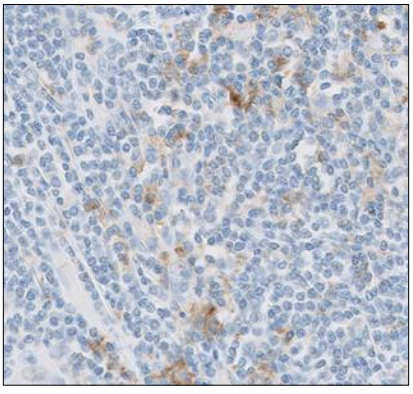


Figure 26b: Tumor cells are absent from this 20× field containing PD-L1 staining mononuclear inflammatory cells, thus none of these cells should be included in the numerator (20× magnification).

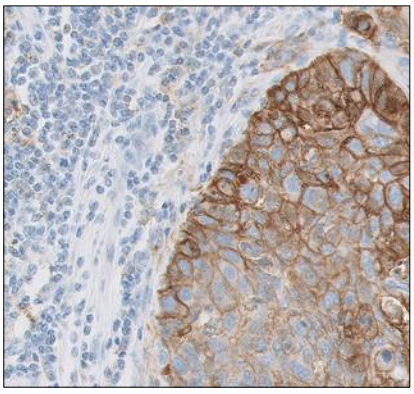


Figure 26c: When positioning the tumor cells in the approximate center of a 20× field, PD-L1 staining mononuclear inflammatory cells that are present within the same field should be included in the numerator (20× magnification).

Tumor Cell Size

ESCC includes different morphologies and tumor cell sizes that can impact the Combined Positive Score (CPS) by increasing or decreasing the total number of tumor cells that are included in the denominator. Well-differentiated squamous cell carcinoma may exhibit larger tumor cells with abundant keratinous cytoplasm, and will commonly have fewer cells per 20× field. Alternatively, a poorly-differentiated, basaloid pattern will commonly have a higher number of tumor cells per 20× field due to the smaller size and scant cytoplasm of the tumor cells. The more tumor cells included in the denominator, the greater the number of PD-L1 staining tumor cells, lymphocytes, and macrophages that
are needed in the numerator to bring the overall score to CPS 10 or above. As a guideline, if tumor cells are 20 µm in diameter and fill a 20× field, there would be approximately 2500 tumor cells in that field.

Small Cell Size

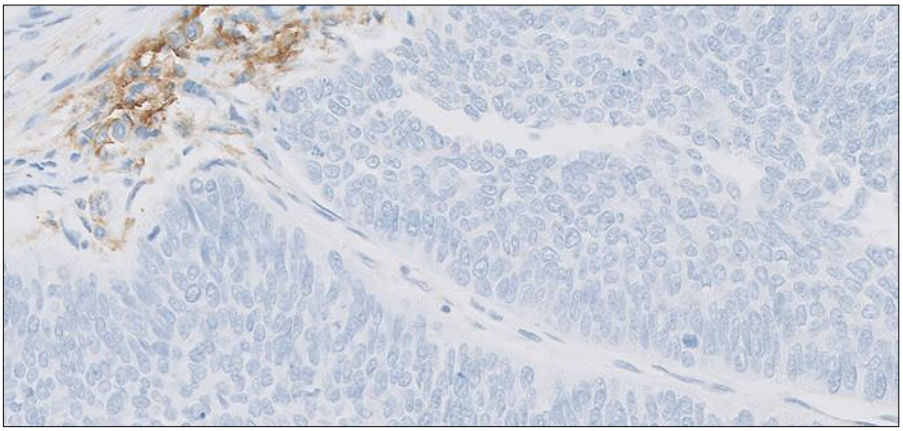


Figure 27: ESCC specimen with small tumor cells (20× magnification).

Medium Cell Size

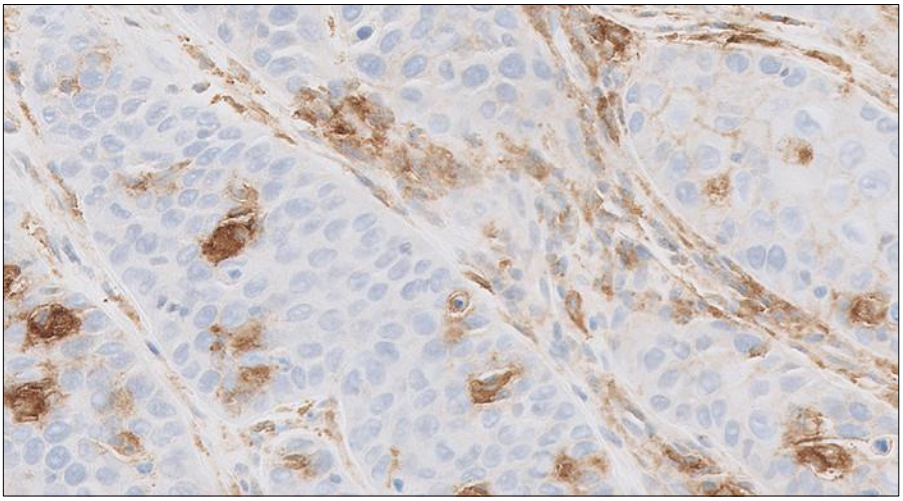


Figure 28: ESCC specimen with medium tumor cells (20× magnification).

Large Cell Size

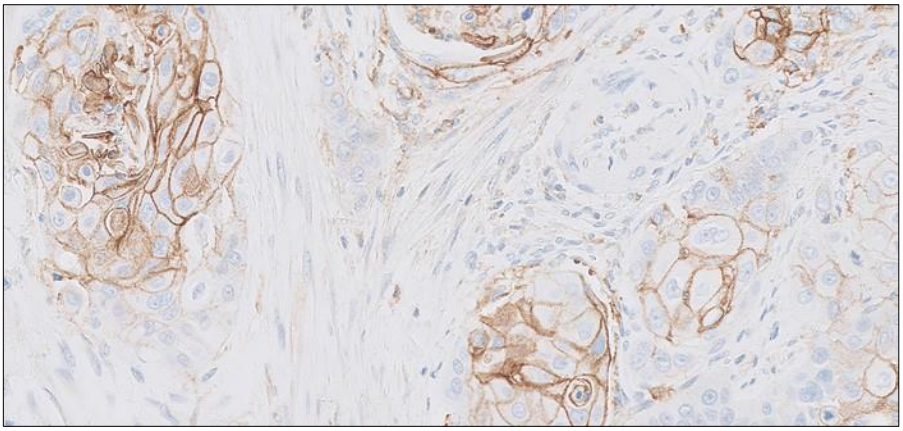


Figure 29: ESCC specimen with large tumor cells (20× magnification).

Key Point

The size of tumor cells can impact the CPS by increasing or decreasing the total number of tumor cells in the denominator

Cells Excluded from CPS

Only tumor cells exhibiting PD-L1 membrane staining and MICs exhibiting PD-L1 membrane and/or cytoplasmic staining should be included in the CPS numerator. Below are cells that can exhibit staining but should be excluded from the CPS calculation (CPS numerator and/or denominator).

Note: Images that follow represent the most common exclusion elements, therefore not all exclusions are represented by images in this manual. Please refer to Tables 1 and 2 on page 26 to view all exclusion criteria.

Tumor Cells with Only Cytoplasmic Staining

Tumor cells exhibiting only cytoplasmic staining are excluded from the CPS numerator. They should, however, still be included in the CPS denominator.

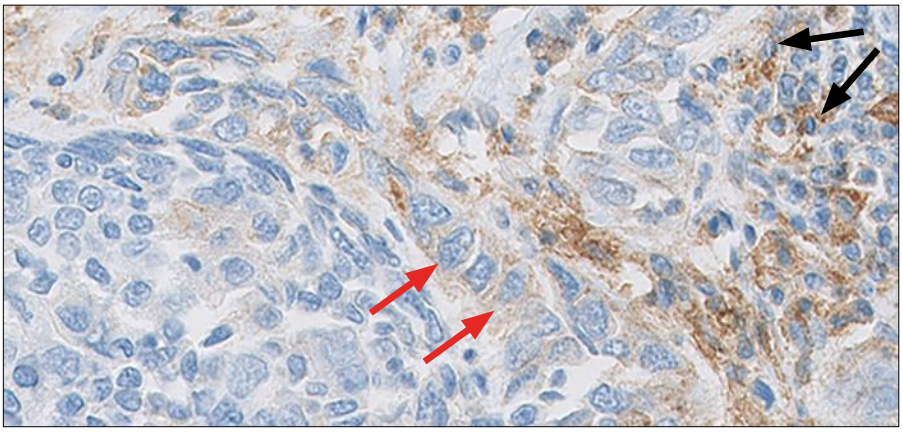


Figure 30: ESCC specimen stained with PD-L1 primary antibody exhibiting only cytoplasmic staining of tumor cells (red arrows) (20× magnification). **Note:** Tumor-associated mononuclear inflammatory cells in the upper right corner exhibit cytoplasmic PD-L1 staining and should be included in the numerator (black arrows).

Key Point

Tumor cells exhibiting only cytoplasmic staining should not be included in the CPS numerator

Benign Cells

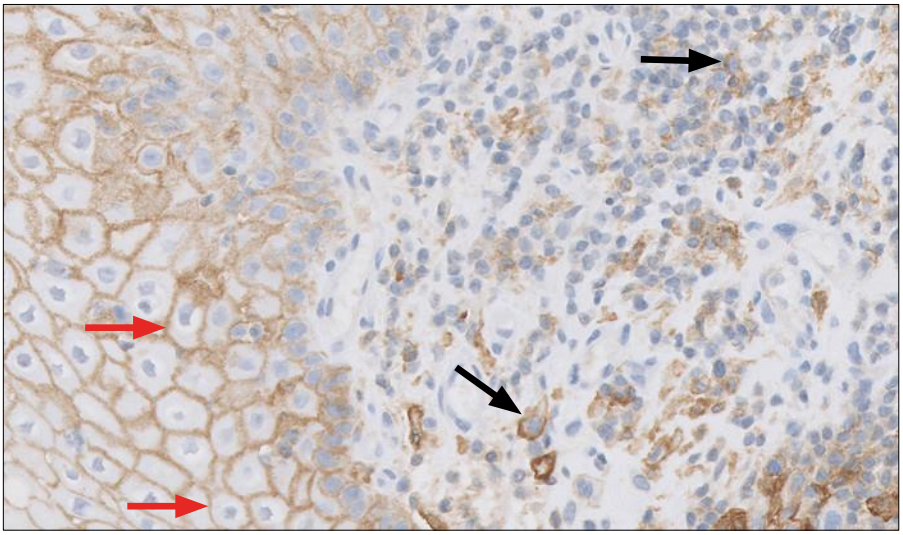


Figure 31: ESCC specimen stained with PD-L1 primary antibody exhibiting staining of benign epithelial cells (red arrows) and associated mononuclear inflammatory cells (black arrows), both of which should be excluded from the score (20× magnification).

Key Point

Benign cells and MICs associated with the benign component may exhibit PD-L1 staining and should be excluded from the score

Carcinoma In Situ (CIS)

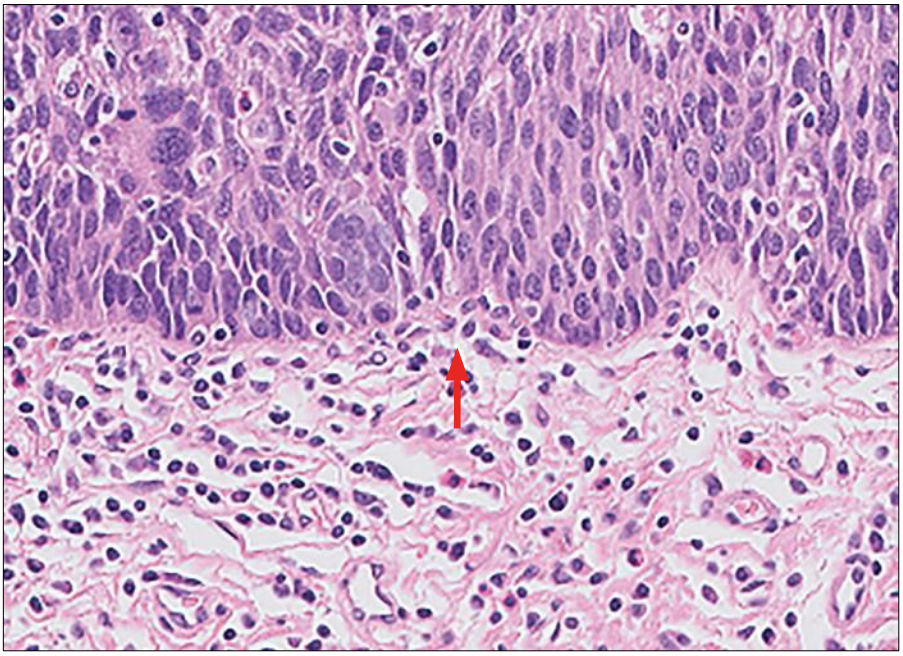


Figure 32a: Hematoxylin and eosin (H&E) section demonstrating ESCC in situ (CIS) (arrow) (10× magnification).

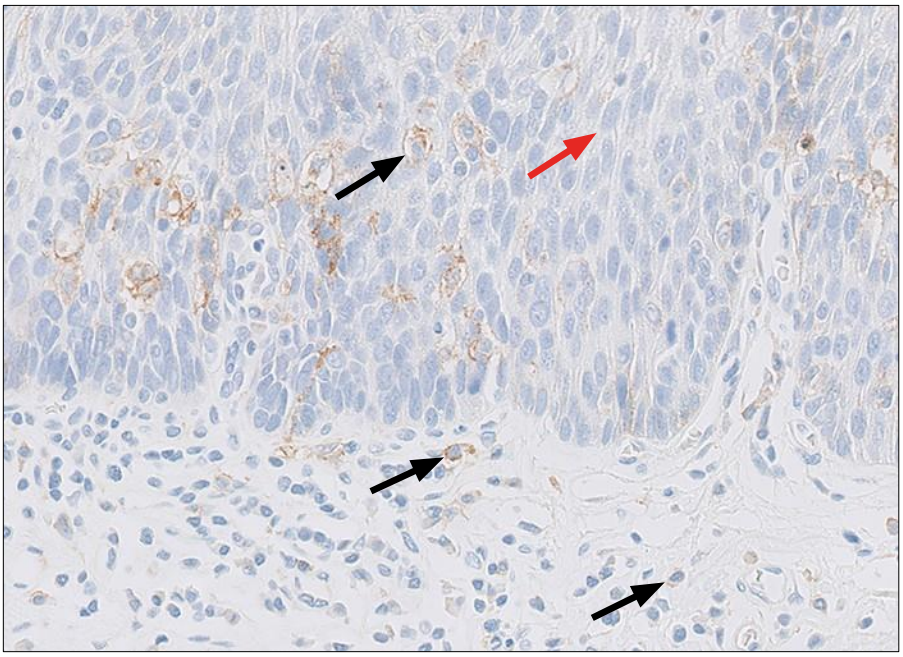


Figure 32b: Any tumor cells that are part of the CIS component should be excluded from the numerator and denominator (red arrow). Any mononuclear inflammatory cells (MICs) (black arrows) associated with the CIS component should be excluded from the numerator (10× magnification).

Key Point

Any tumor cells and MICs associated with the CIS component should be excluded from the score

Stromal Cells

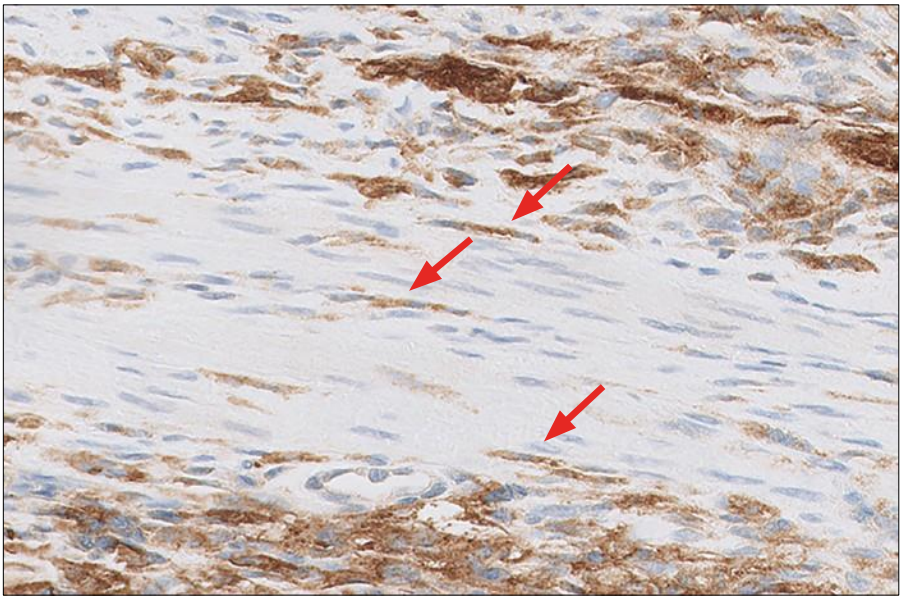


Figure 33: PD-L1 staining on stromal cells (arrows) (20× magnification).

Key Point

Stromal cells exhibiting PD-L1 staining should be excluded from the score

Other Immune Cells Excluded from CPS

Various types of immune cells can exhibit PD-L1 staining, but only tumor-associated lymphocytes and macrophages should be included in the CPS calculation. Refer to page 41 for the immune cell inclusion/exclusion 20× rule. PD-L1 staining neutrophils, eosinophils, and plasma cells should be excluded from the score.

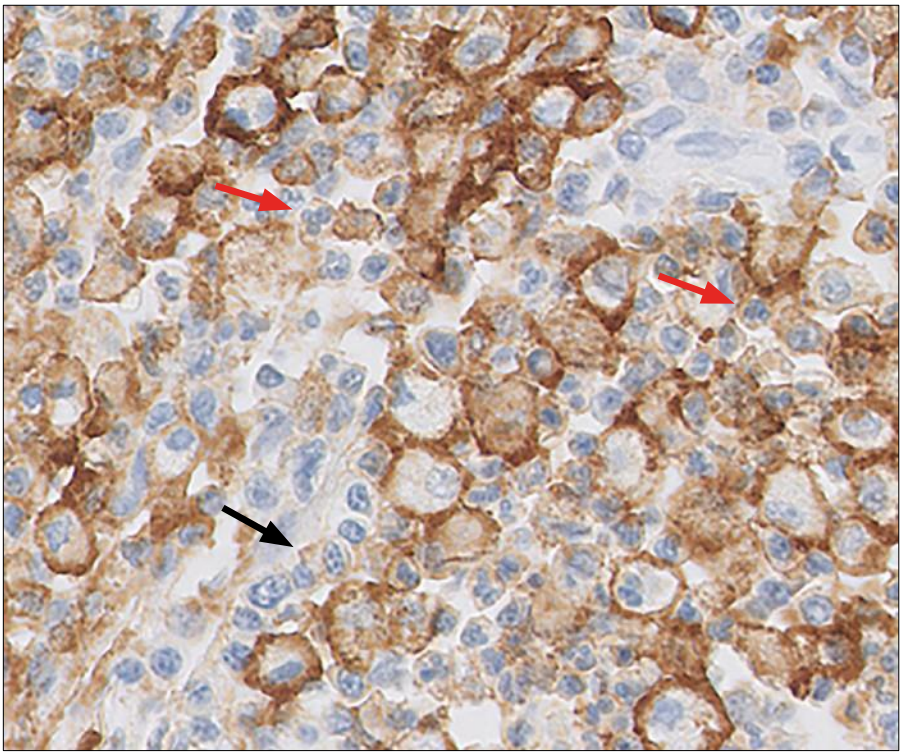


Figure 34: PD-L1 staining on neutrophils (red arrows) and plasma cell (black arrow) (20× magnification). **Note:** Esophageal adenocarcinoma is depicted.

Key Point

PD-L1 staining neutrophils, eosinophils, and plasma cells should be excluded from the score

Artifacts

The following pages provide examples of artifacts you may see when staining with PD-L1 IHC 22C3 pharmDx.

Non-specific Background Staining

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

- Pre-analytic fixation and processing of the specimen
- Incomplete removal of paraffin from the section
- Incomplete rinsing of slides during staining
- Drying of slides; ensure slides remain wet with buffer while loading onto Autostainer Link 48 and prior to initiating run
- Improper deparaffinization procedure
- Incomplete rinsing of reagents from slides

The non-specific background staining of the NCR-stained test section is useful in determining the level of background staining in the PD-L1 stained test section. All specimens must have ≤ 1+ non-specific background staining.

The use of fixatives other than neutral buffered formalin may be a source of background staining and is not recommended. Background staining with PD-L1 IHC 22C3 pharmDx is rare.

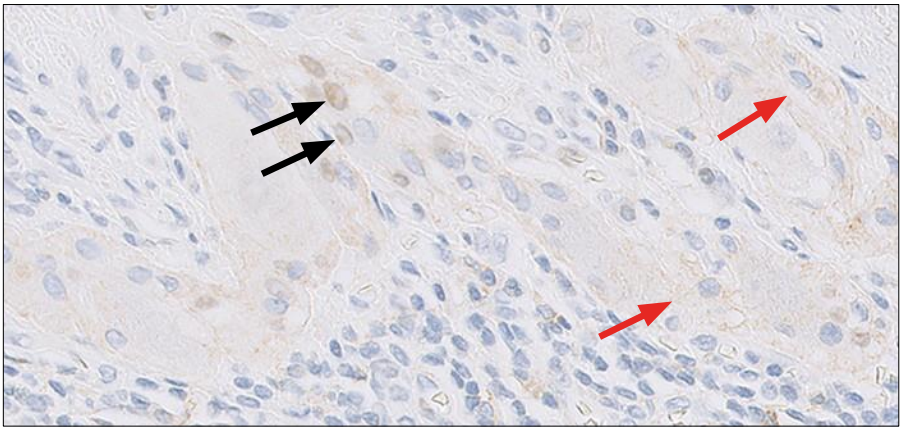


Figure 35a: ESCC specimen stained with PD-L1 primary antibody exhibiting non-specific staining; non-specific background staining (red arrows) should be excluded from the score. Weak nuclear staining is also present and should be ignored (black arrows) (20× magnification).

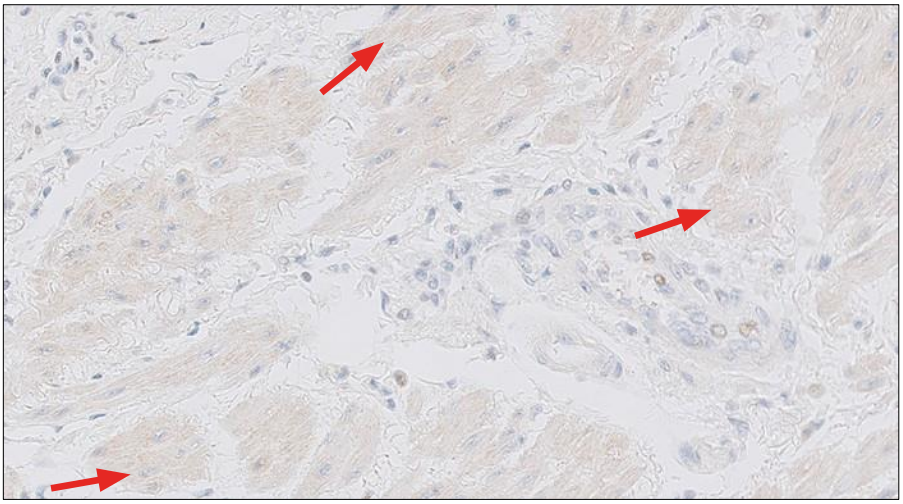


Figure 35b: Negative Control Reagent (NCR) exhibiting non-specific background staining in esophageal adenocarcinoma (arrows) (20× magnification).

Key Point

All specimens must have ≤ 1+ non-specific background staining

Edge Artifact

Commonly, edge artifact is linked to the following pre-analytic factors:

- Thick tissue sections
- Drying of tissue prior to fixation or during staining procedure

Both factors can lead to accentuation of staining at the periphery of the section, and minimal staining or non-staining in the central portion. In this case, only PD-L1 staining at the edge of the tissue section is excluded from scoring.

Note: Although edge artifact can be present, it is not as commonly seen as in other IHC stains.

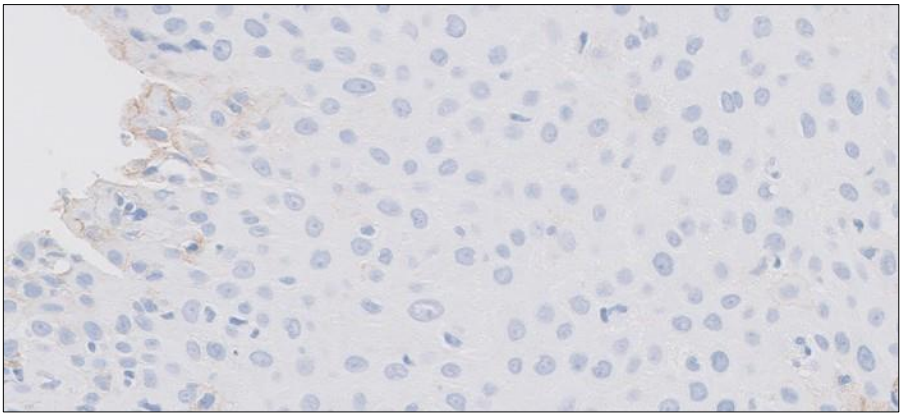


Figure 36: Edge staining should be excluded from the score (20× magnification). **Note:** Squamous cell carcinoma from the cervix is depicted.

Key Point

Scoring of the edge of a specimen should be avoided if staining is inconsistent with the rest of the specimen

Crush Artifact

Areas of the examined section exhibiting cytologically and morphologically distorted secondary crush artifact may show exaggerated staining and should be excluded from the score.

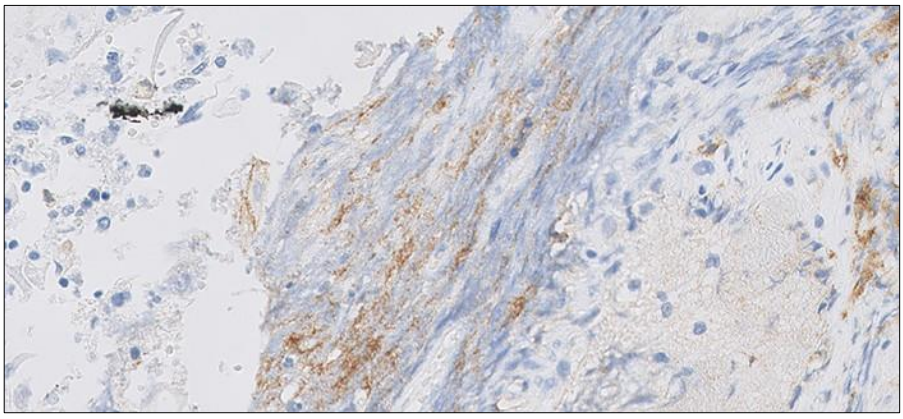


Figure 37: Esophageal adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting crush artifact; crush artifact should be excluded from the score (20× magnification).

Key Point

Scoring of crush artifact should be avoided

Poor Fixation

Standardization of fixation is very important when using PD-L1 IHC 22C3 pharmDx. Suboptimal fixation of tissues may give erroneous results.

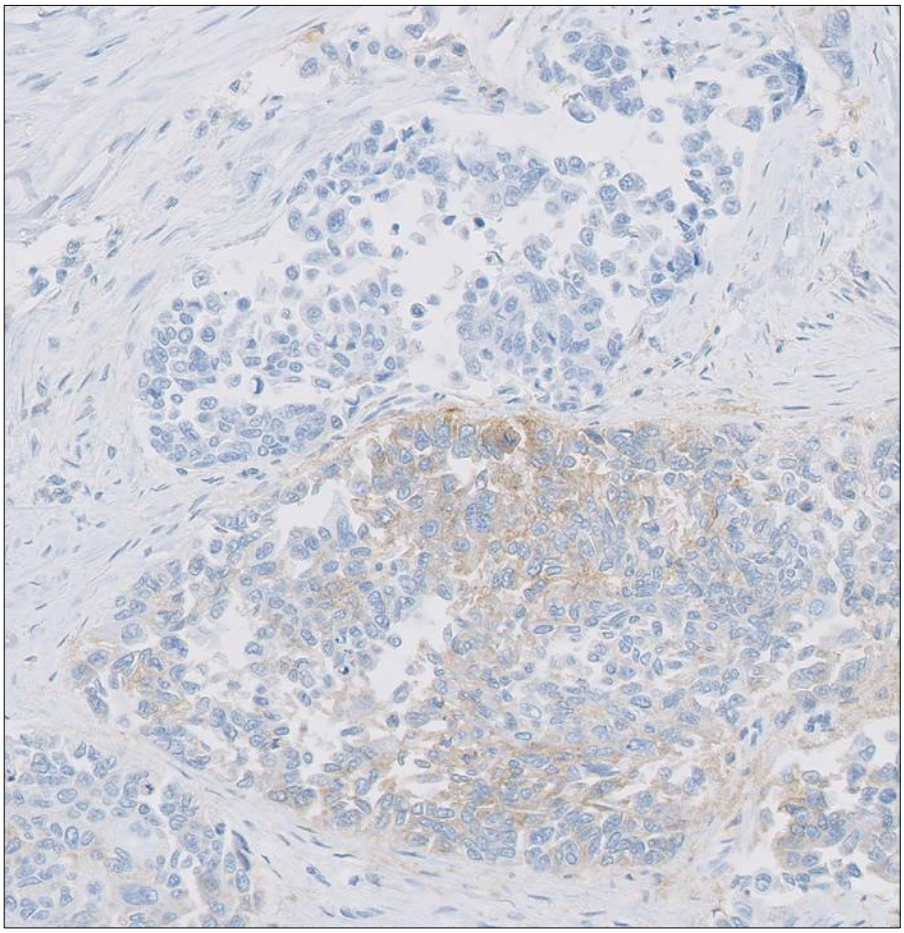


Figure 38: ESCC specimen exhibiting poor tissue fixation (20× magnification).

Key Point

Proper fixation is important for accurate PD-L1 assessment

Necrosis

Necrosis can be described as morphological changes indicative of cell death with undefined cellular detail. PD-L1 staining necrosis is often present in ESCC specimens and should be excluded from the score.

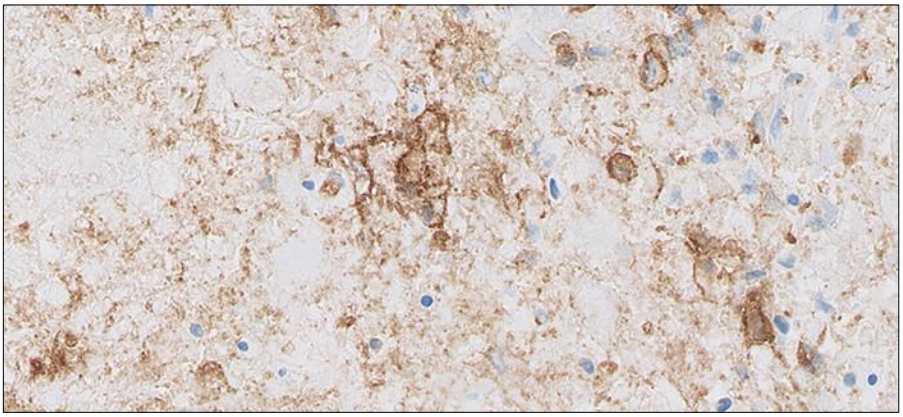


Figure 39: ESCC specimen stained with PD-L1 primary antibody exhibiting staining of necrosis; necrosis staining should be excluded from the score (20× magnification).

Key Point

Scoring of necrotic areas should be excluded from the CPS calculation

PD-L1 IHC 22C3 pharmDx CPS Case Examples

CPS < 10 Case Examples

Case 1: CPS < 10

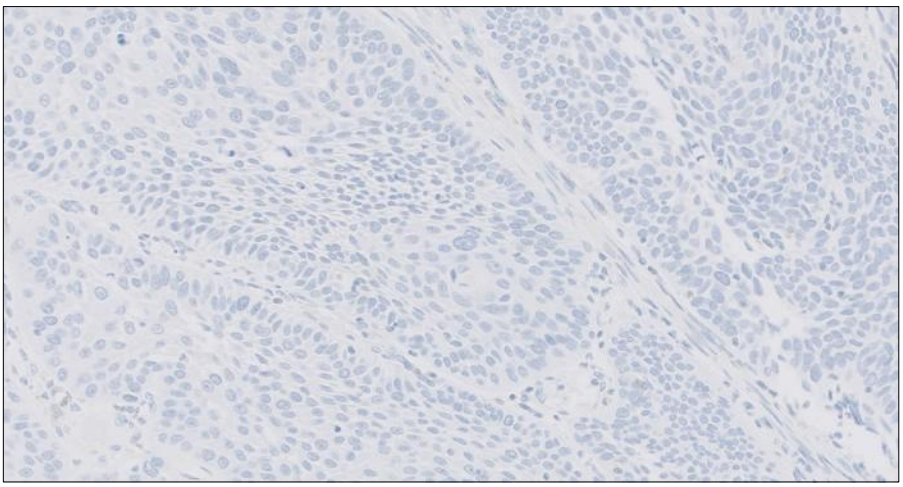


Figure 40: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 0 (20× magnification).

Case 2: CPS < 10

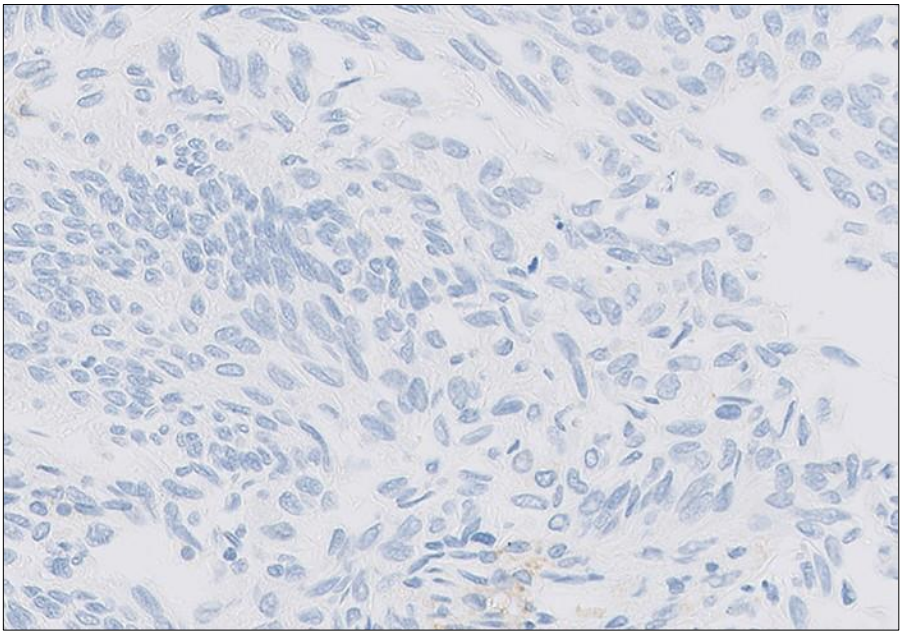


Figure 41: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 0 (20× magnification).

Case 3: CPS < 10

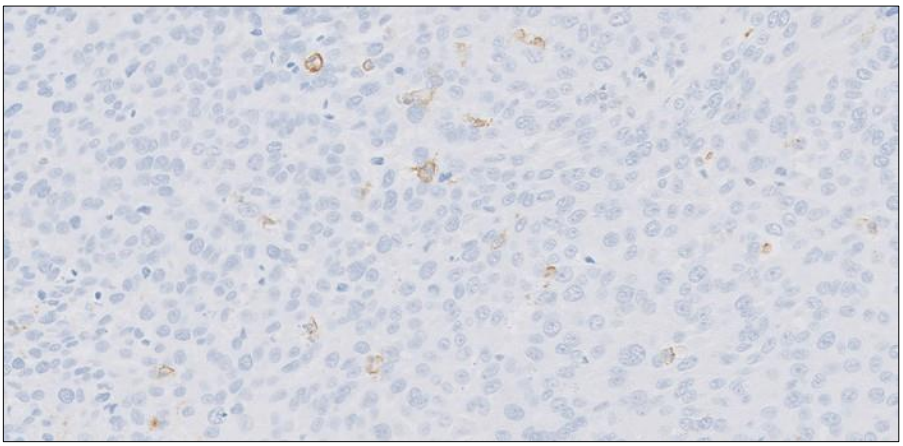


Figure 42: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 3, however any numerical CPS between 2–4 could be assigned to this image (20× magnification).

Case 4: CPS < 10

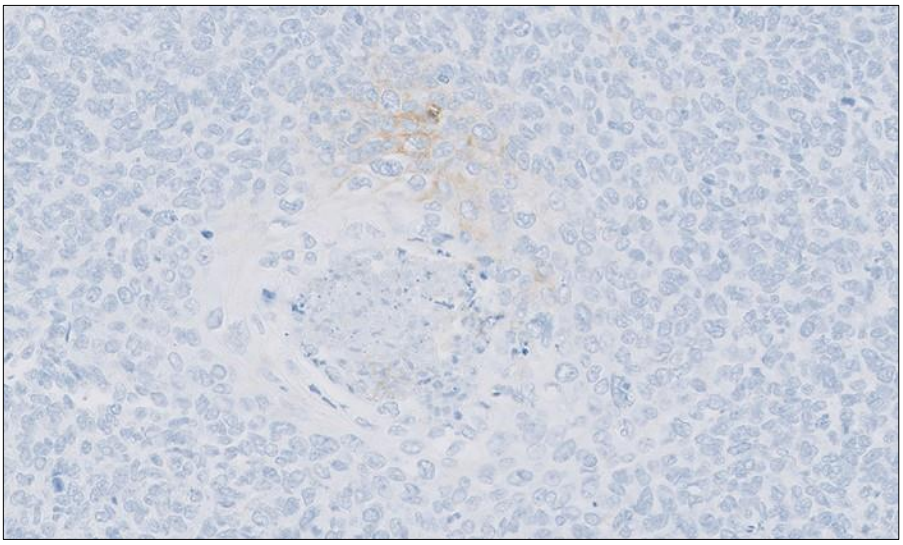


Figure 43: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 4, however any numerical CPS between 2–4 could be assigned to this image (20× magnification).

CPS ≥ 10 Case Examples

Case 5: CPS ≥ 10

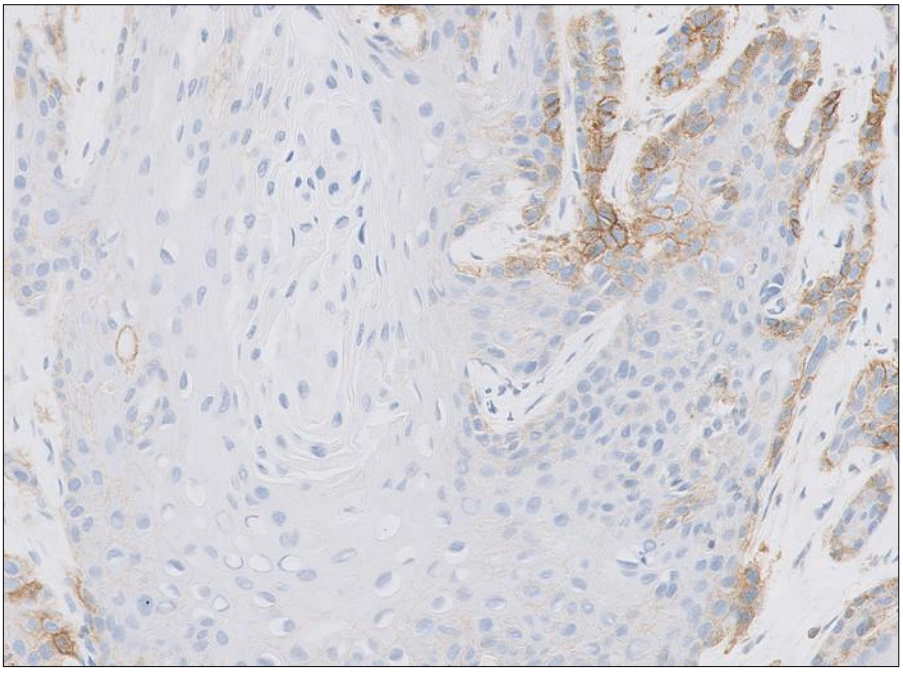


Figure 44: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 23, however any numerical CPS between 20–30 could be assigned to this image (20× magnification).

Case 6: CPS ≥ 10

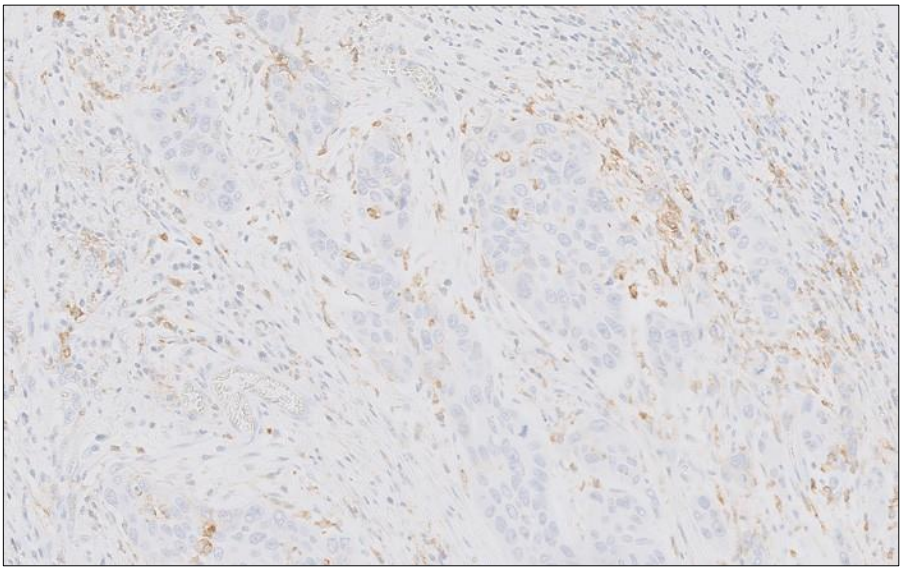


Figure 45: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 40, however any numerical CPS between 35–45 could be assigned to this image (20× magnification).

Case 7: CPS ≥ 10

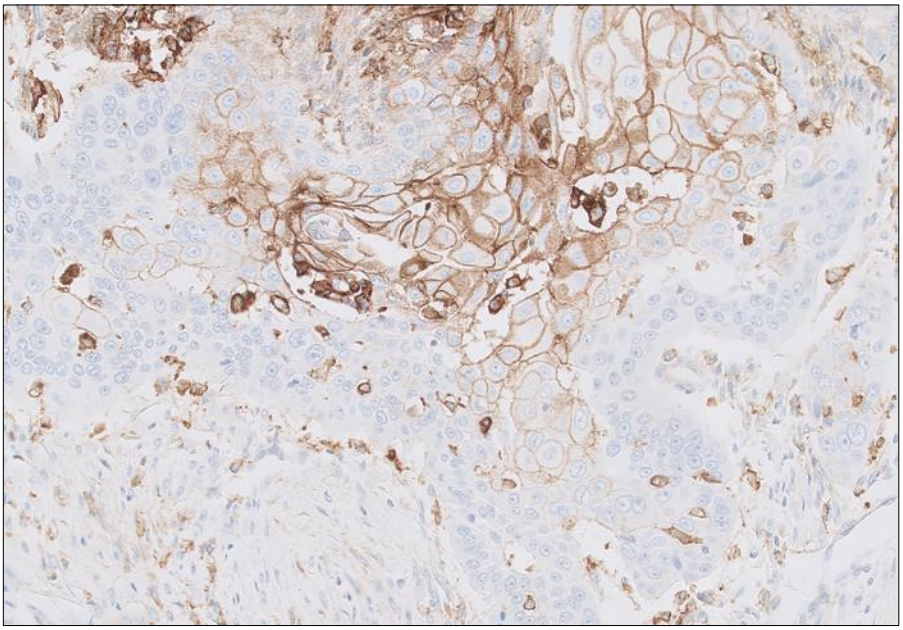


Figure 46: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 45, however any numerical CPS between 40–50 could be assigned to this image (20× magnification).

Case 8: CPS ≥ 10

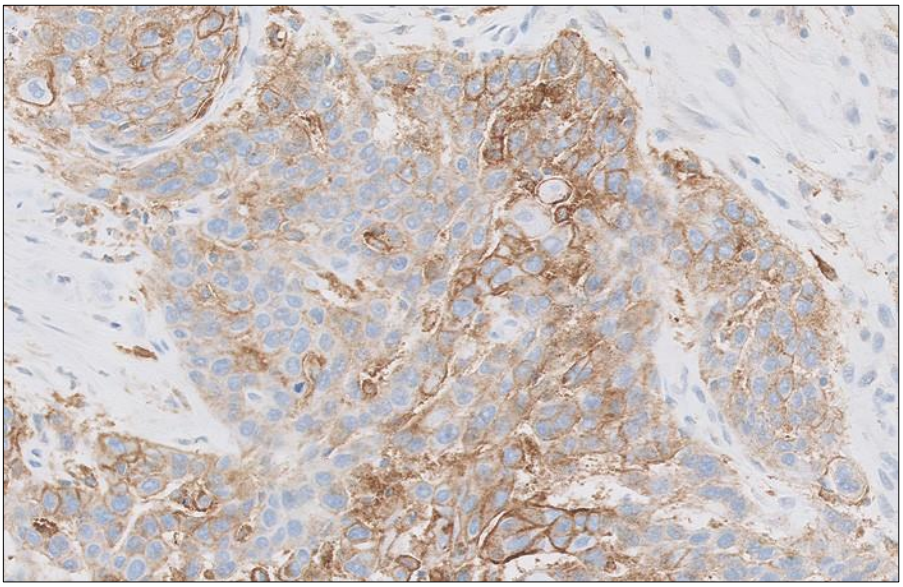


Figure 47: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 72, however any numerical CPS between 70–80 could be assigned to this image (20× magnification).

Near Cut-off Case Examples (CPS Range of Greater Than or Equal to 1 but Less Than 10)

Challenging Case 1: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

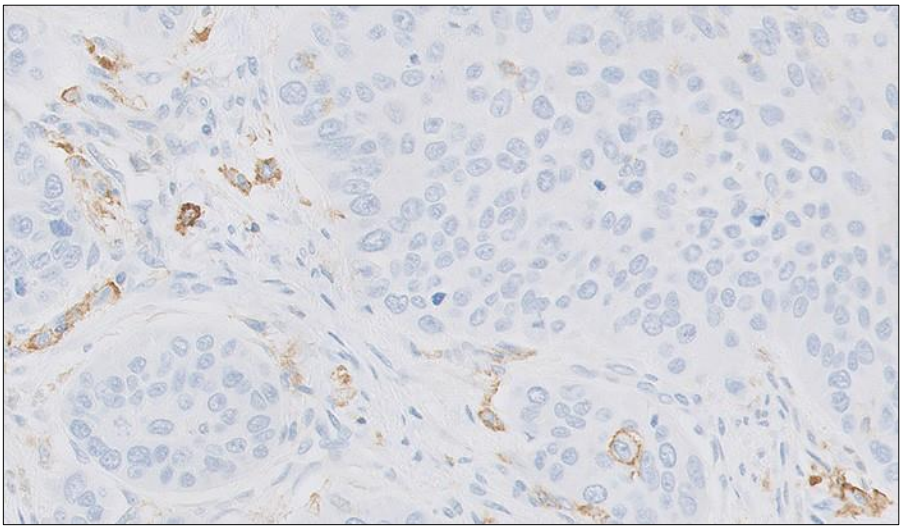


Figure 48: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).

Challenging Case 2: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

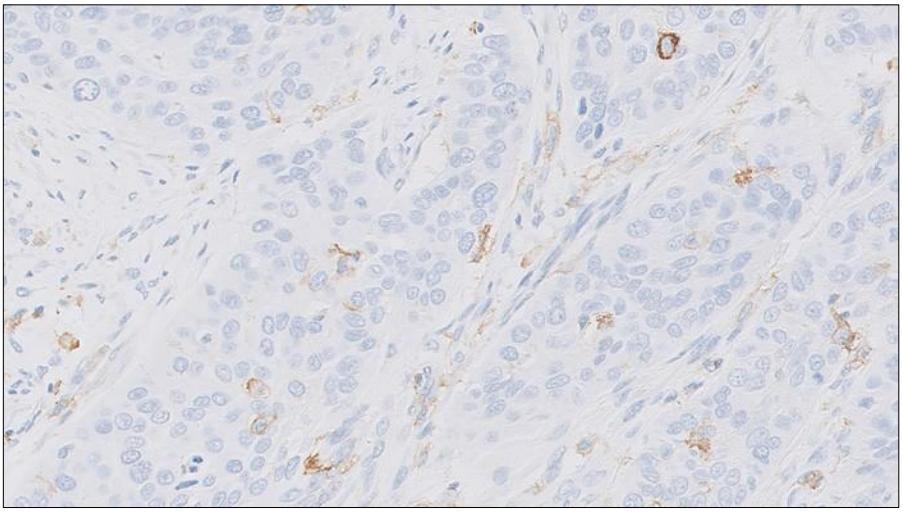


Figure 49: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).

Challenging Case 3: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

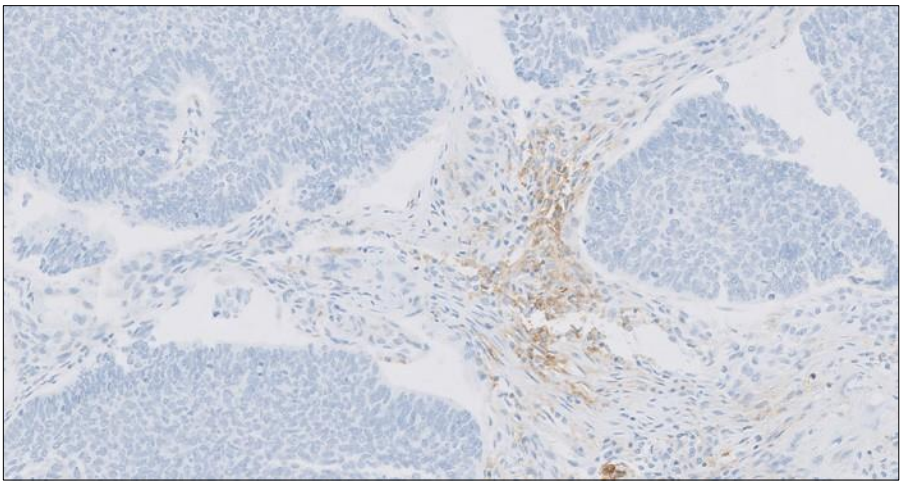


Figure 50: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).

Challenging Case 4: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

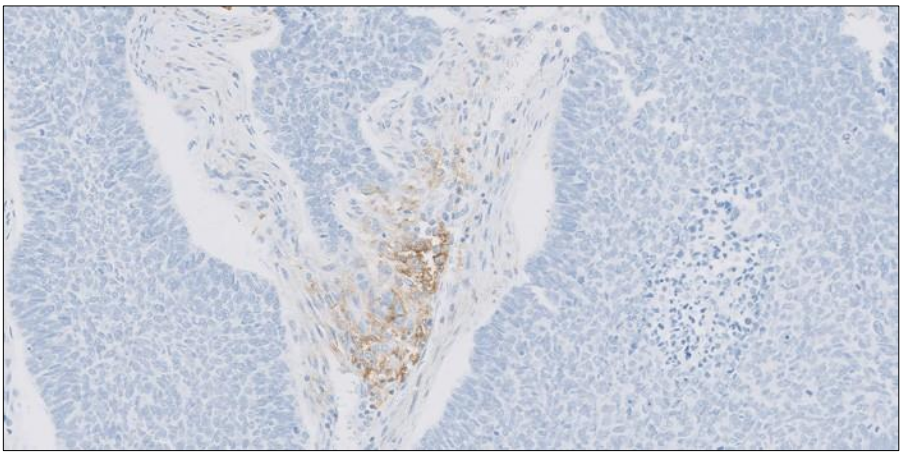


Figure 51: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 4, however any numerical CPS between 2–6 could be assigned to this image (20× magnification).

Challenging Case 5: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

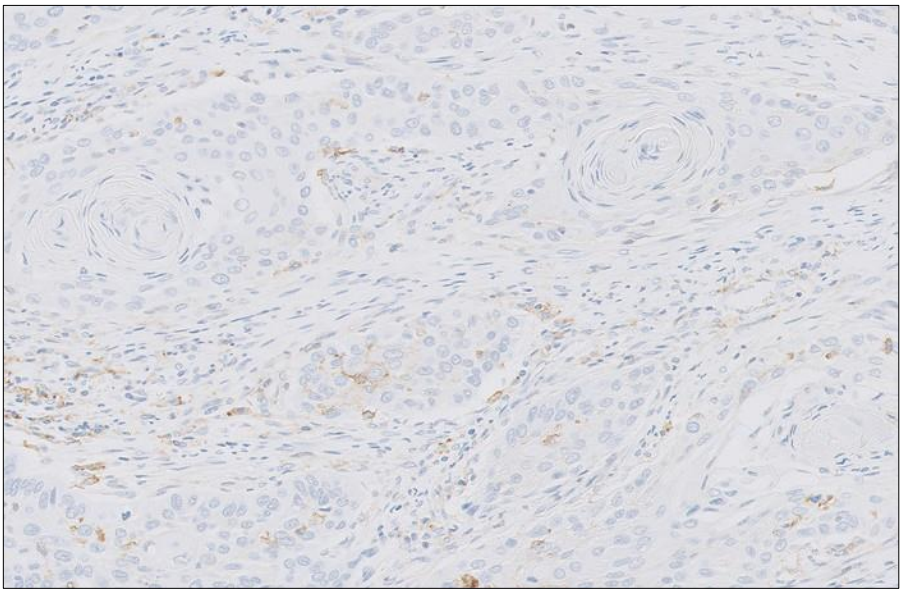


Figure 52: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 8, however any numerical CPS between 6–9 could be assigned to this image (20× magnification).

Challenging Case 6: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

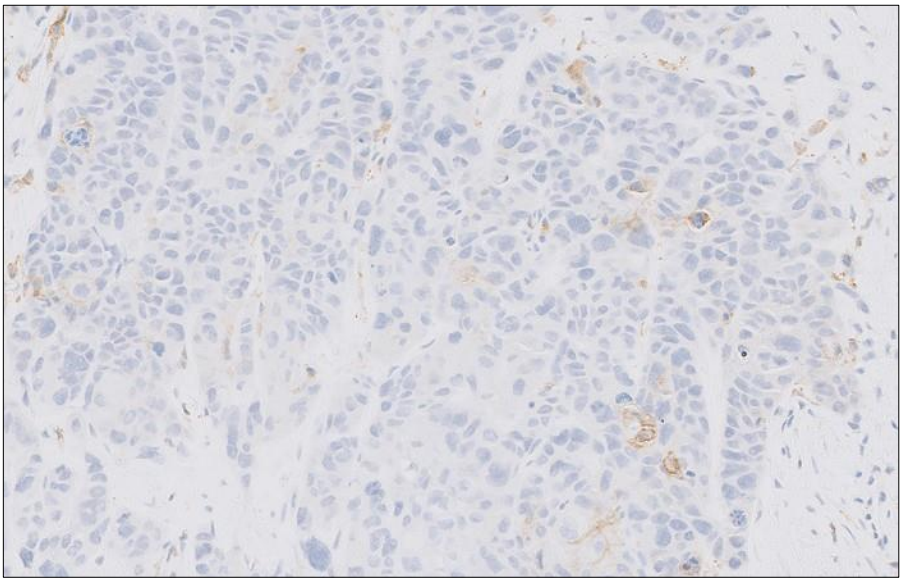


Figure 53: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 5, however any numerical CPS between 3–7 could be assigned to this image (20× magnification).

Near Cut-off Case Examples (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

Challenging Case 7: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

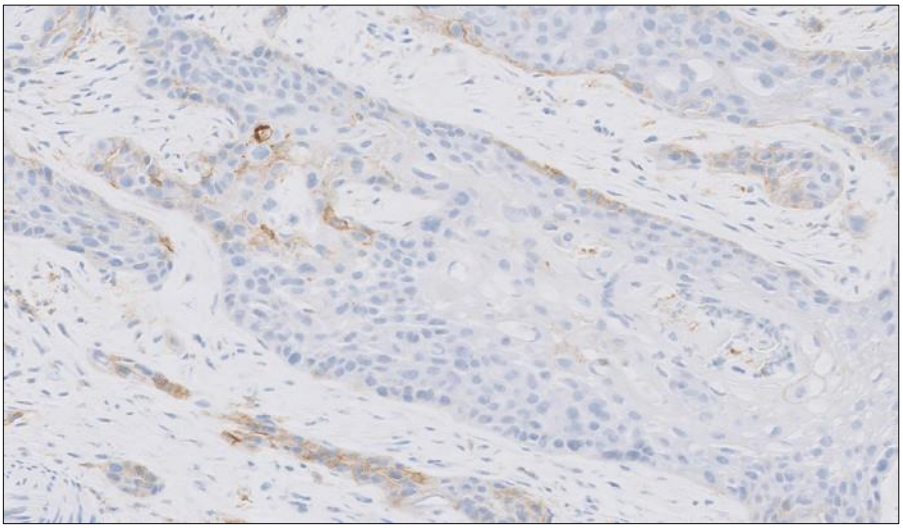


Figure 54: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 14, however any numerical CPS between 12–16 could be assigned to this image (20× magnification).

Challenging Case 8: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

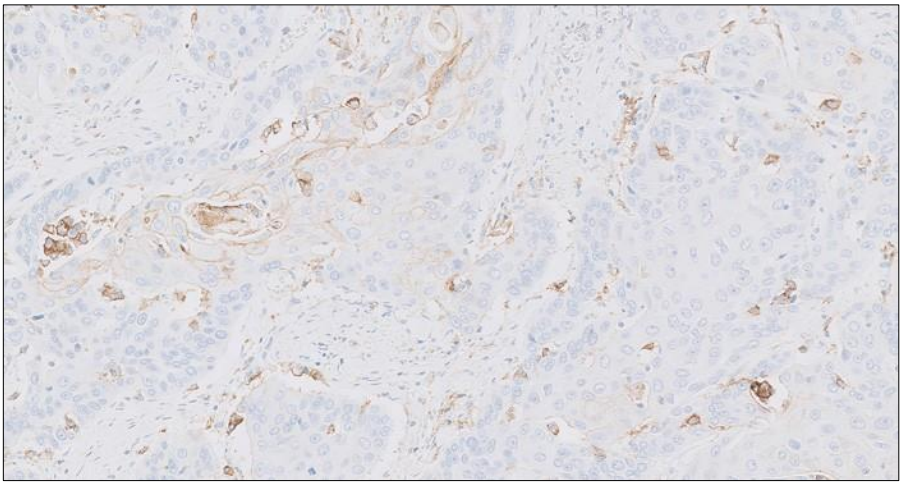


Figure 55: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 13, however any numerical CPS between 10–15 could be assigned to this image (20× magnification).

Challenging Case 9: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

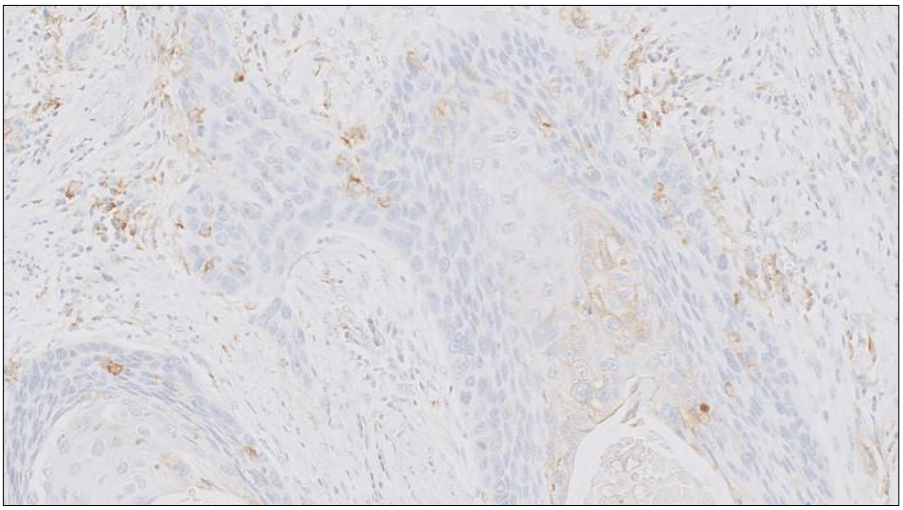


Figure 56: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 16, however any numerical CPS between 13–19 could be assigned to this image (20× magnification).

Challenging Case 10: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

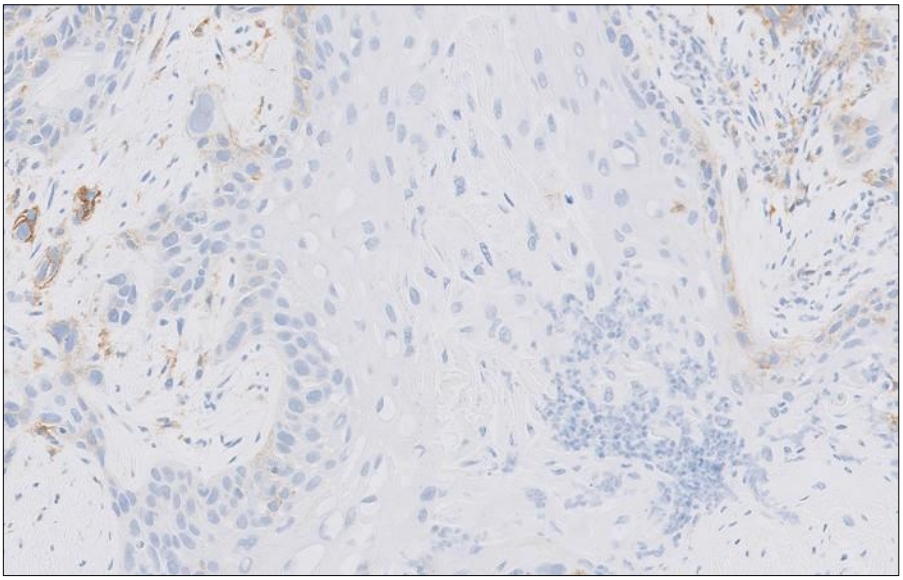


Figure 57: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 16, however any numerical CPS between 13–19 could be assigned to this image (20× magnification).

Troubleshooting Guide

Troubleshooting Guidelines for PD-L1 IHC 22C3 pharmDx

For further troubleshooting help, contact your local Agilent representative.

Problem	Probable Cause	Suggested Action
No staining of slides	Programming error	Verify that the PD-L1 IHC 22C3 pharmDx program was selected for programming of slides
	Lack of reaction with DAB+ Substrate-Chromogen Solution (DAB)	Verify that DAB+ Substrate-Chromogen Solution was prepared properly
	Sodium azide in wash buffer	Use only Dako Wash Buffer (Code K8007)
	Degradation of Control Slide	Check kit expiration date and kit storage conditions on outside of package
Weak staining of specimen slides	Inappropriate fixation method used	Ensure that only neutral buffered formalin fixative and approved fixation methods are used
	Insufficient reagent volume applied	Check size of tissue section and reagent volume applied
	Inappropriate wash buffer used	Use only Dako Wash Buffer, Code K8007
Weak staining of specimen slides or of the positive cell line on the Control Cell Line Slide provided with the kit	Inadequate target retrieval	Verify that the 3-in-1 pre-treatment procedure was correctly performed
	Inappropriate wash buffer used	Use only Dako Wash Buffer, Code K8007
Excessive background staining of slides	Paraffin incomplet ely removed	Verify that the 3-in-1 pre-treatment procedure was correctly performed
	Slides dried while loading onto Autostainer Link 48	Ensure slides remain wet with buffer while loading and prior to initiating run
	Nonspecific binding of reagents to tissue section	Check for proper fixation of the specimen and/or the presence of necrosis
	Inappropriate fixation method used	Ensure that only neutral buffered formalin fixative and recommended fixation methods are used
Tissue detached from slides	Use of incorrect microscope slides	Use Dako FLEX IHC Microscope Slides, (Code K8020), or Superfrost Plus slides
	Inadequate preparation of specimens	Cut sections should be placed in a 58 ± 2 °C oven for 1 hour prior to staining
Excessively strong specific staining	Inappropriate fixation method used	Ensure that only approved fixatives and fixation methods are used
	Inappropriate wash buffer used	Only use Dako Wash Buffer, Code K8007
Target Retrieval Solution is cloudy in appearance when heated	When heated, the Target Retrieval Solution turns cloudy in appearance	This is normal and does not influence staining

Note: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call Agilent Technical Support for further assistance. Additional information on staining techniques and specimen preparation can be found in Dako Education Guide: Immunohistochemical Staining Methods (available from Agilent).

Clinical Performance Evaluation

The efficacy of KEYTRUDA was investigated in KEYNOTE-181 (NCT02564263), a multicenter, randomized, open-label, active-controlled trial that enrolled 628 patients with recurrent locally advanced or metastatic esophageal cancer who progressed on or after one prior line of systemic treatment for advanced disease. Patients with HER2/neu positive esophageal cancer were required to have received treatment with approved HER2/neu targeted therapy. All patients were required to have tumor specimens for PD-L1 testing at a central laboratory; PD-L1 status was determined using the PD-L1 IHC 22C3 pharmDx kit. Patients with a history of non-infectious pneumonitis that required steroids or current pneumonitis, active autoimmune disease, or a medical condition that required immunosuppression were ineligible.

Patients were randomized (1:1) to receive either KEYTRUDA 200 mg every 3 weeks or investigator's choice of any of the following chemotherapy regimens, all given intravenously: paclitaxel 80–100 mg/m^2 on Days 1, 8, and 15 of every 4 week cycle, docetaxel 75 mg/m^2 every 3 weeks, or irinotecan 180 mg/m^2 every 2 weeks. Randomization was stratified by tumor histology (esophageal squamous cell carcinoma [ESCC] vs. esophageal adenocarcinoma [EAC]/ Siewert type I EAC of the gastroesophageal junction [GEJ]), and geographic region (Asia vs. ex-Asia). Treatment with KEYTRUDA or chemotherapy continued until unacceptable toxicity or disease progression. Patients randomized to KEYTRUDA were permitted to continue beyond the first RECIST v1.1 (modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ)-defined disease progression if clinically stable until the first radiographic evidence of disease progression was confirmed at least 4 weeks later with repeat imaging. Patients treated with KEYTRUDA without disease progression could be treated for up to 24 months. Assessment of tumor status was performed every 9 weeks. The major efficacy outcome measure was OS evaluated in the following co-primary populations: patients with ESCC, patients with tumors expressing PD-L1 CPS ≥ 10, and all randomized patients. Additional efficacy outcome measures were PFS, ORR, and DoR, according to RECIST v1.1, modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ, as assessed by BICR.

A total of 628 patients were enrolled and randomized to KEYTRUDA (n=314) or investigator's treatment of choice (n=314). Of these 628 patients, 167 (27%) had ESCC that expressed PD L1 with a CPS ≥ 10. Of these 167 patients, 85 patients were randomized to KEYTRUDA and 82 patients to investigator's treatment of choice [paclitaxel (n=50), docetaxel (n=19), or irinotecan (n=13)]. The baseline characteristics of these 167 patients were: median age of 65 years (range: 33 to 80), 51% age 65 or older; 84% male; 32% White and 68% Asian; 38% had an ECOG PS of 0 and 62% had an ECOG PS of 1. Ninety percent had M1 disease and 10% had M0 disease.

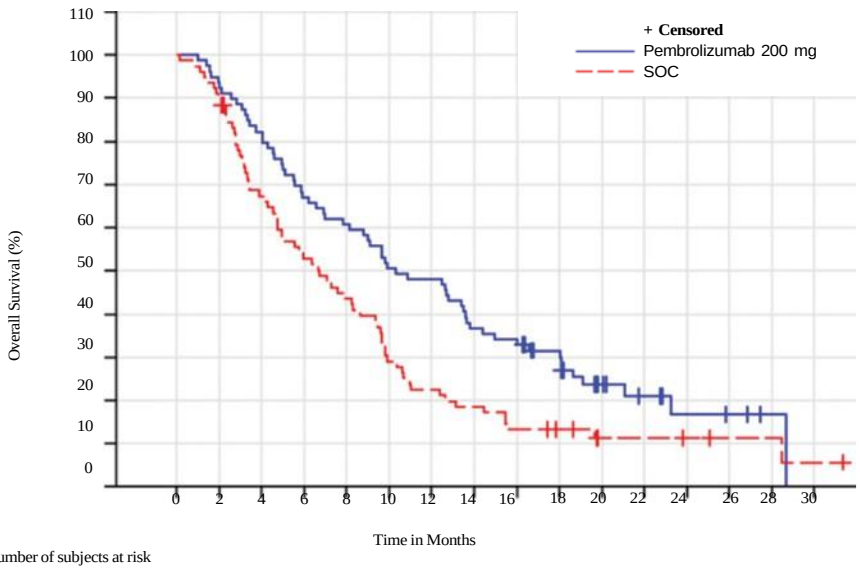
The trial did not meet the pre-specified threshold to demonstrate a statistically significant improvement in OS in any of the three pre-specified co-primary patient populations, with observed hazard ratios of 0.77 (95% CI: 0.63, 0.96) in patients with ESCC, 0.70 (95% CI: 0.52, 0.94) in patients with tumors expressing PD-L1 CPS ≥ 10, and 0.89 (95% CI: 0.75, 1.05) in all randomized patients. In an exploratory analysis conducted in patients whose ESCC tumors expressed PD-L1 (CPS ≥ 10), an improvement in OS was observed among patients randomized to KEYTRUDA as compared with chemotherapy (hazard ratio of 0.64, 95% CI: 0.46, 0.90).

Ten (10) of the 167 ESCC participants with tumors expressing PD-L1 CPS ≥ 10 had specimens stained outside the stability window. These 10 participants have been excluded from the efficacy results summarized in Table 5 and the Kaplan-Meier curve for OS shown in Figure 58. The efficacy results for the population excluding the 10 participants with specimens outside the stability window are consistent with the efficacy conclusions detailed above.

Table 5: Efficacy Results in Patients with Recurrent or Metastatic ESCC (CPS ≥ 10) in KEYNOTE-181 (Excluding 10 Participants with Specimens Outside the Stability Window)

Endpoint	KEYTRUDA 200 mg every 3 weeks n=79	Chemotherapy n=78
OS		
Number (%) of patients with event	62 (78.5)	68 (87.2)
Median in months (95% CI)	10.3 (7.8, 13.6)	6.7 (4.8, 9.3)
Hazard ratio* (95% CI)	0.63 (0.45, 0.90)	
PFS		
Number (%) of patients with event	70 (88.6)	72 (92.3)
Median in months (95% CI)	3.2 (2.1, 4.3)	2.6 (2.1, 3.7)
Median follow-up (95% CI), months	3.2 (2.1, 4.4)	2.8 (2.1, 4.0)
Hazard ratio* (95% CI)	0.67 (0.48, 0.95)	
Objective Response Rate		
ORR (95% CI)	21.5 (13.1, 32.2)	7.7 (2.9, 16.0)
Number (%) of complete responses	3 (3.8)	1 (1.3)
Number (%) of partial responses	14 (17.7)	5 (6.4)
Median duration of response in months (range)	10.3 (2.8, 18.8+)	7.7 (4.3, 16.8+)

** Cox proportional hazards model stratified by geographic region (Asia vs ex-Asia) "+" indicates there is no progressive disease by the time of last disease assessment*



Number of subjects at risk

Pembrolizumab 200 mg :	79	74	65	53	48	40	38	29	27	21	11	7	4	3	1	0
SOC:	78	70	51	40	33	22	17	14	10	8	4	4	3	2	2	1

Figure 58: Kaplan-Meier Curve for Overall Survival in KEYNOTE-181 (ESCC CPS ≥ 10). (Excluding 10 Participants with Specimens Outside the Stability Window)

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- Data on file. Agilent Technologies, Inc.

Notes

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1. PD-L1 IHC 22C3 pharmDx [package insert]. Carpinteria, CA: Dako, Agilent Pathology Solutions; 2019. **2.** Keytruda [package insert]. Kenilworth, NJ: Merck & Co., Inc.; 2019.
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