PD-L1 IHC 22C3 pharmDx Interpretation Manual – Esophageal Cancer

CE-IVD–marked for in vitro diagnostic use
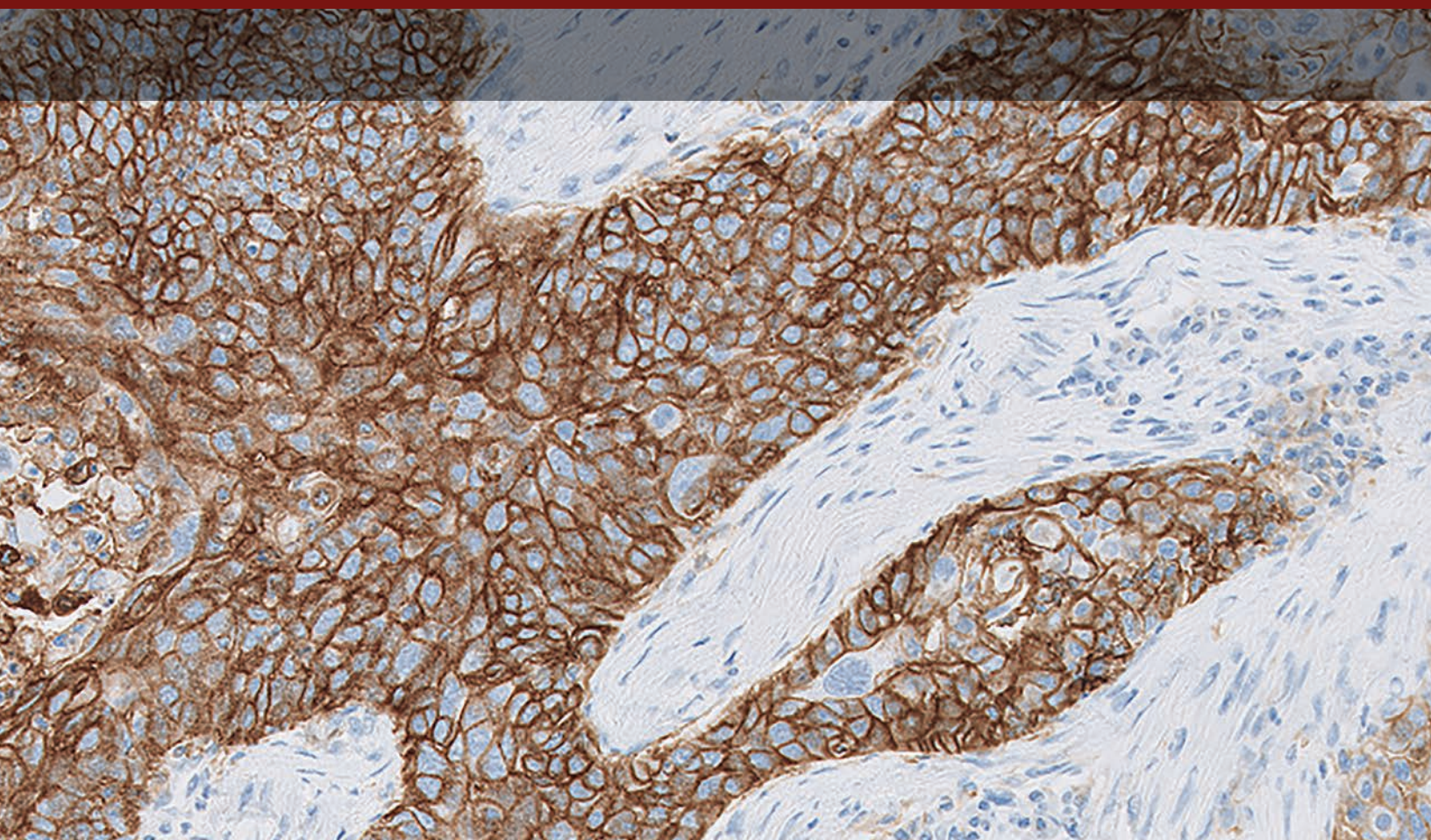
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Intended Use

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) esophageal cancer tissue using EnVision FLEX visualization system on Autostainer Link 48.

Esophageal Cancer

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

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

For descriptions of the intended use in other indications, please refer to the current version of the Instructions for Use (IFU) for PD-L1 IHC 22C3 pharmDx, Code SK006.

KEYTRUDA® is a registered trademark of Merck Sharp & Dohme LLC, a subsidiary of Merck & Co., Inc., Rahway, NJ, USA.
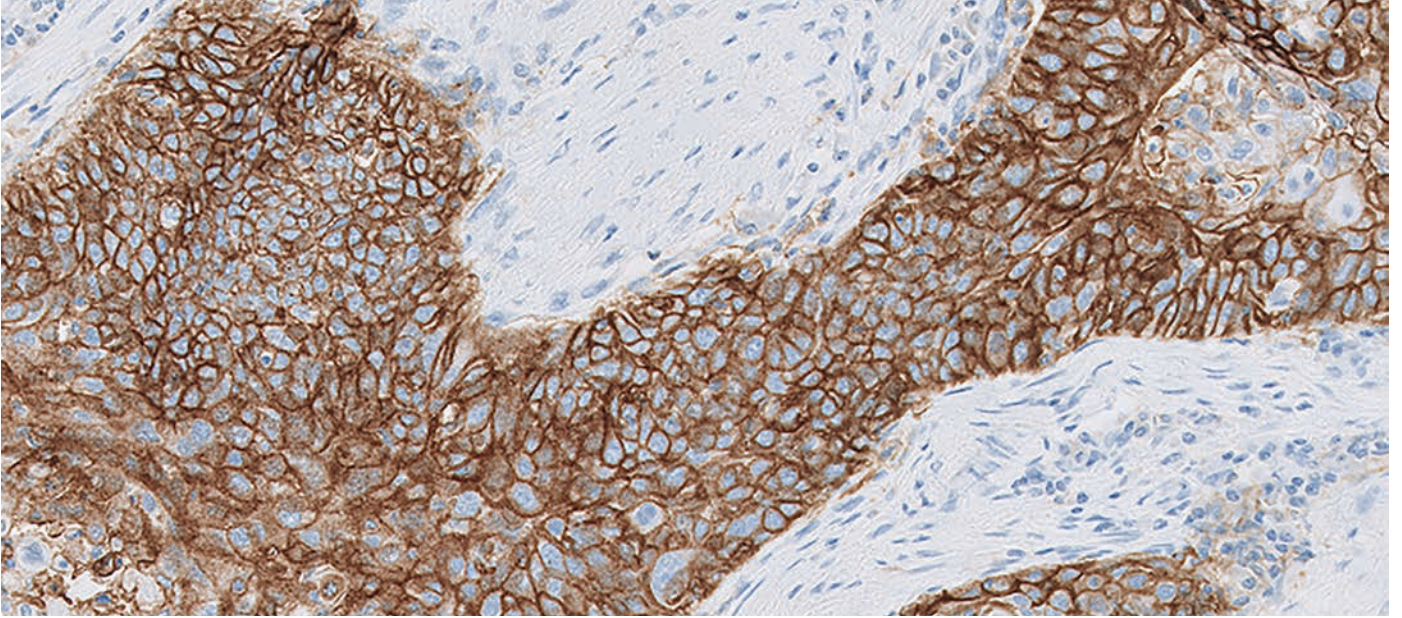
Introduction

PD-L1 IHC 22C3 pharmDx is the only clinical trial-proven companion diagnostic CE-IVD–marked as an aid in identifying patients with esophageal cancer for treatment with KEYTRUDA® (pembrolizumab).[2] 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 esophageal cancer 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 esophageal cancer specimens stained 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 esophageal cancer specimens stained with PD-L1 IHC 22C3 pharmDx can help individual laboratories achieve reproducible and reliable results.

PD-L1 IHC 22C3 pharmDx is considered a qualitative immunohistochemical assay. PD-L1 expression in esophageal cancer is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100.

Esophageal cancer 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 for PD-L1 IHC 22C3 pharmDx, Code SK006.

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 esophageal cancer, except for Figure 37 which is squamous cell carcinoma from the cervix.

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

Tissue samples supplied by BioIVT (Hicksville, NY, USA).

Data and biospecimens used in this project were provided by US Biolab, Rockville, MD and 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 Esophageal Cancer Specimens

PD-L1 upregulation in esophageal cancer 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-590) to evaluate the relationship between PD-L1 expression and clinical efficacy. KEYTRUDA is a humanized monoclonal PD-1-blocking antibody.
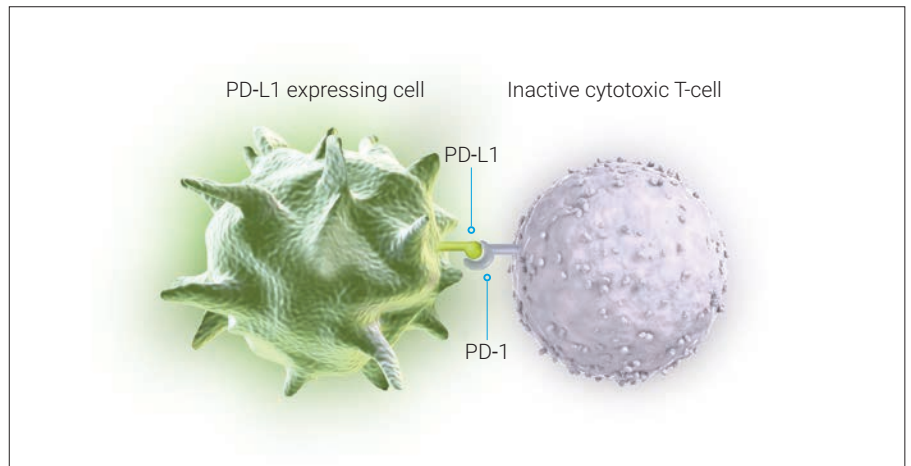
The PD-1/PD-L1 Pathway

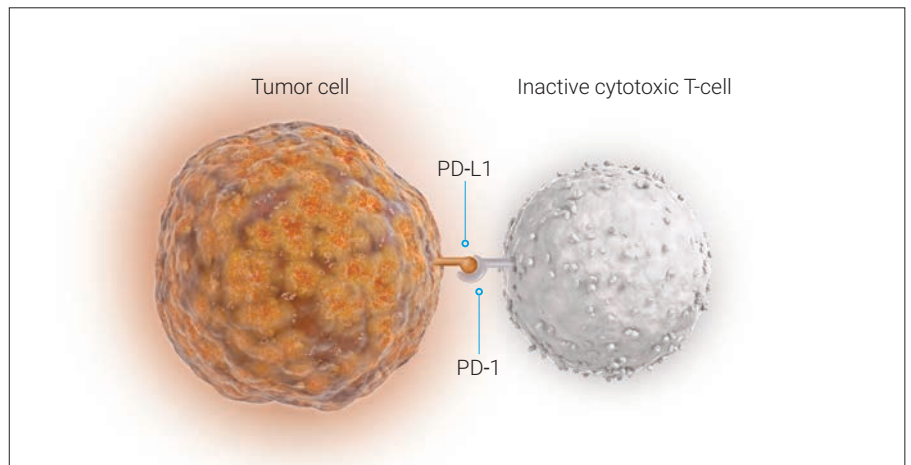**Figure 1:** Inactivation of T-cells limits damage to normal tissue.

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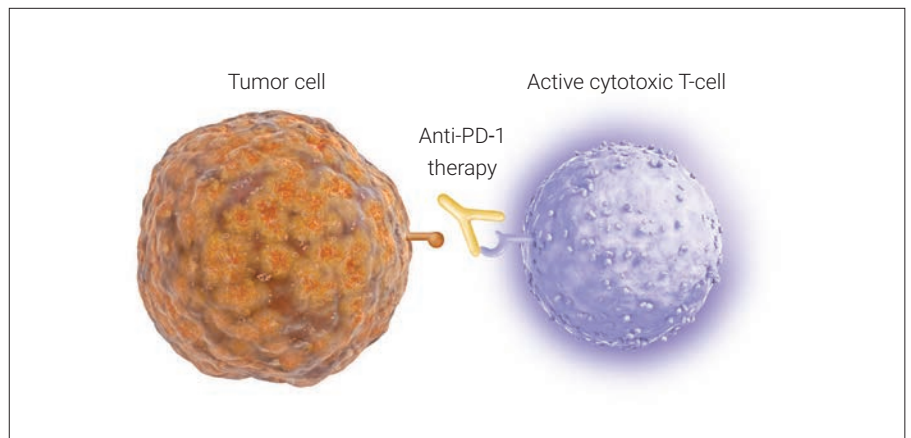

Figure 3: Blocking the PD-1/PD-L1 interaction helps to enable active T-cells and tumor cell death and elimination.

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 esophageal cancer 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 esophageal cancer 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.
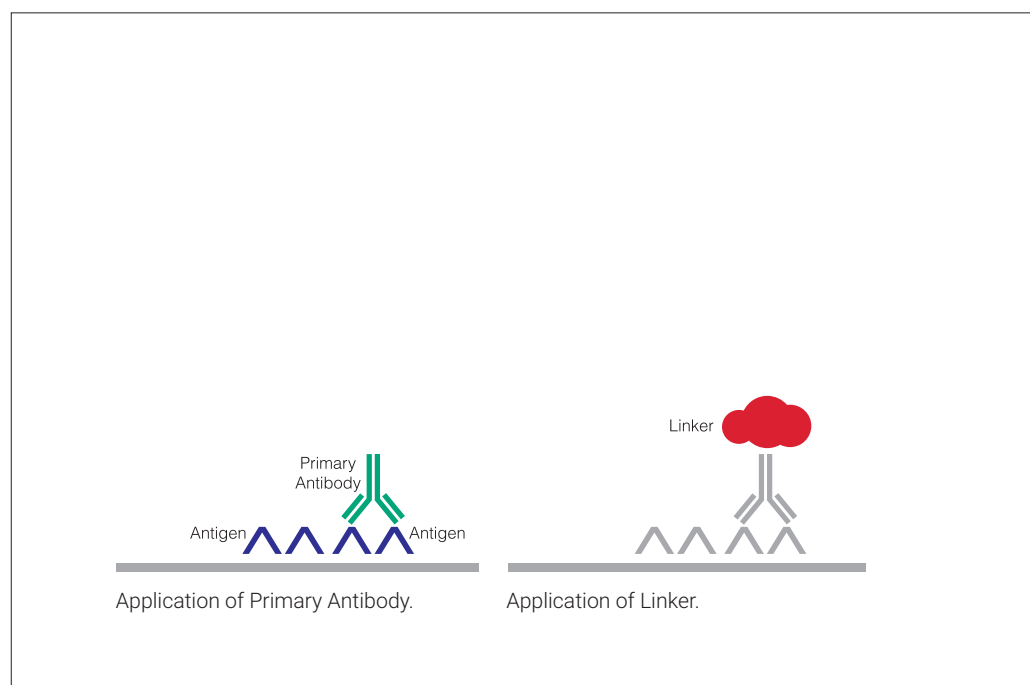

Primary Antibody

Antigen Antigen

Application of Primary Antibody.

Linker

Application of Linker.

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

Kit Configuration (SK006)

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

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

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-HRP
7. DAB+ Substrate Buffer
8. DAB+ Chromogen
9. DAB Enhancer
10. PD-L1 IHC 22C3 pharmDx Control Cell Line Slides*

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

Application of Visualization Reagent.

Application of DAB+ Substrate Chromogen Solution.

Application of DAB Enhancer.
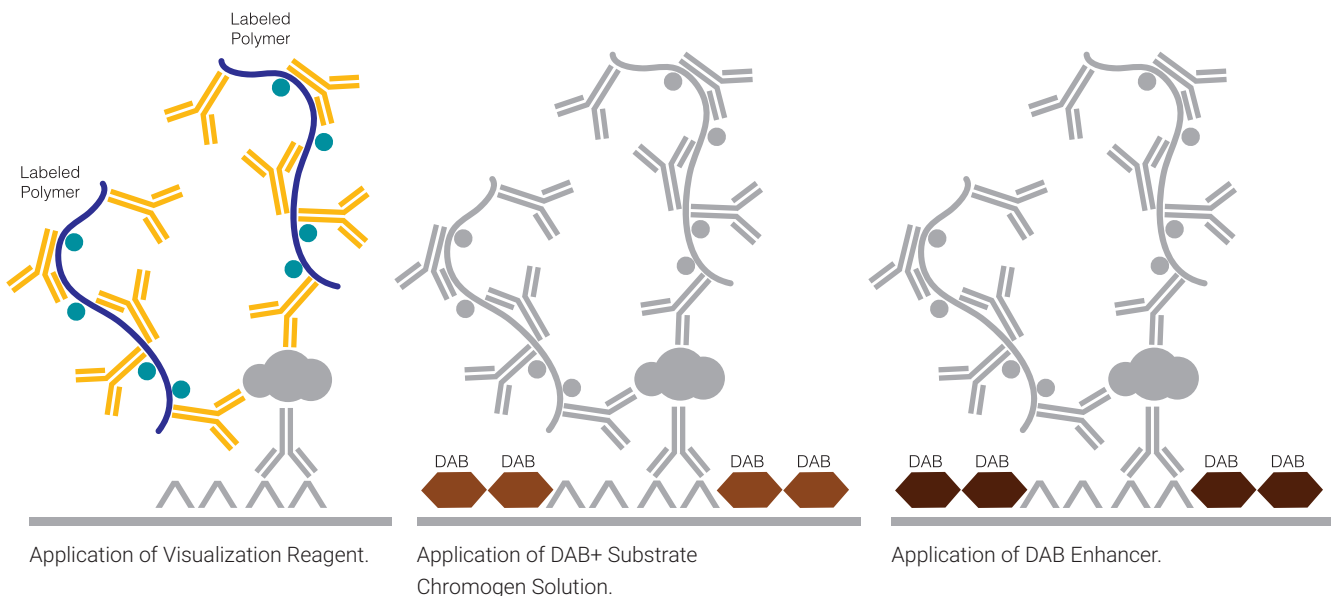
Technical Considerations

Technical problems related to PD-L1 IHC 22C3 pharmDx may arise and can be attributed to two areas: 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.

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 and with the Negative Control Reagent
- Serial section of each patient specimen stained with the NCR

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 tissues in each staining run, in addition to the PD-L1 IHC 22C3 pharmDx Control Cell Line Slide.

Select positive and negative control tissues from fresh specimens of the same tumor indication as the patient specimen. Fix, process, and embed the control tissues 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-grade 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.

EnVision FLEX Wash Buffer

Dilute EnVision FLEX Wash Buffer (20×) 1:20 using distilled or deionized water (reagent-grade water). Store unused 1× EnVision FLEX Wash 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 Solution 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

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 1× EnVision FLEX Target Retrieval Solution, Low pH to 65 °C

– Immerse Autostainer racks containing mounted, FFPE tissue sections into the preheated 1× EnVision FLEX 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 Hematoxylin (Link) (Code K8008)

Mounting

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

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 esophageal cancer 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?	☐	☐
1× EnVision FLEX Target Retrieval Solution, Low pH is prepared properly? pH of 1× EnVision FLEX Target Retrieval Solution must be 6.1 ± 0.2.	☐	☐
1× EnVision FLEX Wash Buffer is prepared properly?	☐	☐
DAB+ Substrate-Chromogen Solution is prepared properly?	☐	☐
Slides are counterstained with Hematoxylin (Link) (Code K8008)?	☐	☐
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 retain the same favorable tissue quality.

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.
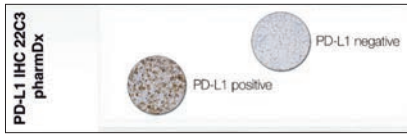
Evaluating Controls

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 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

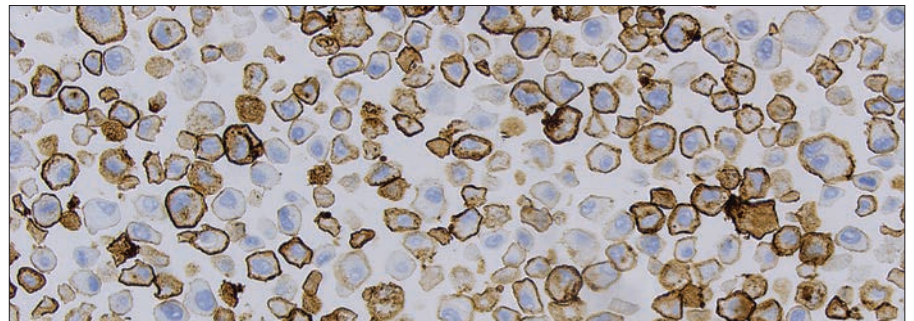– Non-specific staining < 1+ intensity

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):

– 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
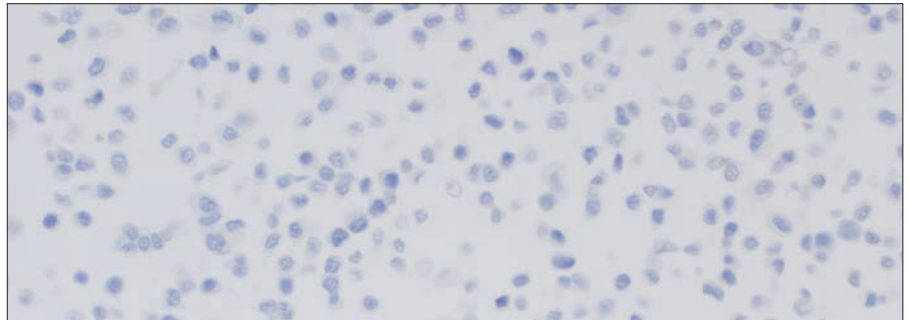

Figure 8: Negative cell pellet with no staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20× magnification).

See the Control Cell Line (CCL) Appendix on page 64 for images of passing, borderline, and failing control cell line staining.

Positive and Negative In-house Control Tissue (Esophageal Cancer)

Examine the esophageal cancer Positive Control Tissue Slides to verify that the fixation method and epitope retrieval process are effective. The Positive Control Tissue Slides should be stained with both PD-L1 primary antibody and Negative Control Reagent. 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). Known positive tissue controls should only be utilized for monitoring the correct performance of processed tissues and test reagents, not as an aid in formulating a specific diagnosis of patient samples. If staining of positive in-house control tissue is not satisfactory, all results with the patient specimen should be considered invalid.

– Requirements for slide stained with PD-L1: Presence of brown plasma membrane staining should be observed. Non-specific staining should be ≤ 1+

– Requirements for slide stained with Negative Control Reagent: No membrane staining. Non-specific staining should be ≤ 1+
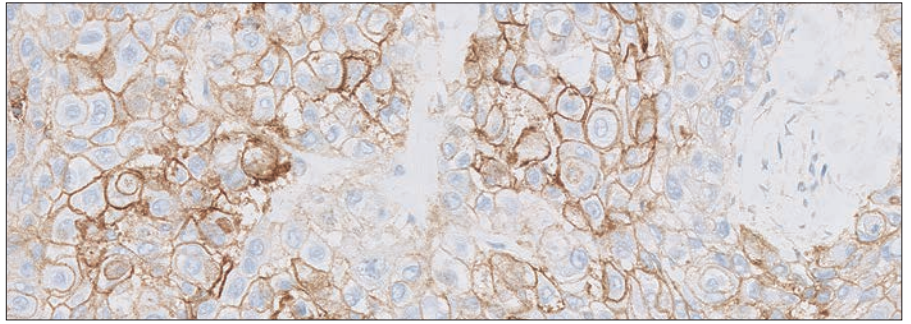
Figure 9: Positive in-house control tissue (20× magnification).

Examine the esophageal cancer Negative Control Tissue Slides to verify the specificity of the labeling of the target antigen by the primary antibody. The Negative Control Tissue Slides (known to be PD-L1 negative) should be stained with both PD-L1 primary antibody and Negative Control Reagent. The ideal esophageal cancer 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.

Note: As an alternative, negative portions of the Positive Control Tissue may serve as the Negative Control Tissue, but this should be verified by the user.

If staining of negative in-house control tissue is not satisfactory, all results with the patient specimen should be considered invalid.

– Requirements for slide stained with PD-L1: No membrane staining in tumor cells. Non-specific staining should be ≤ 1+

– Requirements for slide stained with Negative Control Reagent: No membrane staining. Non-specific staining should be ≤ 1+
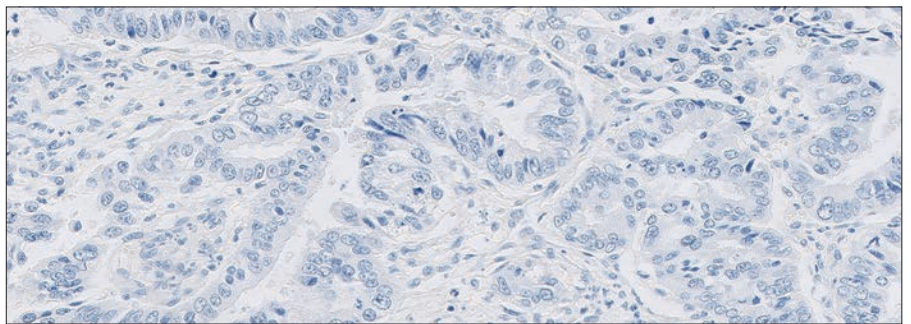

Figure 10: Negative in-house control tissue demonstrating lack of staining of tumor cells and MICs (20× magnification).

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).

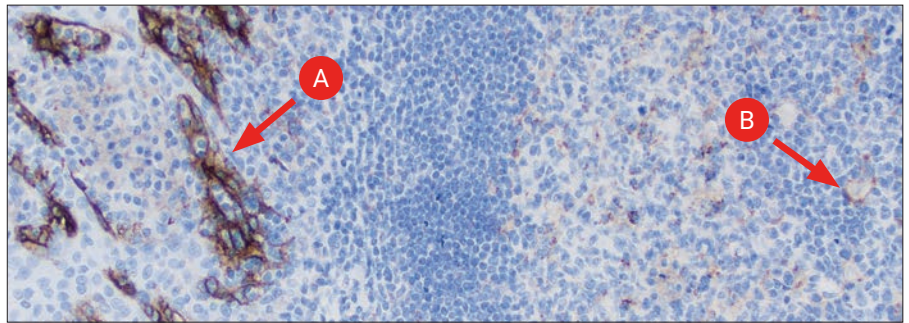PD-L1 expression of the endothelium, fibroblasts, and the surface epithelium should be absent.

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 0 specific staining and ≤ 1+ non-specific 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.
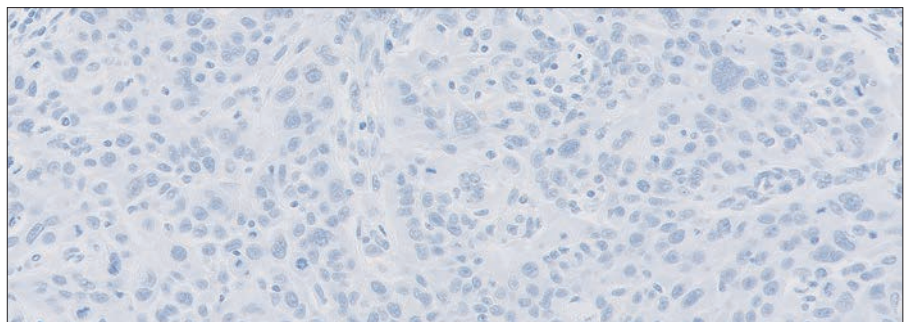

Figure 12: Esophageal cancer tissue specimen stained with NCR (20× magnification).

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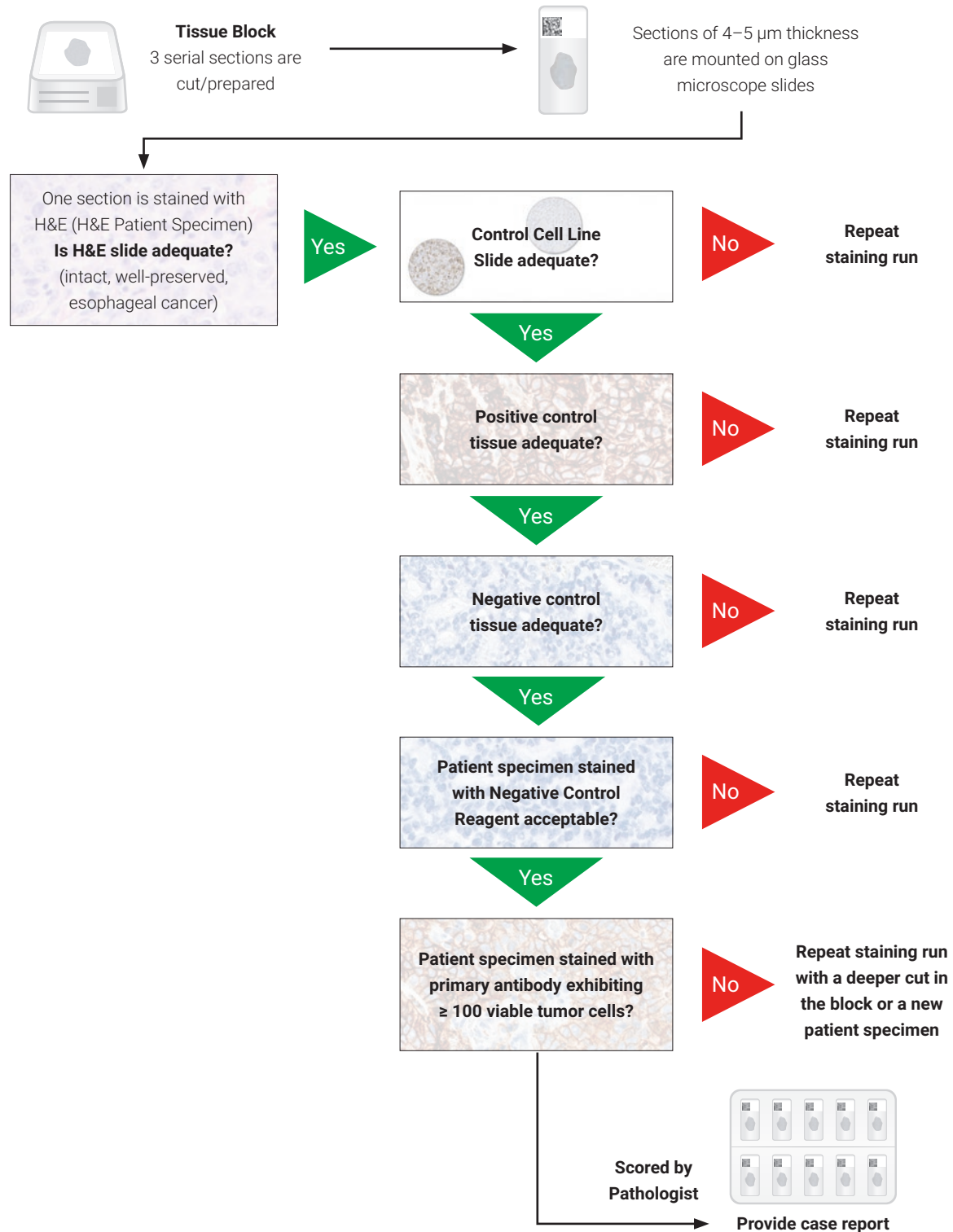
Slide Evaluation Flowchart

Tissue Block
3 serial sections are cut/prepared

Sections of 4−5 μm thickness are mounted on glass microscope slides

One section is stained with H&E (H&E Patient Specimen)
Is H&E slide adequate?
(intact, well-preserved, esophageal cancer)

Yes

Control Cell Line Slide adequate?

No → Repeat staining run

Yes

Positive control tissue adequate?

No → Repeat staining run

Yes

Negative control tissue adequate?

No → Repeat staining run

Yes

Patient specimen stained with Negative Control Reagent acceptable?

No → Repeat staining run

Yes

Patient specimen stained with primary antibody exhibiting ≥ 100 viable tumor cells?

No → Repeat staining run with a deeper cut in the block or a new patient specimen

Scored by Pathologist

Provide case report

Figure 13: Recommended order of slide evaluation.

Combined Positive Score

Definition of Combined Positive Score (CPS)

PD-L1 expression in esophageal cancer is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages*) divided by the total number of viable tumor cells, multiplied by 100. Although the result of the calculation can exceed 100, the maximum score is defined as CPS 100.

CPS is defined accordingly:

* Macrophages and histiocytes are considered the same cells

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

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 for Esophageal Cancer

Tissue Elements	Included in the Numerator	Excluded from the Numerator
Tumor Cells	Convincing partial or complete cell membrane staining (at any intensity) of viable invasive esophageal tumor cells: adenocarcinoma (including intramucosal component) and squamous cell carcinoma	− Non-staining tumor cells − Tumor cells with only cytoplasmic staining − Any non-invasive neoplasia including glandular and squamous dysplasia (e.g. high grade glandular dysplasia and squamous cell carcinoma in situ)
Immune Cells	Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma[†], such as: − Lymphocytes (including lymphocyte aggregates) − Macrophages[‡] Only MICs directly associated with the response to the tumor are scored	− Non-staining MICs − MICs associated with non-invasive neoplasia − MICs associated with benign structures − MICs (including lymphoid aggregates) not directly associated with the response to the tumor − Neutrophils, eosinophils and plasma cells
Other Cells	None	− Benign epithelial cells − Stromal cells (including fibroblasts) − Necrotic cells and/or cellular debris

In MICs membrane and cytoplasmic staining are often indistinguishable due to a high nuclear to cytoplasmic ratio. Therefore, membrane and/or cytoplasmic staining of MICs is included in the score
[†] *Adjacent MICs are defined as being within the same 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 for Esophageal Cancer

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

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)*
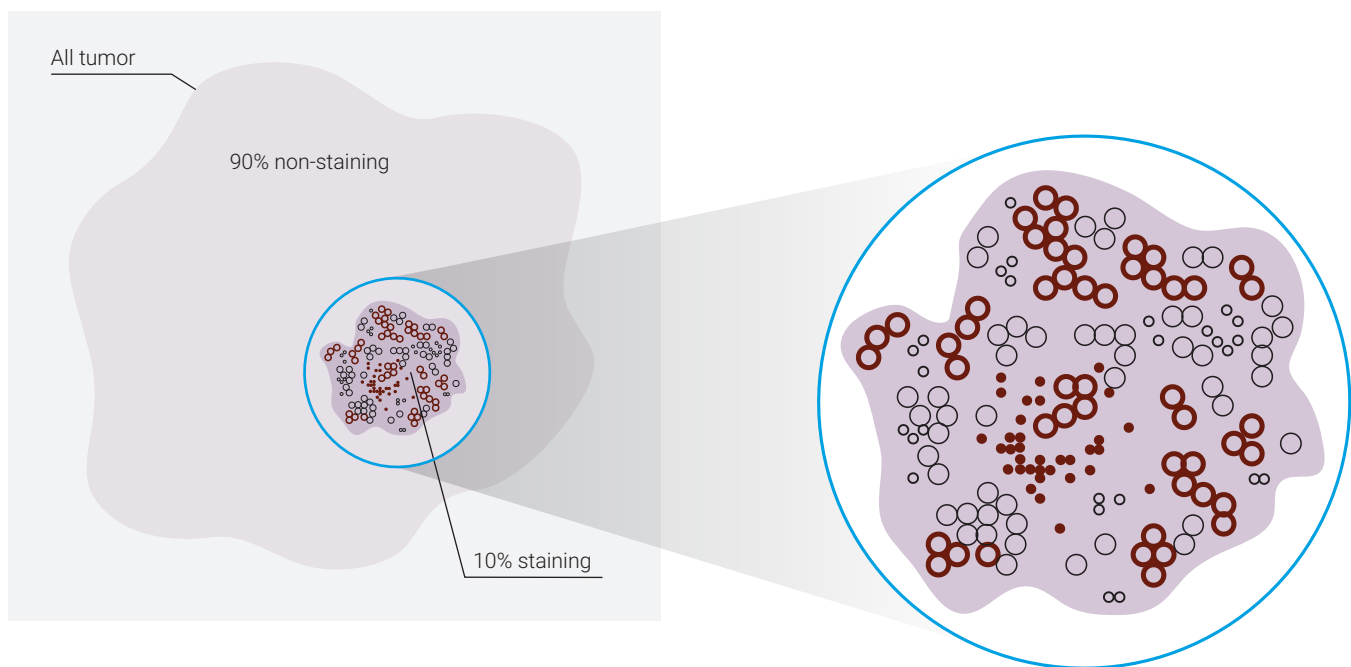
Calculate the Combined Positive Score of the entire tumor area:

Assessment:

CPS of area with staining:

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

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

Clinical Interpretation: CPS < 10

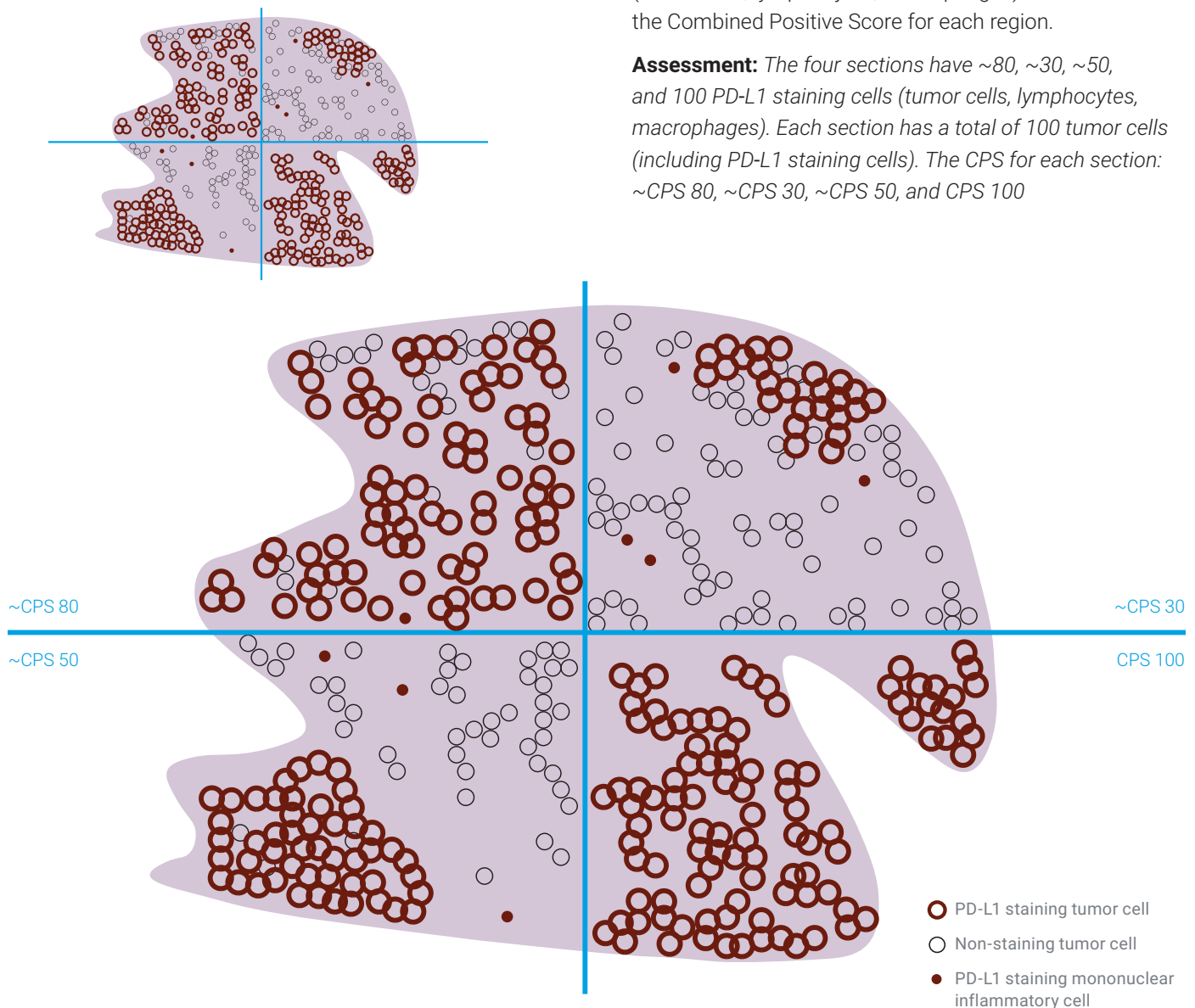§ *Including tumor cells, lymphocytes, macrophages*

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*

~CPS 80 ~CPS 30
~CPS 50 CPS 100

○ PD-L1 staining tumor cell
○ Non-staining tumor cell
● PD-L1 staining mononuclear inflammatory cell

Calculate the Combined Positive Score of the entire tumor area:

Assessment:
Combined Positive Score:
(80 + 30 + 50 + 100) / 4 ≈ CPS 65

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

Clinical Interpretation: CPS ≥ 10

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

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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*
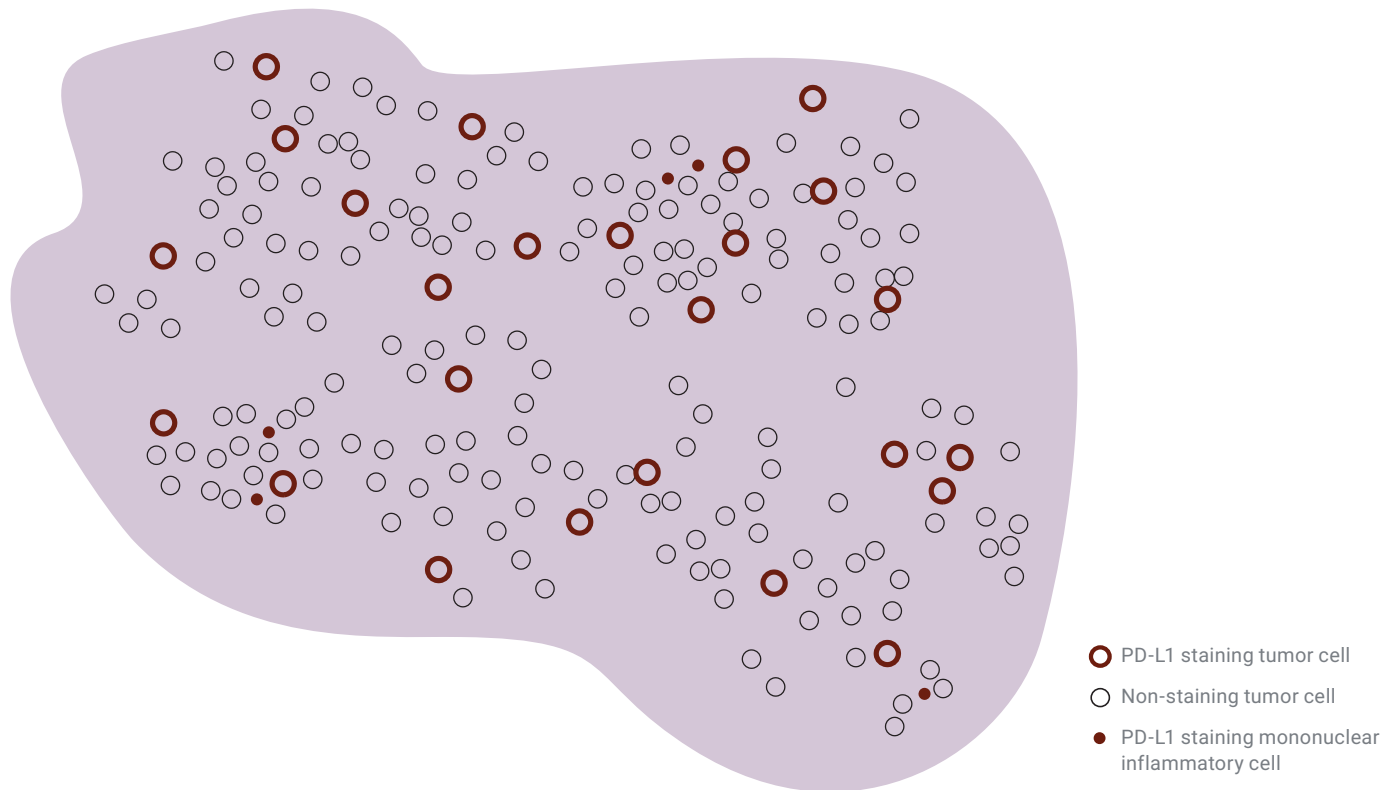
○ PD-L1 staining tumor cell

○ Non-staining tumor cell

● PD-L1 staining mononuclear inflammatory cell

Calculate the Combined Positive Score of the entire tumor area:

Assessment:

Combined Positive Score:

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

Clinical Interpretation: CPS ≥ 10
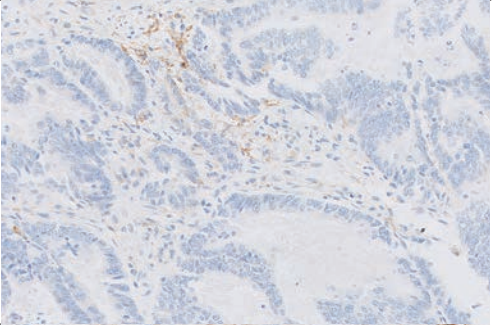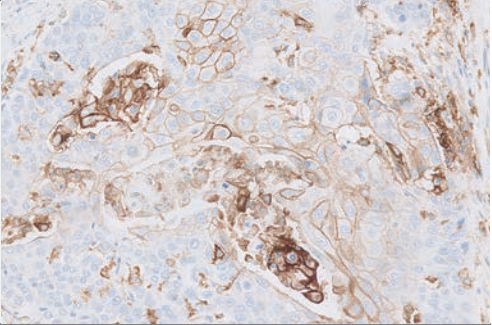
* Including tumor cells, lymphocytes, macrophages

Figure 16: Example of near cut-off specimen.

Interpretation of CPS

The Combined Positive Score (CPS) determines the PD-L1 expression level 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 Esophageal Cancer for Treatment

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

Clinical Validation of PD-L1 IHC 22C3 pharmDx in Previously Untreated Patients with Esophageal Cancer

The clinical validity of PD-L1 IHC 22C3 pharmDx in evaluating PD-L1 expression in previously untreated patients with esophageal cancer is based on the KEYTRUDA KEYNOTE-590 study sponsored by Merck Sharp & Dohme Corp. Specimens from patients with locally advanced unresectable or metastatic esophageal carcinoma or HER-2 negative gastroesophageal junction carcinoma were tested for PD-L1 expression using PD-L1 IHC 22C3 pharmDx. Fifty-one percent of enrolled patients had tumors 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 77–79.

Table 4: PD-L1 Prevalence in Patients with Locally Advanced Unresectable or Metastatic Esophageal Cancer or HER-2 Negative Gastroesophageal Junction Carcinoma Enrolled in KEYNOTE-590

PD-L1 Expression	CPS < 10	CPS ≥ 10
Prevalence % (n)	49% (366)	51% (383)

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.

Locally advanced unresectable or metastatic esophageal cancer patient specimen

Use PD-L1 IHC 22C3 pharmDx to determine PD-L1 expression

Pathologist reports numerical CPS and PD-L1 expression level

CPS < 10

CPS ≥ 10

Oncologist determines treatment

Patients with locally advanced unresectable or metastatic esophageal carcinoma or HER-2 negative gastroesophageal junction carcinoma with PD-L1 expression level CPS ≥ 10 are indicated for first-line treatment with KEYTRUDA in combination with platinum and fluoropyrimidine based chemotherapy

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), in combination with platinum and fluoropyrimidine based chemotherapy, is indicated for the first-line treatment of patients with locally advanced unresectable or metastatic carcinoma of the oesophagus or HER-2 negative gastroesophageal junction adenocarcinoma in adults whose tumours express PD-L1 with a CPS ≥ 10. See the KEYTRUDA Summary of Product Characteristics 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 esophageal cancer 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 esophageal cancer 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.

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

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

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

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 esophageal cancer 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.
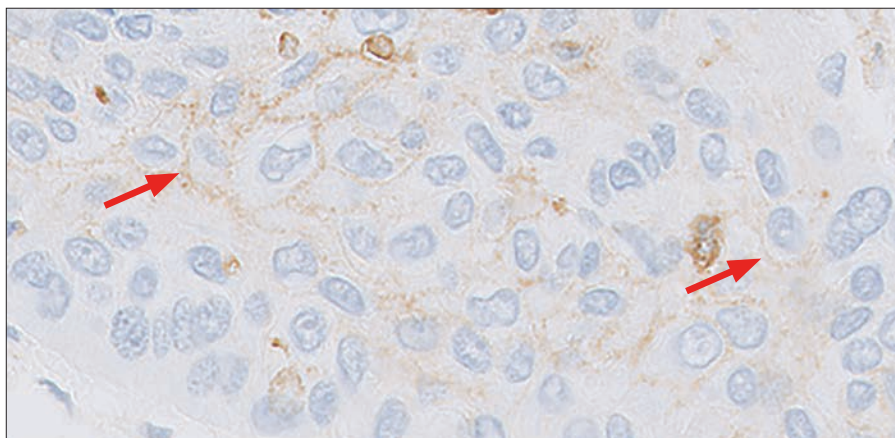
Figure 18a: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting 1+ linear membrane staining of tumor cells (arrows) (20× magnification).
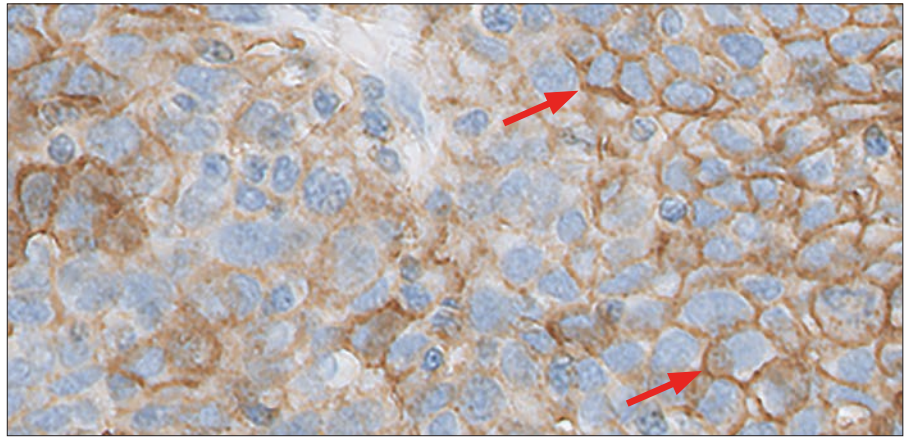
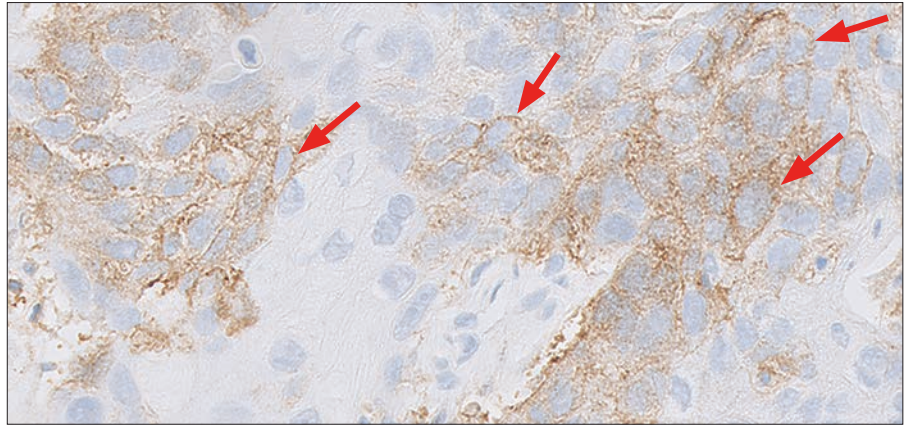**Figure 18b:** Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting 2+ linear membrane staining of tumor cells (arrows) (20× magnification).
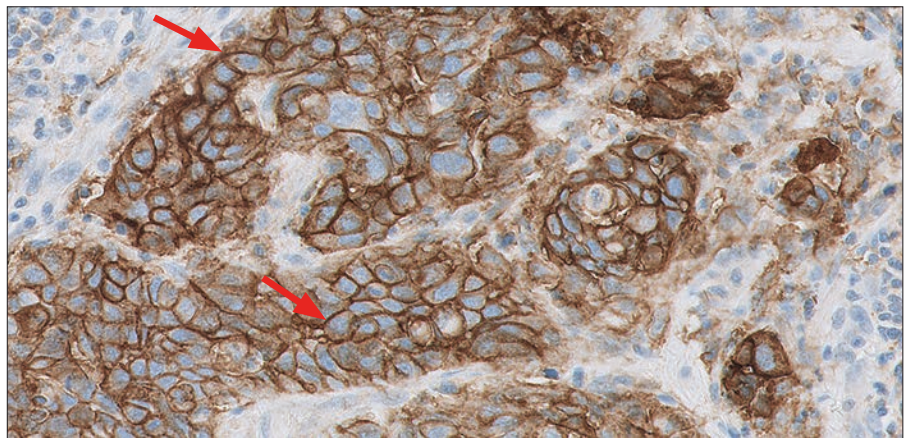
Figure 18c: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting 3+ linear membrane staining of tumor cells (arrows) (20× magnification).

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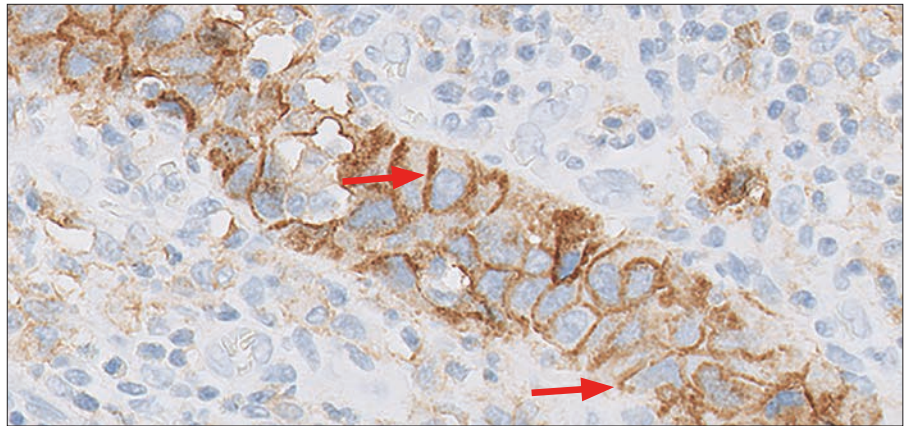
Figure 19: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting partial linear membrane staining of tumor cells (arrows) (20× magnification).

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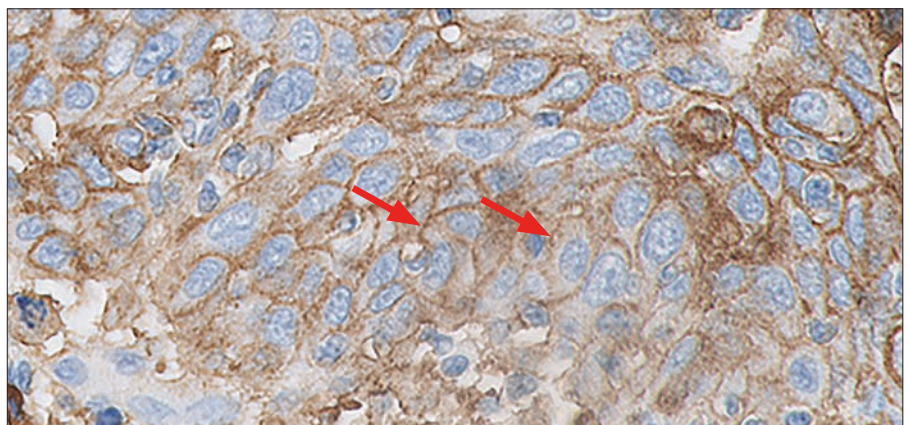
Figure 20: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting linear membrane staining distinct from cytoplasmic staining (arrows) (20× magnification).

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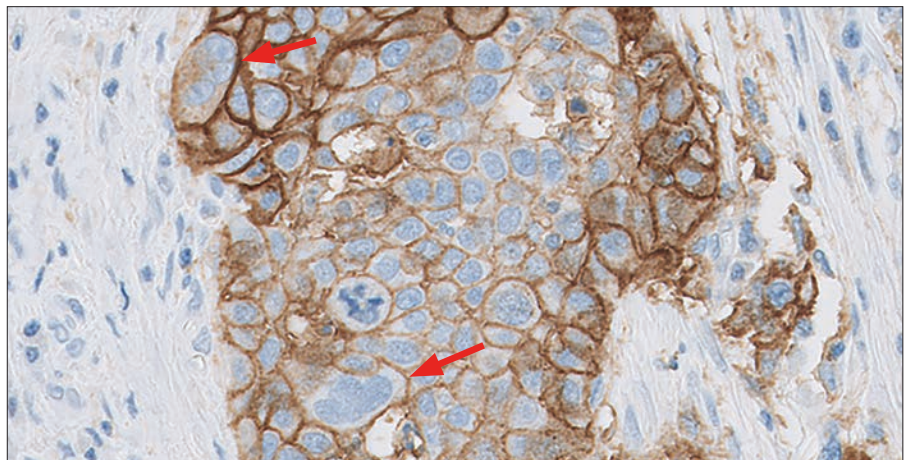
Figure 22: Multinucleate tumor cells (arrows) (20× magnification).

Key Point

Multinucleate tumor cells can be seen in esophageal cancer 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.
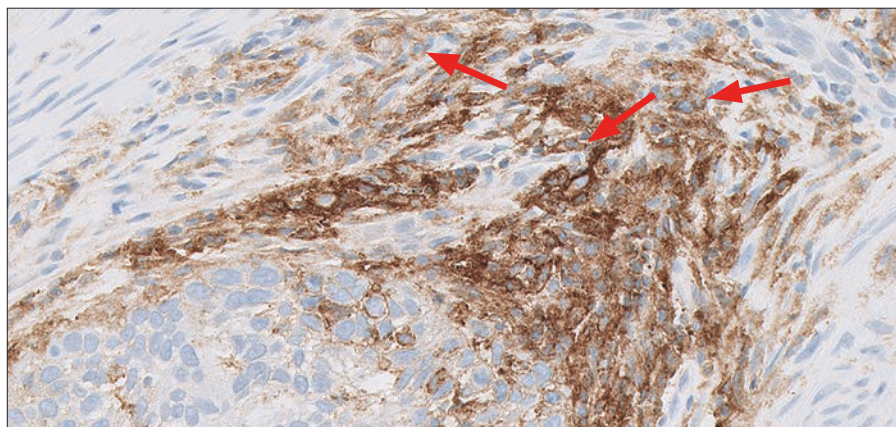
Figure 23a: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting staining of tumor-associated lymphocytes (arrows) (20× magnification).
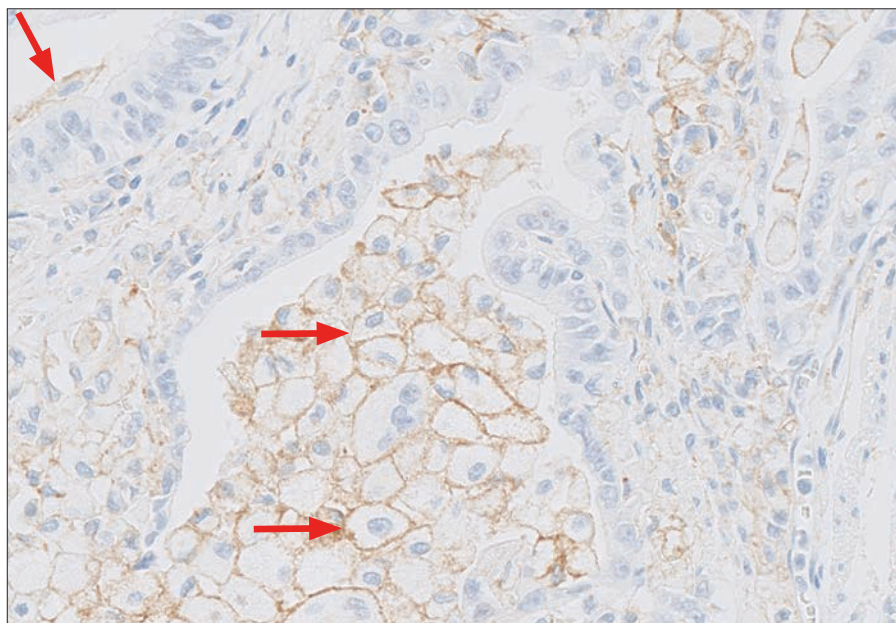
Figure 23b: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting staining of tumor-associated macrophages (arrows) (20× magnification).

Multinucleate Giant Cells

Multinucleate giant cells can be seen in esophageal cancer 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.
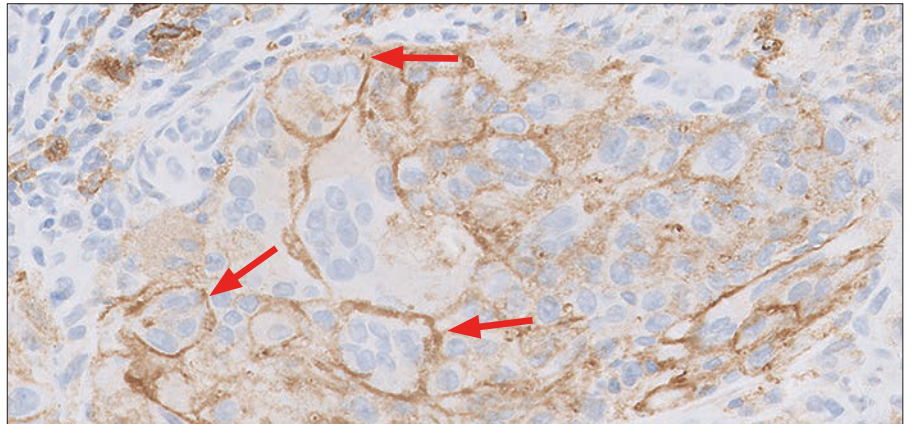
Figure 24: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting staining of multinucleate giant cells (arrows) (20× magnification).

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.
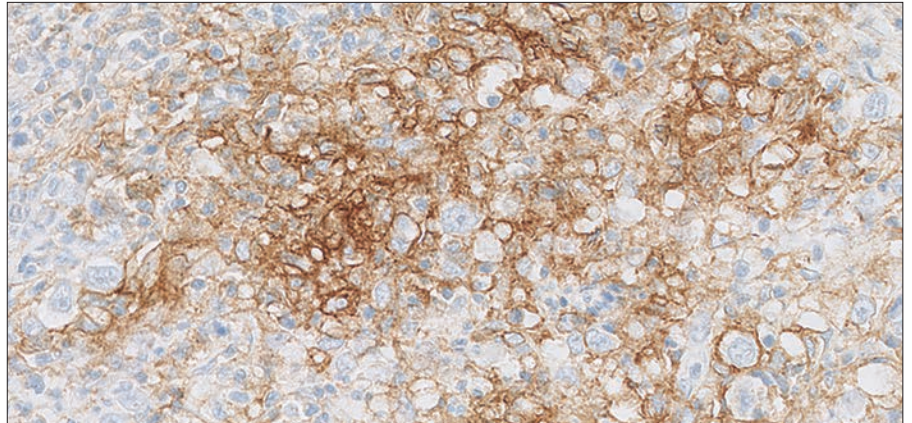
Figure 25a: Tumor and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other and exhibit PD-L1 primary antibody staining (20× magnification).
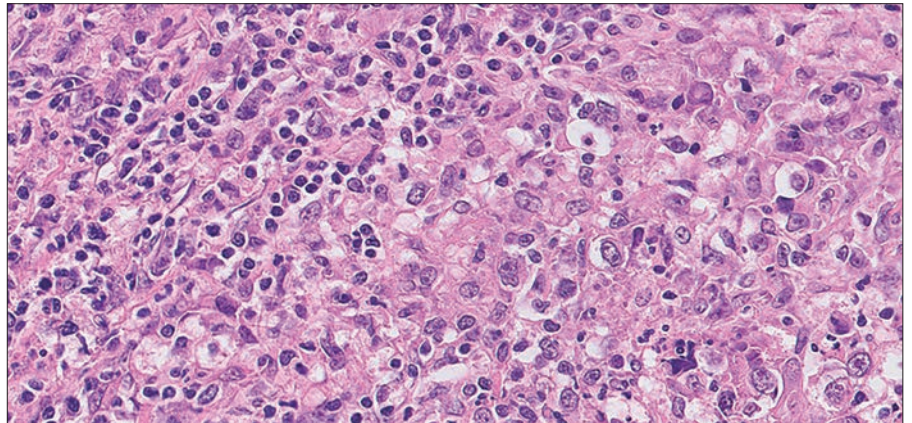
Figure 25b: Corresponding H&E to reference when tumor and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other (20× magnification).

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.
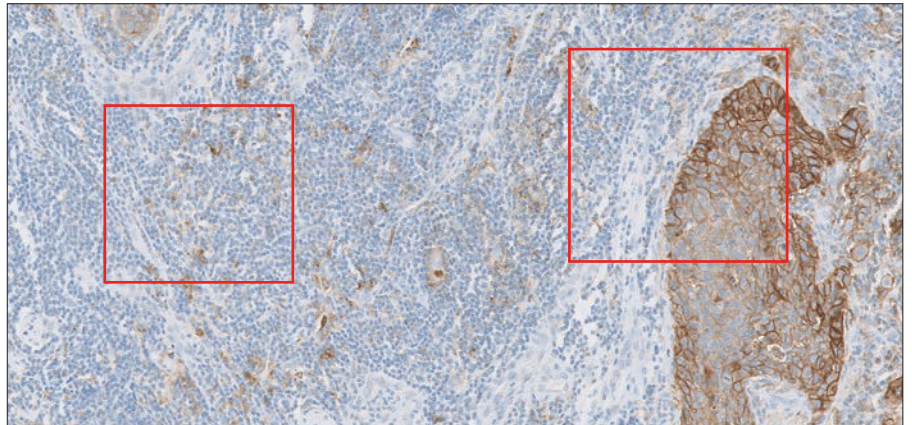
Figure 26a: At 5× magnification, several areas of PD-L1 staining mononuclear inflammatory cells are visible. To demonstrate which immune cells to include in the numerator, zoom in to 20× magnification on the boxed fields (5× magnification).
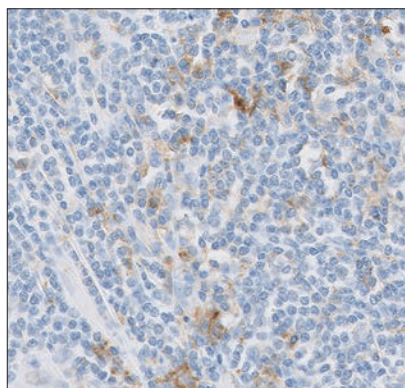
Figure 26b: Tumor cells are absent from this 20× field containing PD-L1 staining mononuclear inflammatory cells, thus none of these cells should be included in the numerator (20× magnification).
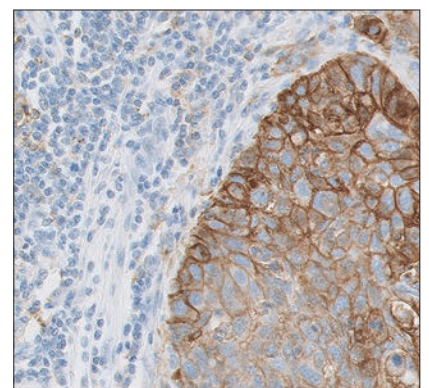
Figure 26c: When positioning the tumor cells in the approximate center of a 20× field, PD-L1 staining mononuclear inflammatory cells that are present within the same field should be included in the numerator (20× magnification).

Tumor Cell Size

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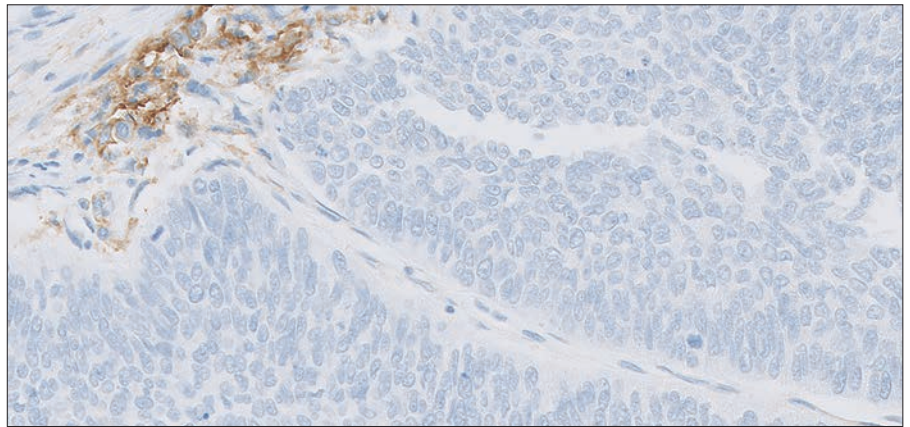
Figure 27: Esophageal cancer specimen with small tumor cells (20× magnification).

Medium Cell Size
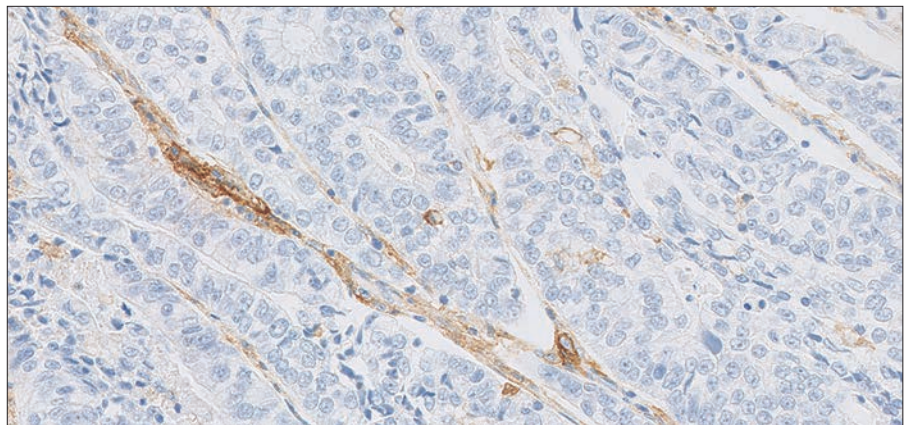
Figure 28: Esophageal cancer specimen with medium tumor cells (20× magnification).

Large Cell Size
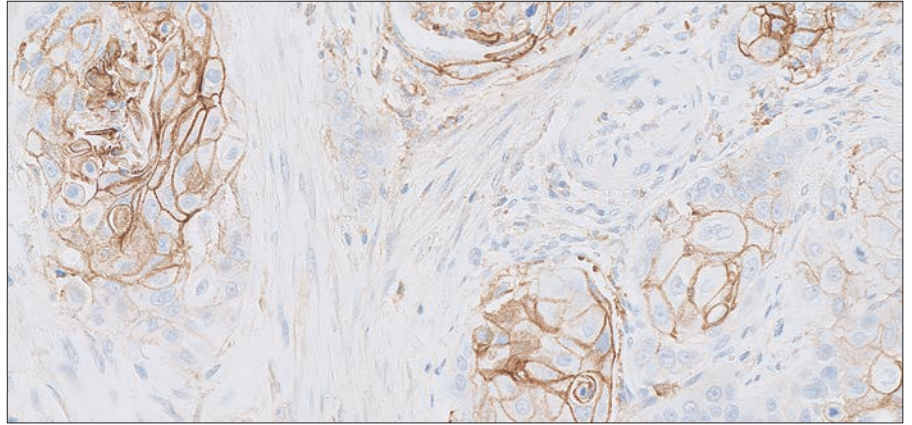
Figure 29: Esophageal cancer specimen with large tumor cells (20× magnification).

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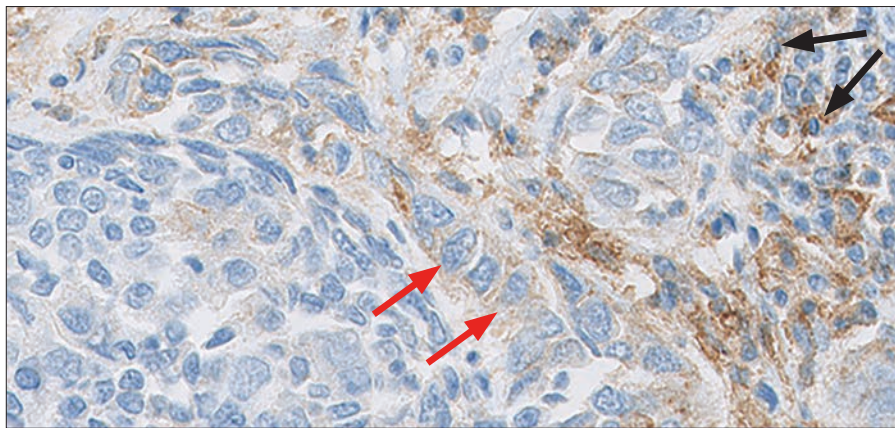
Figure 30: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting only cytoplasmic staining of tumor cells (red arrows) (20× magnification). **Note:** Tumor-associated mononuclear inflammatory cells in the upper right corner exhibit cytoplasmic PD-L1 staining and should be included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

Key Point

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

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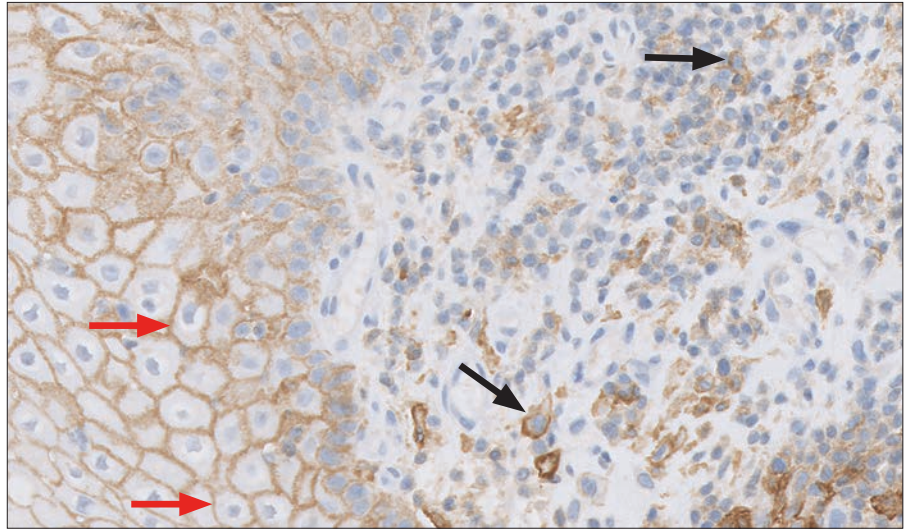
Figure 31: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting staining of benign epithelial cells (red arrows) and associated mononuclear inflammatory cells (black arrows), both of which should be excluded from the score (20× magnification).

Key Point

Benign cells and MICs associated with the benign component may exhibit PD-L1 staining and should be excluded from the score
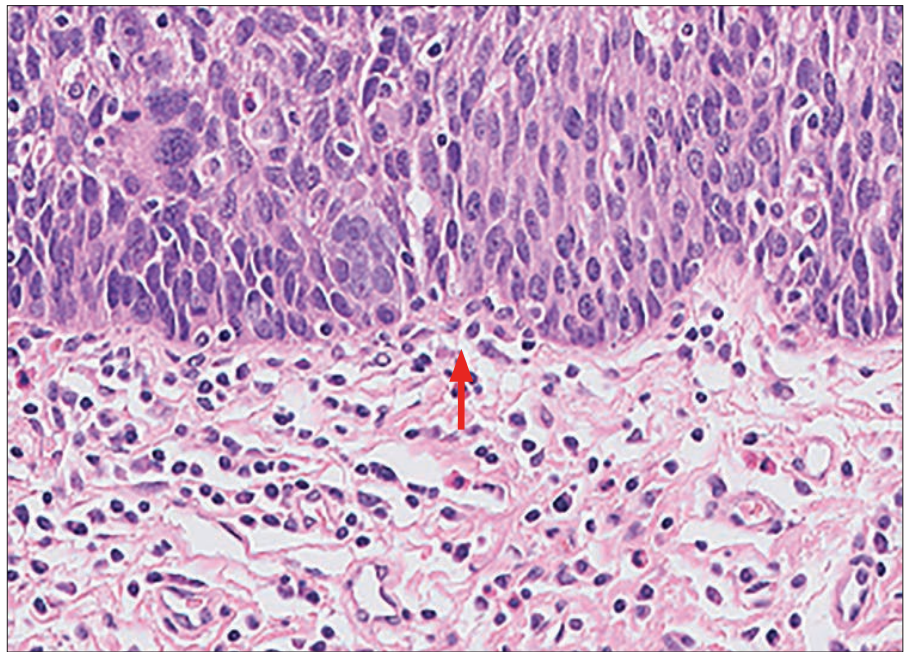
Carcinoma In Situ (CIS)

Figure 32a: Hematoxylin and eosin (H&E) section demonstrating esophageal carcinoma in situ (CIS) (arrow) (10× magnification).
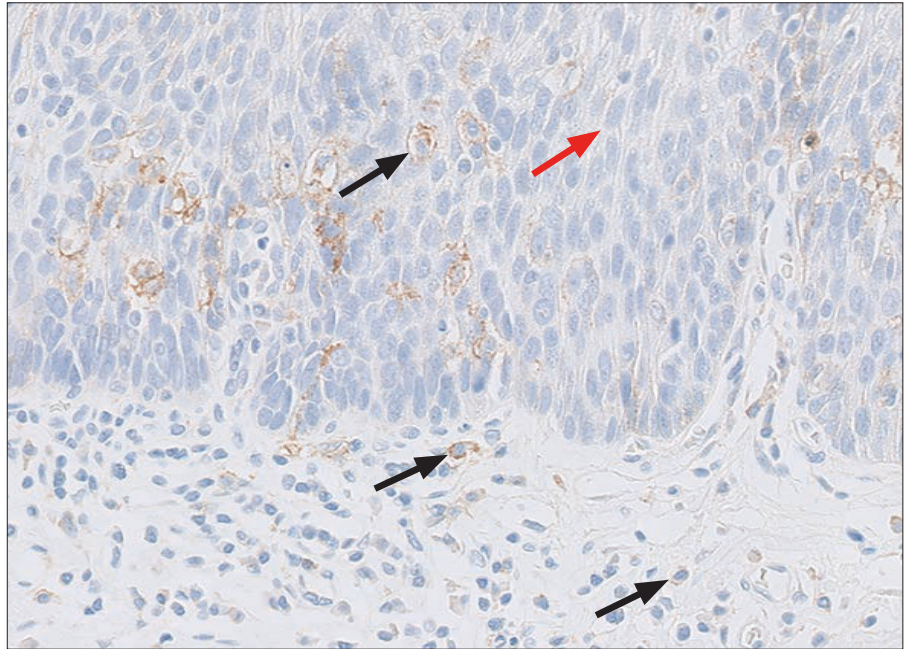
Figure 32b: Any tumor cells that are part of the CIS component should be excluded from the numerator and denominator (red arrow). Any mononuclear inflammatory cells (MICs) (black arrows) associated with the CIS component should be excluded from the numerator (10× magnification).

Key Point

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

Non-Invasive Dysplasia
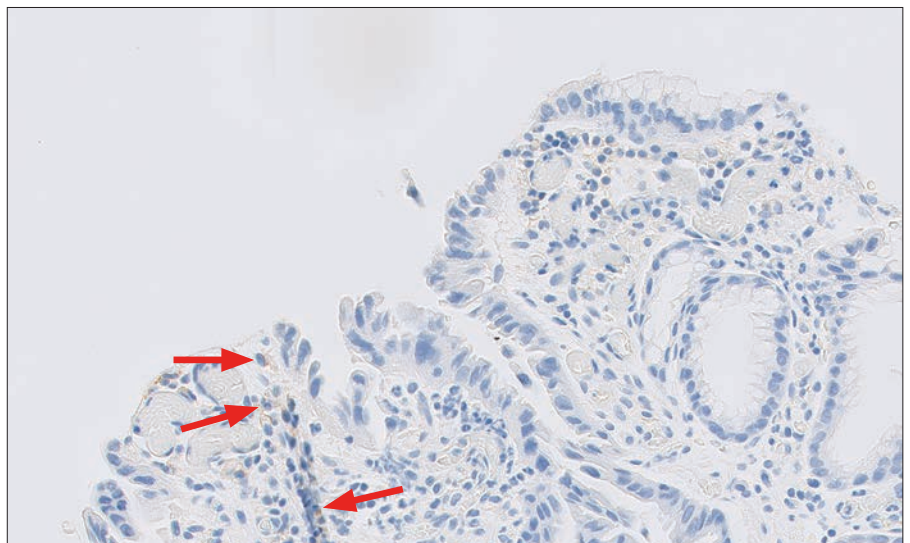
Figure 33: Mononuclear inflammatory cells (MICs) exhibiting PD-L1 staining adjacent to non-invasive dysplasia (arrows) (20× magnification).

Key Point

MICs not associated with invasive carcinoma may exhibit PD-L1 staining and should be excluded from the score

Stromal Cells
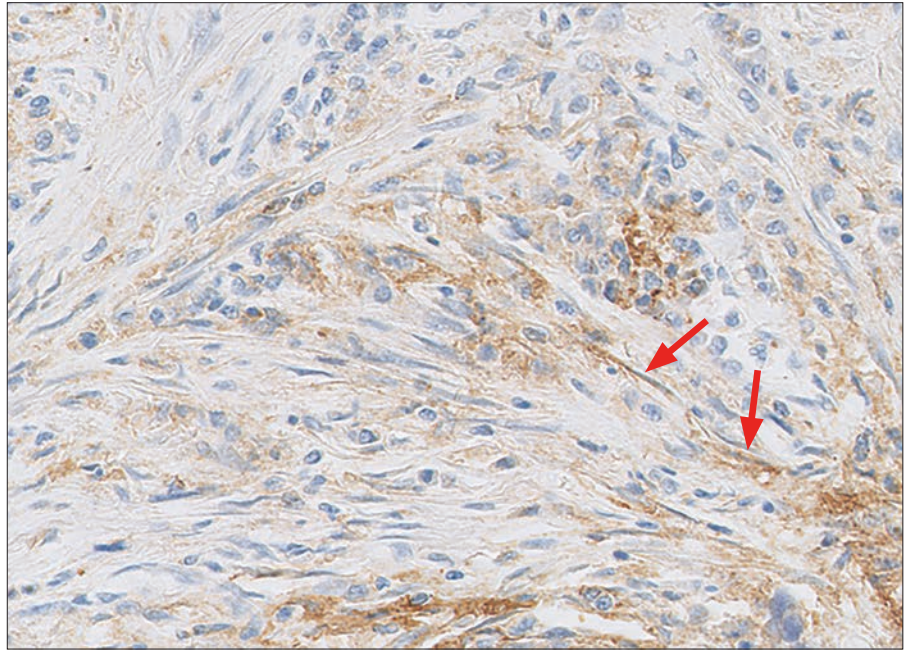
Figure 34: PD-L1 staining on stromal cells (arrows) (20× magnification).

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.
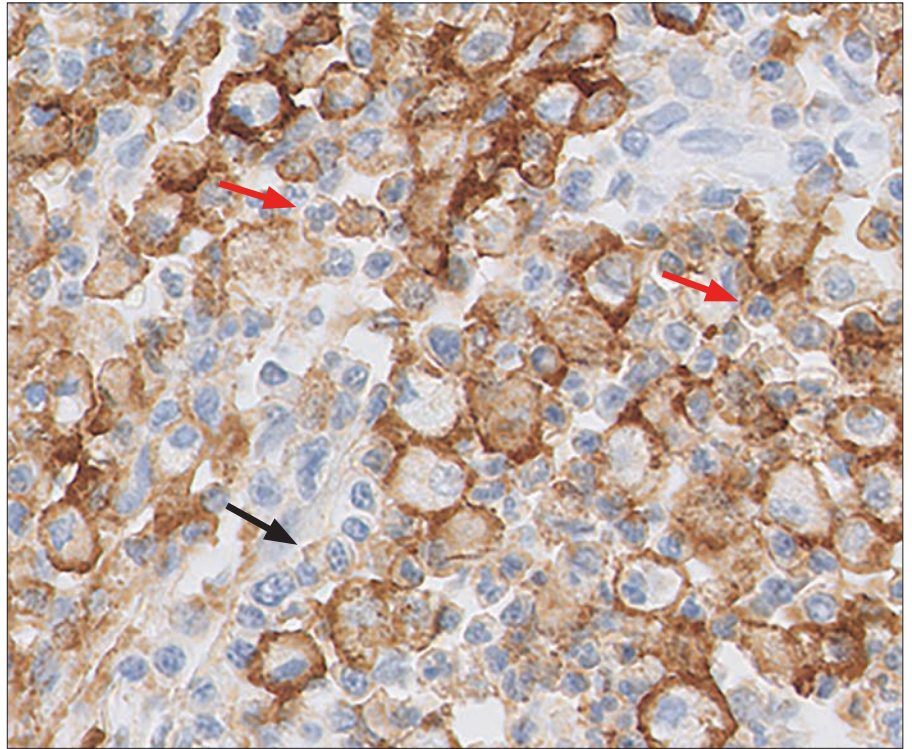
Figure 35: PD-L1 staining on neutrophils (red arrows) and plasma cell (black arrow) (20× magnification).

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.
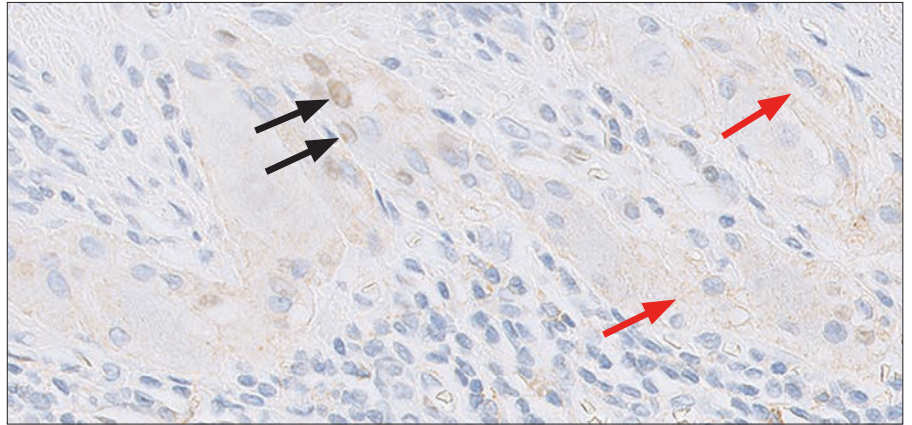
Figure 36a: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting non-specific staining; non-specific background staining (red arrows) should be excluded from the score. Weak nuclear staining is also present and should be ignored (black arrows) (20× magnification).
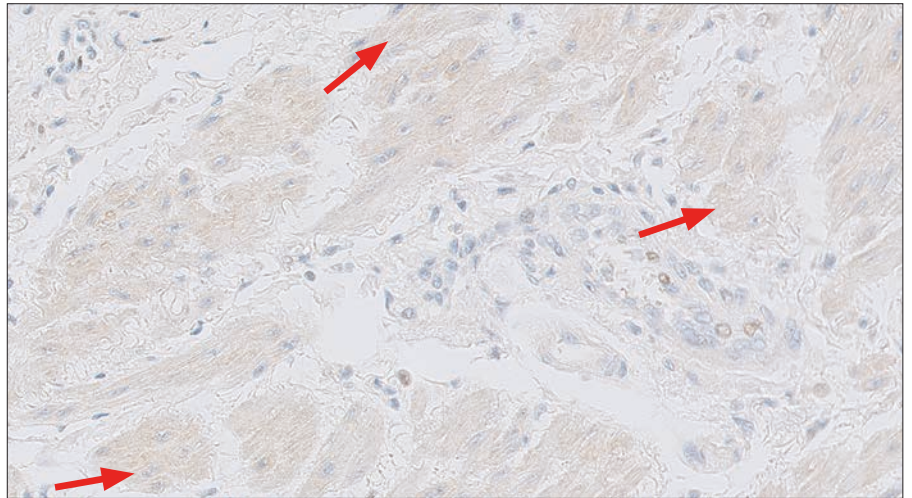

Figure 36b: Negative Control Reagent (NCR) exhibiting non-specific background staining in esophageal cancer (arrows) (20× magnification).

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.
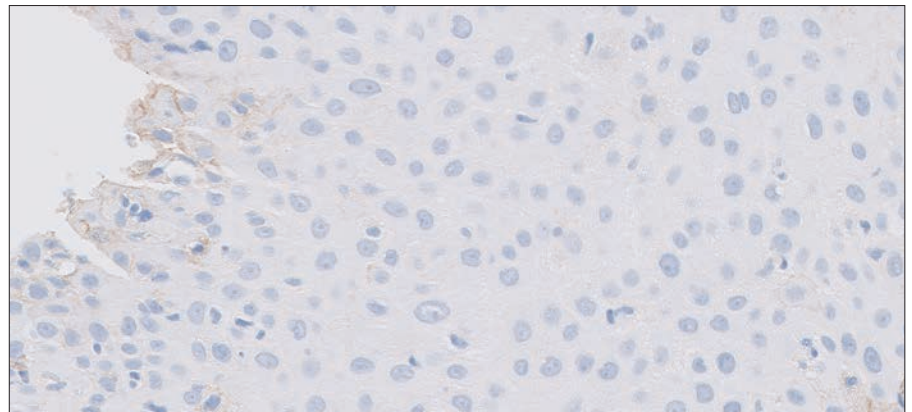
Figure 37: Edge staining should be excluded from the score (20× magnification). **Note:** Squamous cell carcinoma from the cervix is depicted.

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.
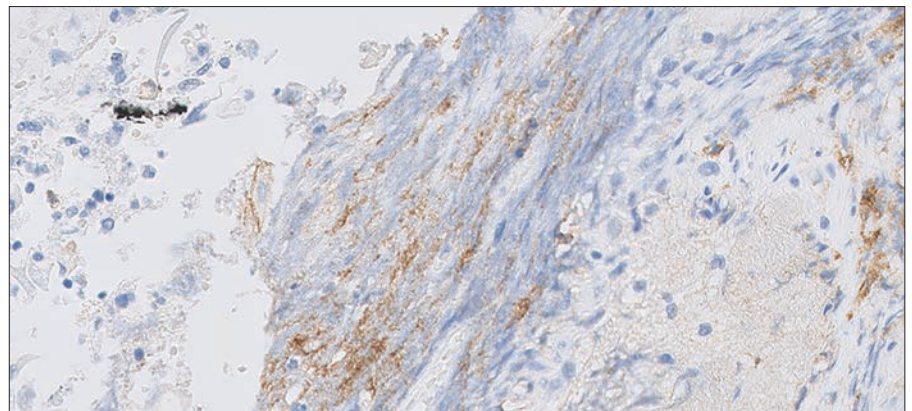

Figure 38: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting crush artifact; crush artifact should be excluded from the score (20× magnification).

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.
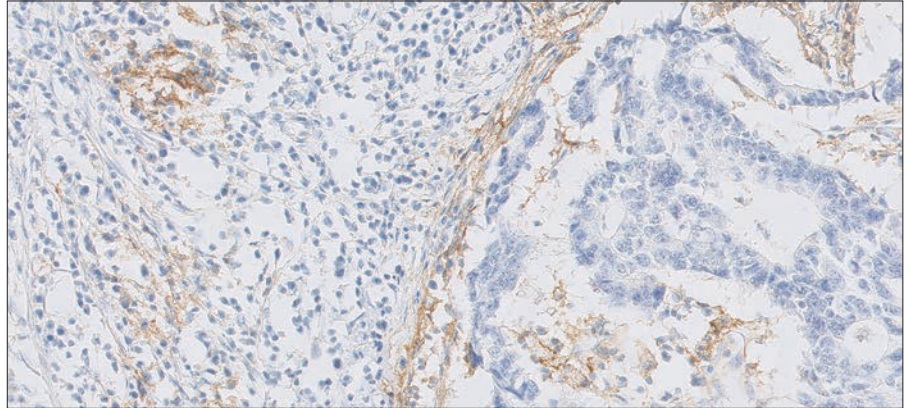

Figure 39: Esophageal cancer specimen exhibiting poor tissue fixation (20× magnification).

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 esophageal cancer specimens and should be excluded from the score.
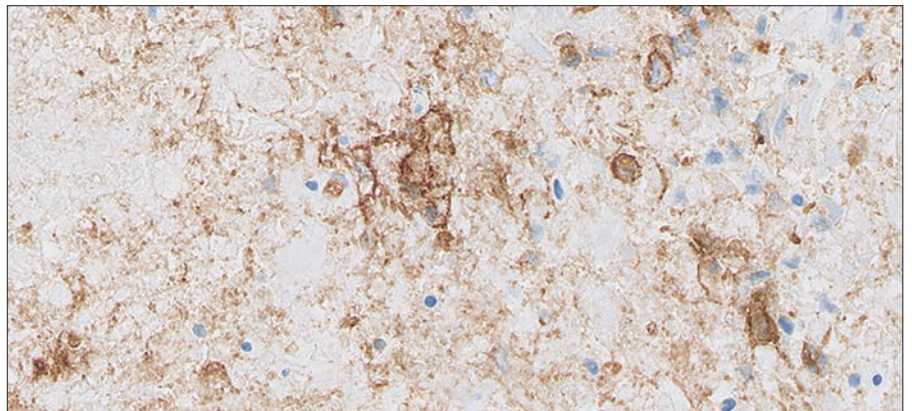
Figure 40: Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting staining of necrosis; necrosis staining should be excluded from the score (20× magnification).

Key Point

Scoring of necrotic areas should be excluded from the CPS calculation

PD-L1 IHC 22C3 pharmDx CPS Case Examples
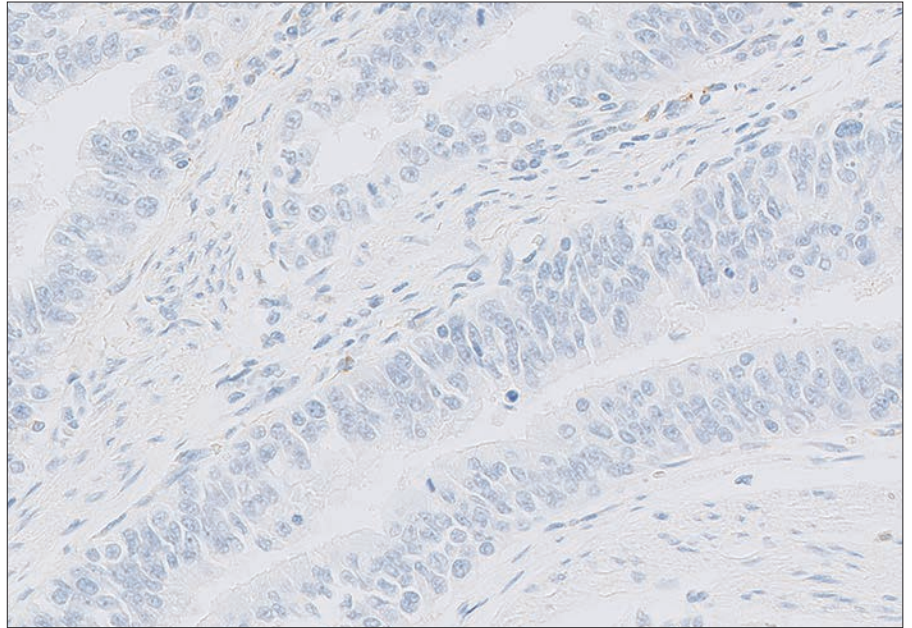
CPS < 10 Case Examples

Case 1: CPS < 10

Figure 41: Esophageal cancer specimen stained with PD-L1 antibody exhibiting a CPS of 0 (20× magnification).
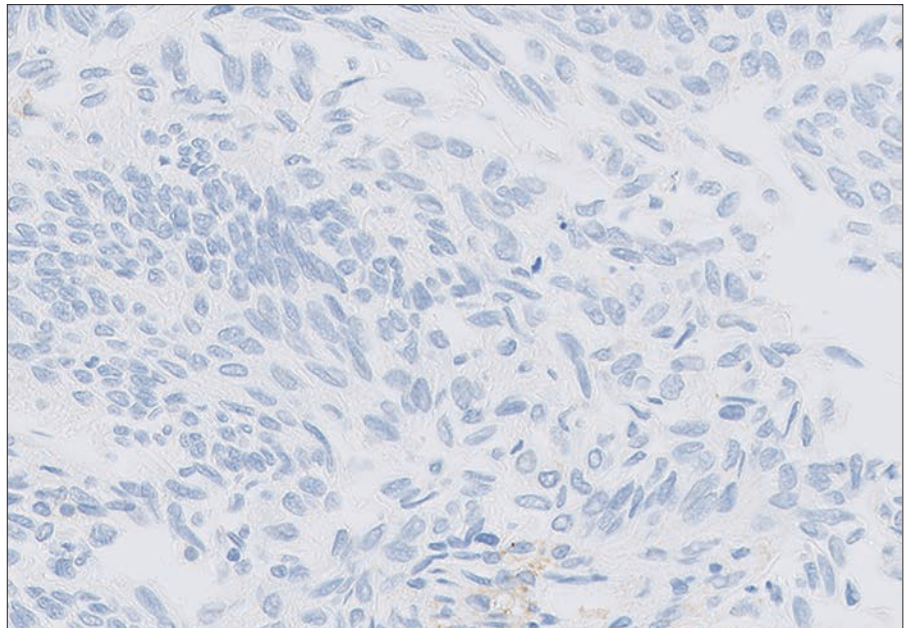
Case 2: CPS < 10

Figure 42: Esophageal cancer specimen stained with PD-L1 antibody exhibiting a CPS of 0 (20× magnification).
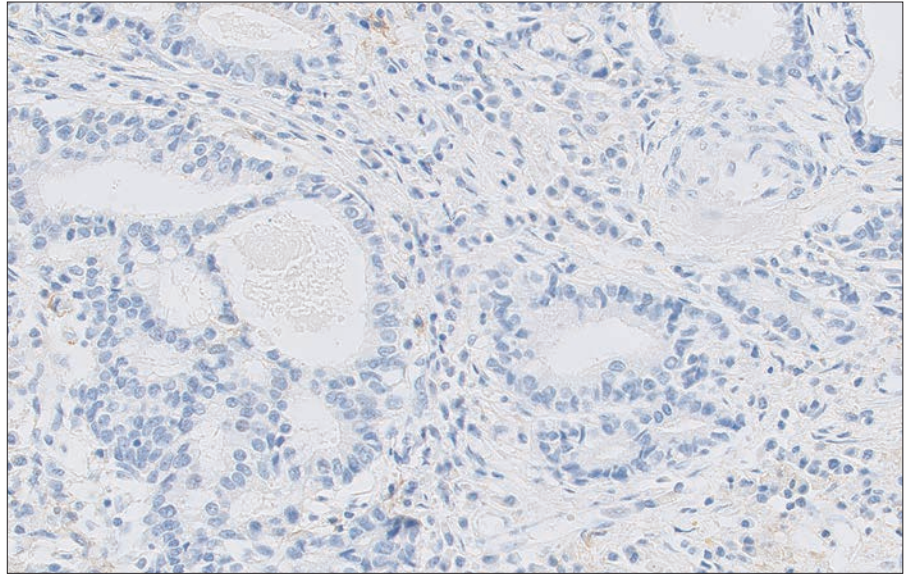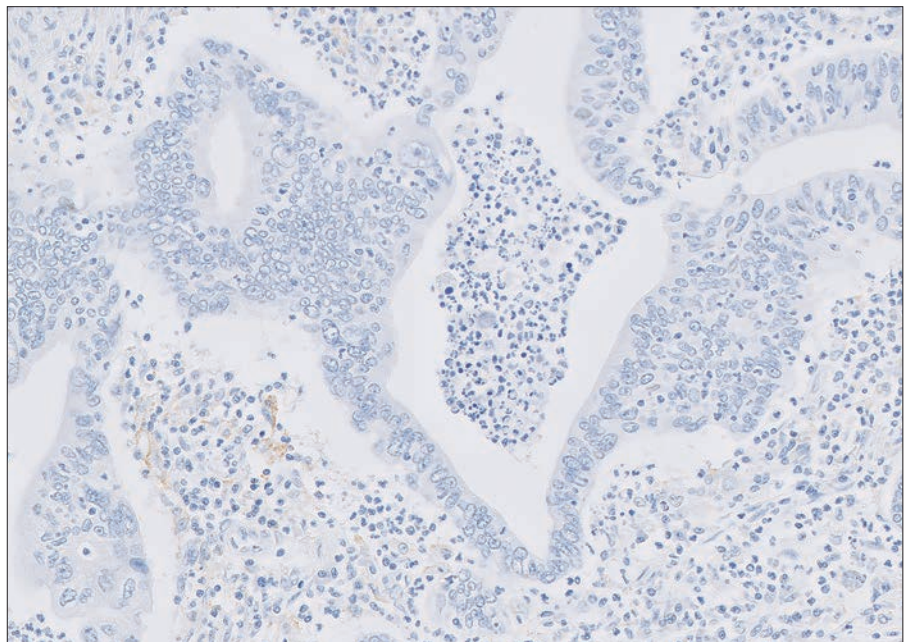
Case 3: CPS < 10

Figure 43: Esophageal cancer specimen stained with PD-L1 antibody exhibiting a CPS of 2, however any numerical CPS between 1–3 could be assigned to this image (20× magnification).
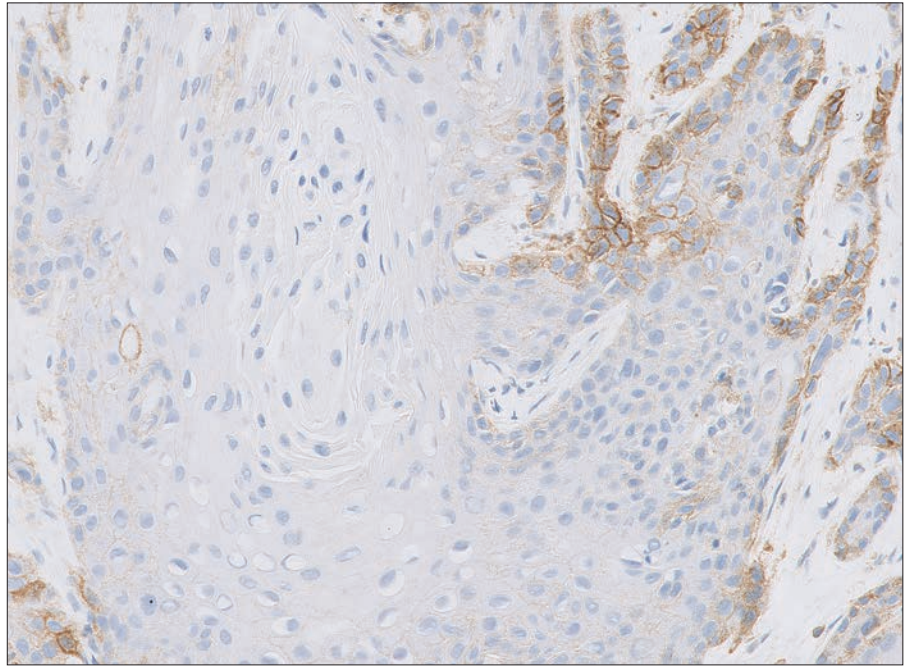
Case 4: CPS < 10

Figure 44: Esophageal cancer specimen stained with PD-L1 antibody exhibiting a CPS of 2, however any numerical CPS between 1–3 could be assigned to this image (20× magnification).

CPS ≥ 10 Case Examples

Case 5: CPS ≥ 10

Figure 45: Esophageal cancer 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).
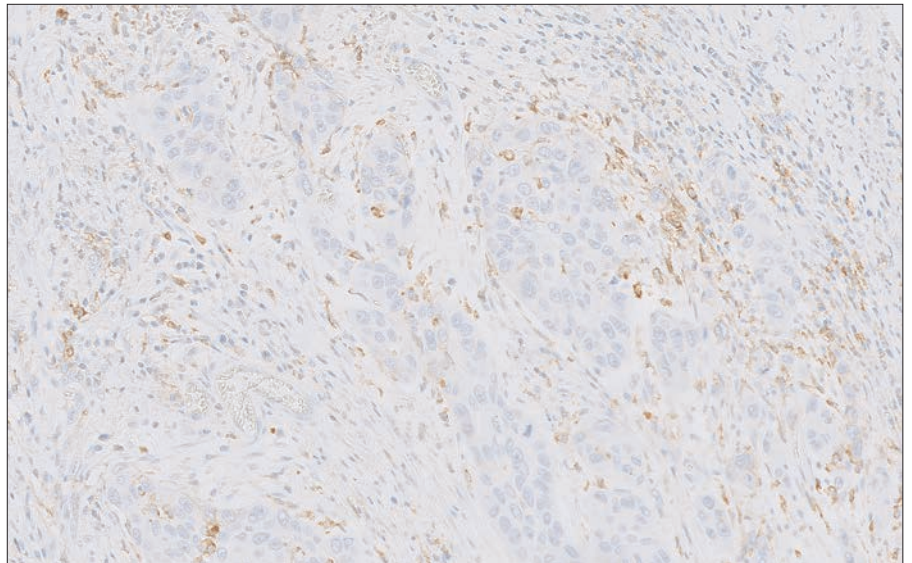
Case 6: CPS ≥ 10

Figure 46: Esophageal cancer 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).
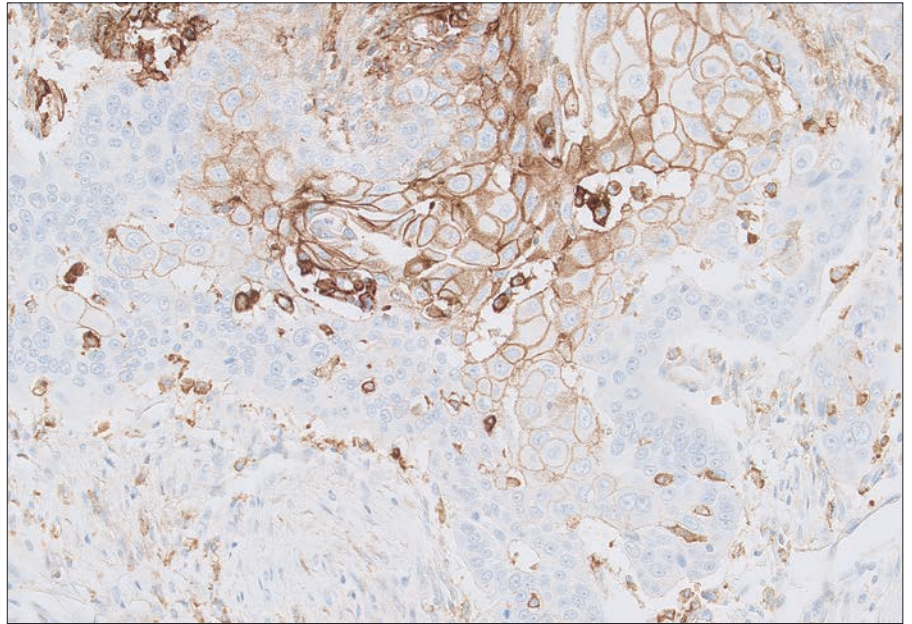
Case 7: CPS ≥ 10

Figure 47: Esophageal cancer 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).
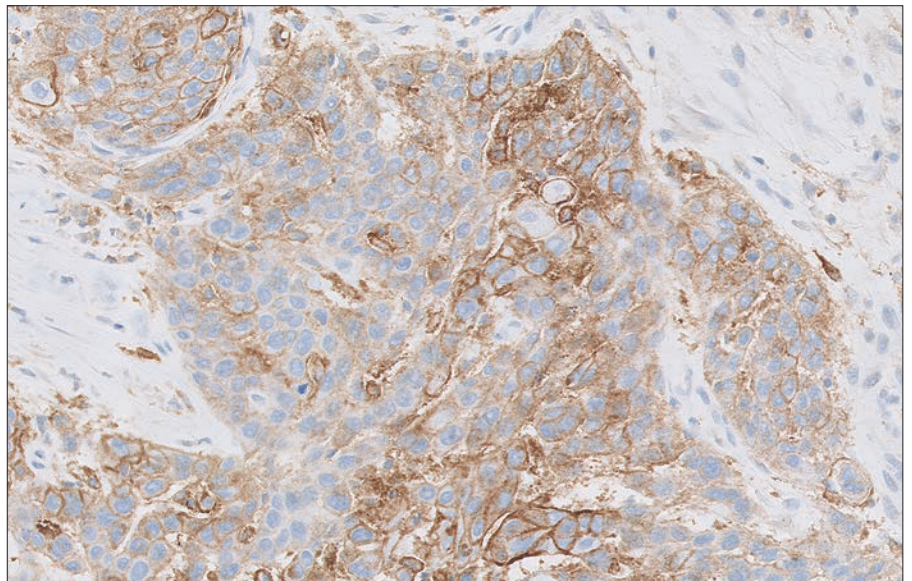
Case 8: CPS ≥ 10

Figure 48: Esophageal cancer 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 Cutoff Case Examples (CPS Range of Greater Than or Equal to 1 but Less Than 10)

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

Figure 49: Esophageal cancer 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 Cutoff (CPS Range of Greater Than or Equal to 1 but Less Than 10)
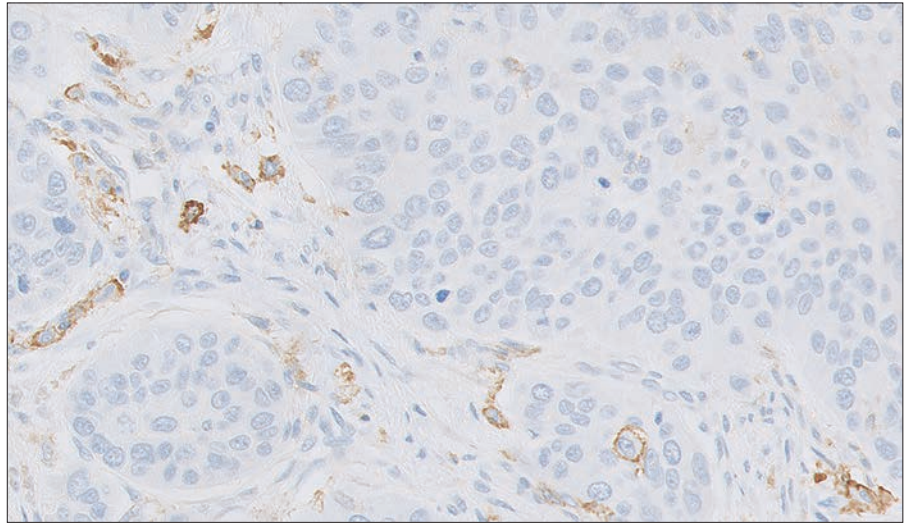

Figure 50: Esophageal cancer specimen stained with PD-L1 antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).

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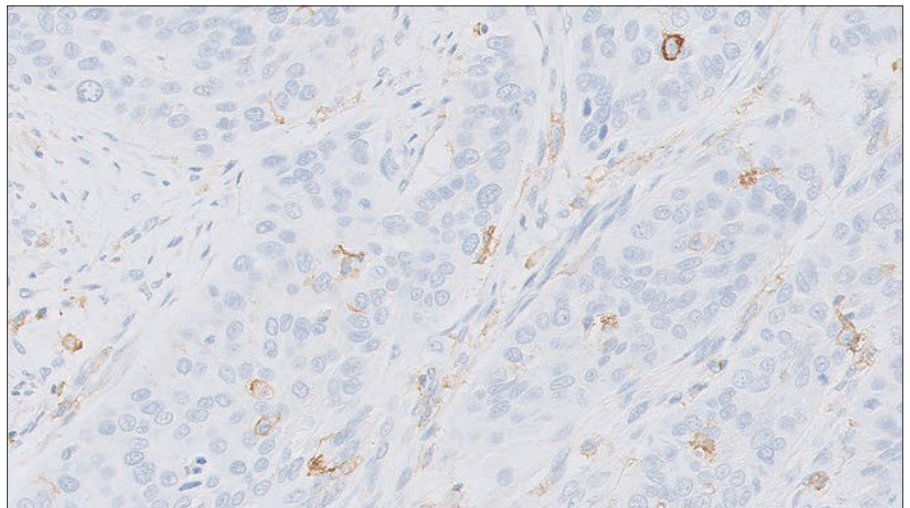

Figure 51: Esophageal cancer specimen stained with PD-L1 antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).

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

Figure 52: Esophageal cancer 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).

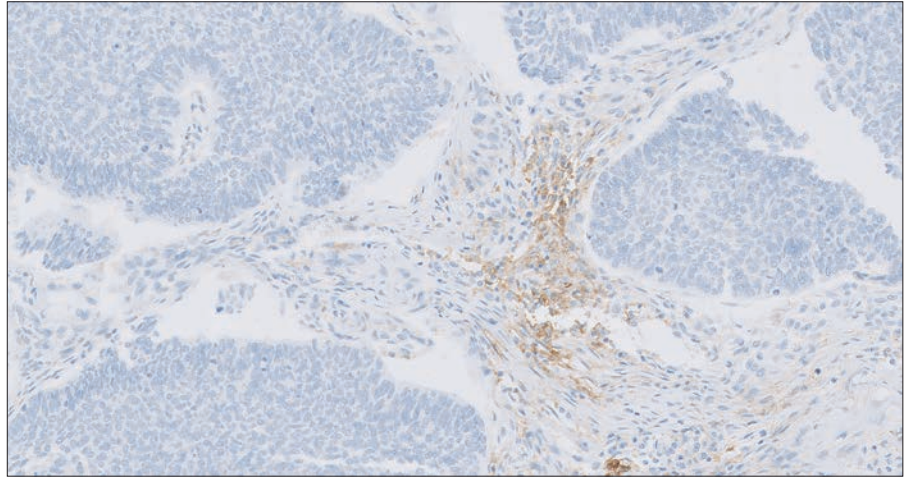## Challenging Case 5: Near Cutoff (CPS Range of Greater Than or Equal to 1 but Less Than 10)

Figure 53: Esophageal cancer 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).

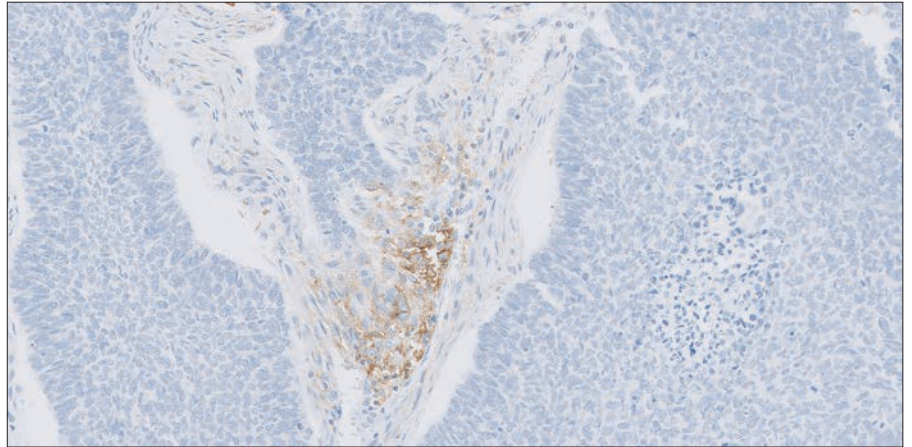## Challenging Case 6: Near Cutoff (CPS Range of Greater Than or Equal to 1 but Less Than 10)

Figure 54: Esophageal cancer 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 Cutoff Case Examples (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

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

Figure 55: Esophageal cancer specimen stained with PD-L1 antibody exhibiting a CPS of 17, however any numerical CPS between 14–20 could be assigned to this image (20× magnification).

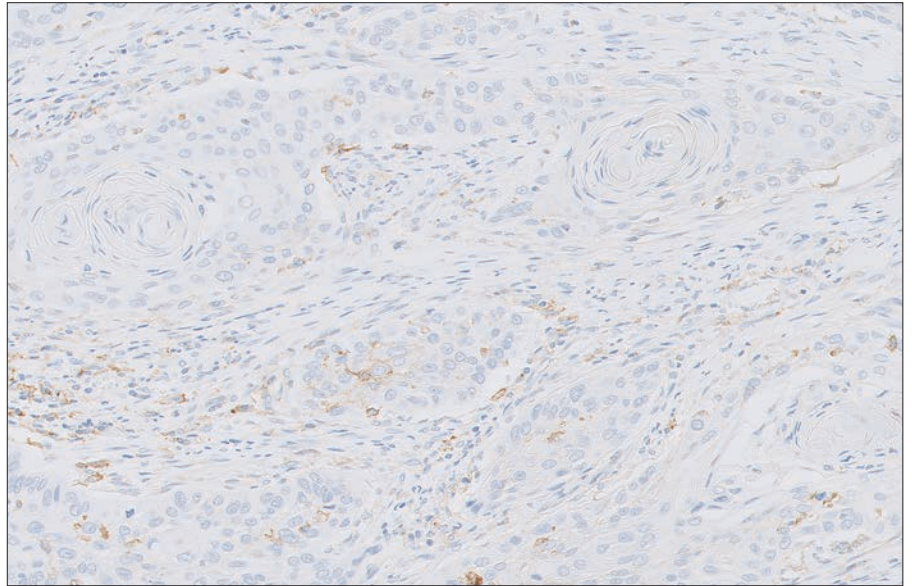### Challenging Case 8: Near Cutoff (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

Figure 56: Esophageal cancer 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).

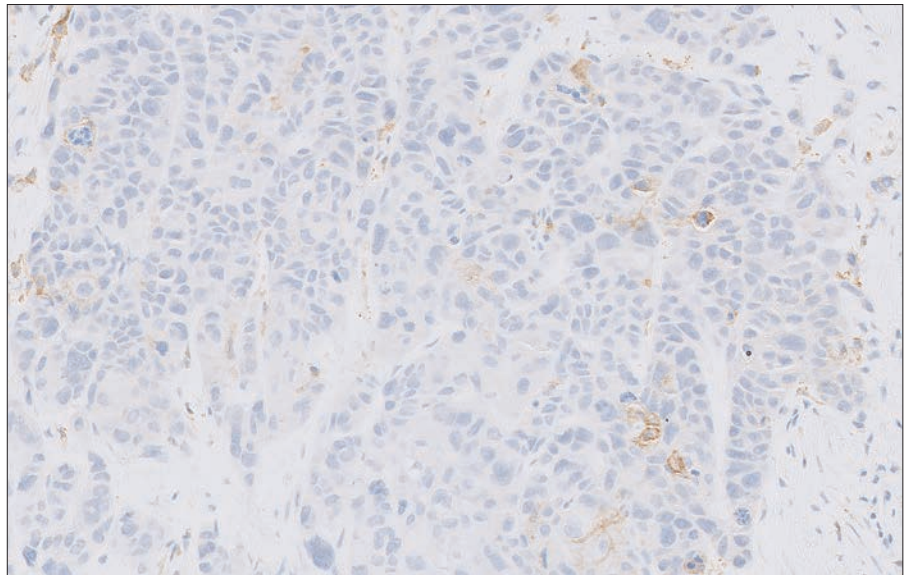## Challenging Case 9: Near Cutoff (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

Figure 57: Esophageal cancer 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 Cutoff (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)
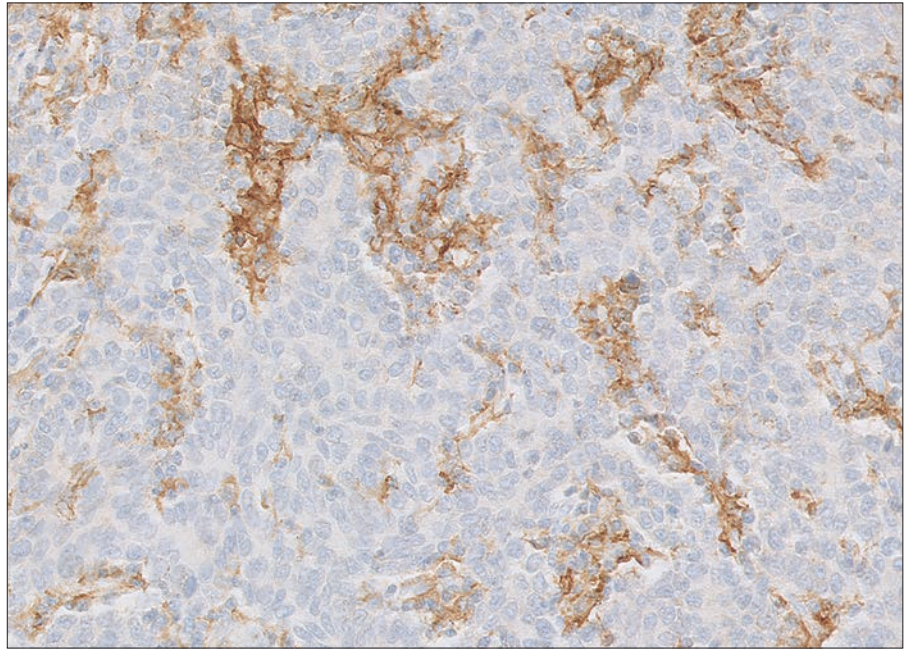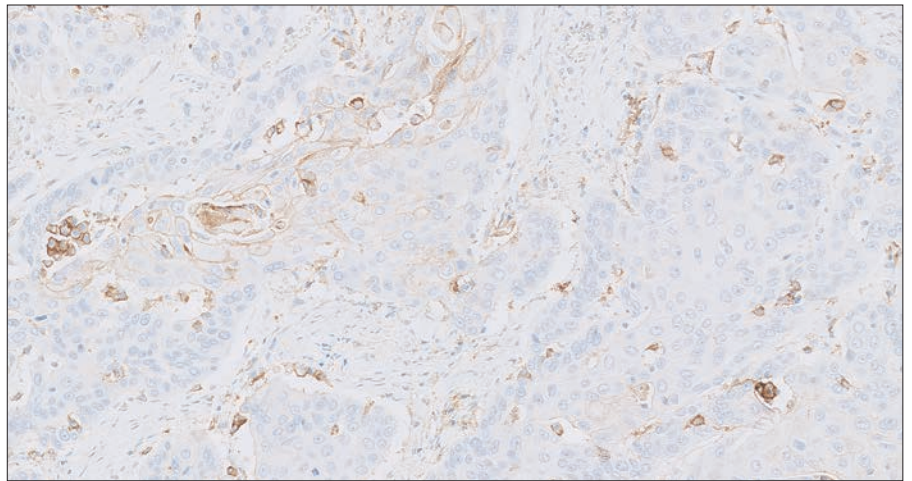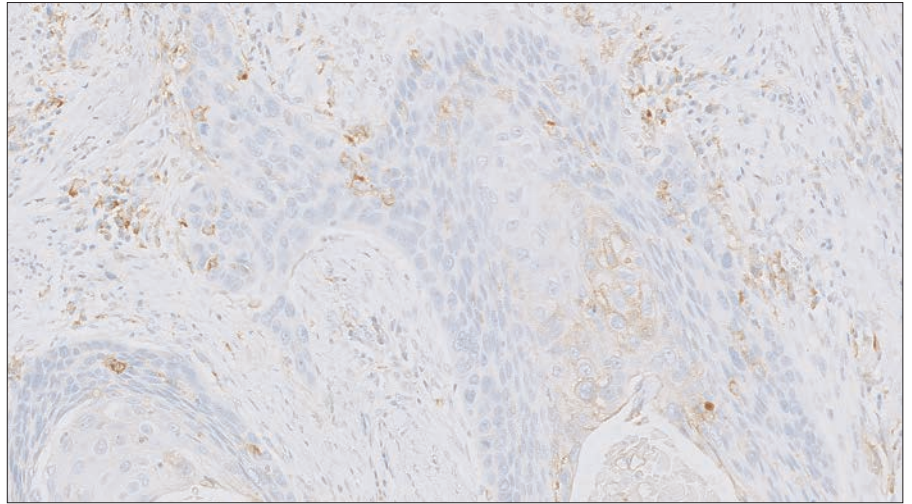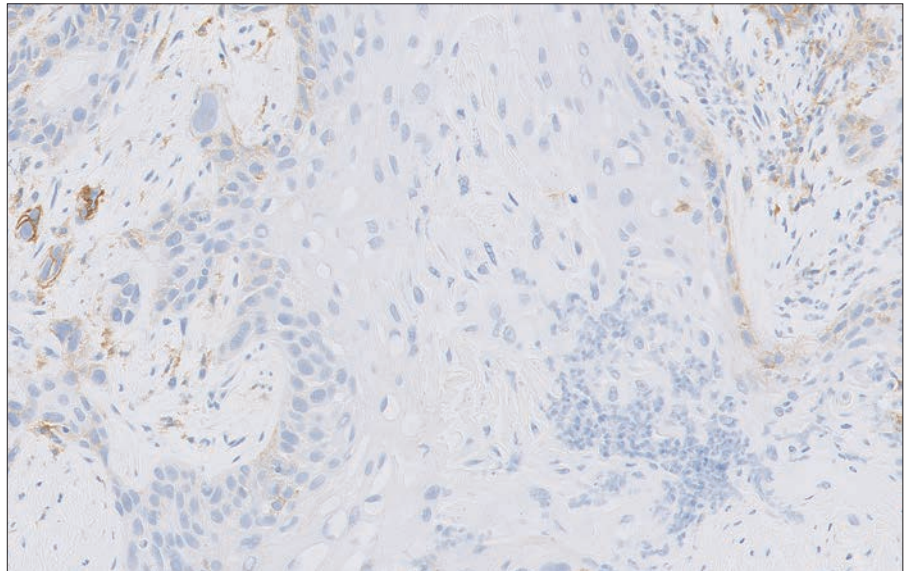

Figure 58: Esophageal cancer specimen stained with PD-L1 antibody exhibiting a CPS of 16, however any numerical CPS between 13–19 could be assigned to this image (20× magnification).

Control Cell Line (CCL) Appendix

Passing CCL

Passing PD-L1 Negative CCL

– No specific staining

– Non-specific staining is < 1+ intensity

– 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
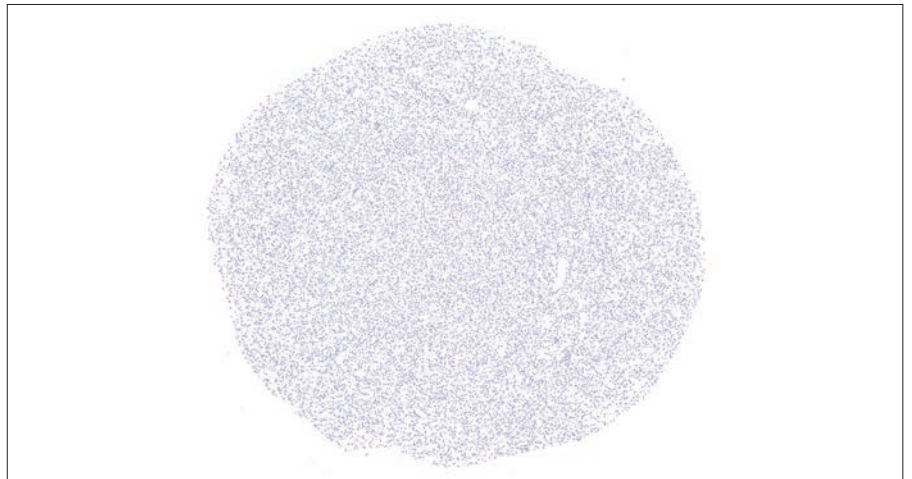
Figure 59: MCF-7 cell pellet (2× magnification).

Passing PD-L1 Positive CCL

– Cell membrane staining of ≥ 70% of cells

– ≥ 2+ average staining intensity

– Non-specific staining is < 1+ intensity

Figure 60: NCI-H226 cell pellet (2× magnification).

Borderline Passing CCL

Borderline Passing vs. Passing PD-L1 Positive CCL

Borderline Passing PD-L1 positive CCL

Figure 61: NCI-H226 cell pellet (2× magnification).

Evaluation Strategy for Borderline Passing PD-L1 Positive CCL

For a borderline PD-L1 positive CCL, to determine the total percentage of cells staining in the cell pellet and the average staining intensity of all cells in the pellet, the cell pellet can be split into quadrants and inspected at 20× magnification.

Quadrant 1

Quadrant 2

Quadrant 3
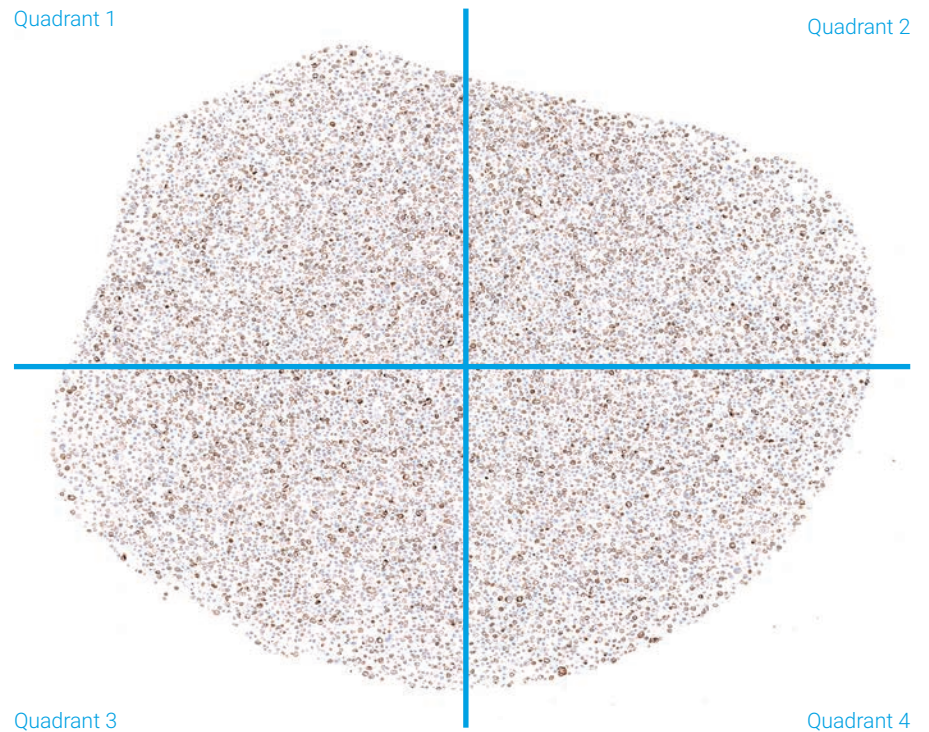
Quadrant 4

Quadrant 1

In Quadrant 1 approximately 70% of cells exhibit membrane staining, and the average staining intensity of all cells in this quadrant is ≥ 2+.

Figure 62: NCI-H226 cell pellet (5× magnification).

Quadrant 2

In Quadrant 2 approximately 75% of cells exhibit membrane staining, and the average staining intensity of all cells in this quadrant is ≥ 2+.
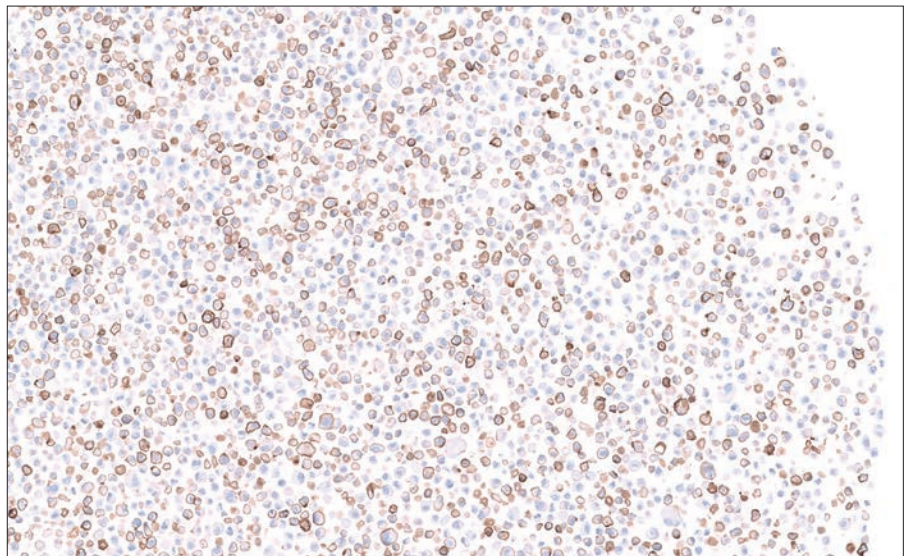
Figure 63: NCI-H226 cell pellet (5× magnification).

Quadrant 3

In Quadrant 3 approximately 70% of cells exhibit membrane staining, and the average staining intensity of all cells in this quadrant is ≥ 2+.
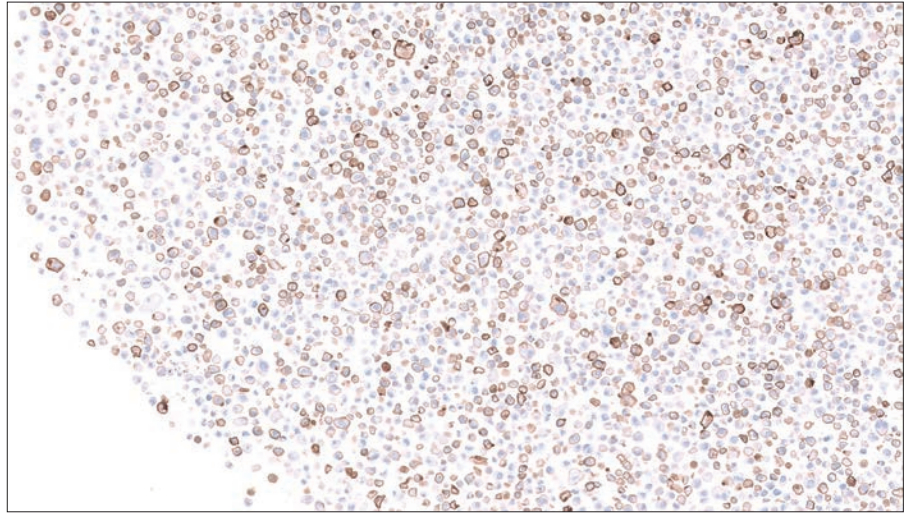
Figure 64: NCI-H226 cell pellet (5× magnification).

Quadrant 4

In Quadrant 4 approximately 65% of cells exhibit membrane staining, and the average staining intensity of all cells in this quadrant is ≥ 2+.
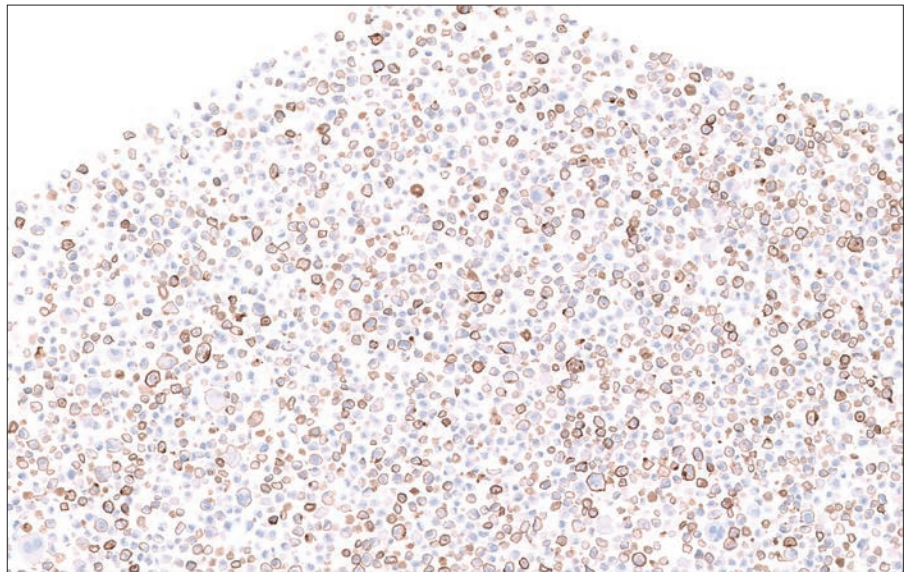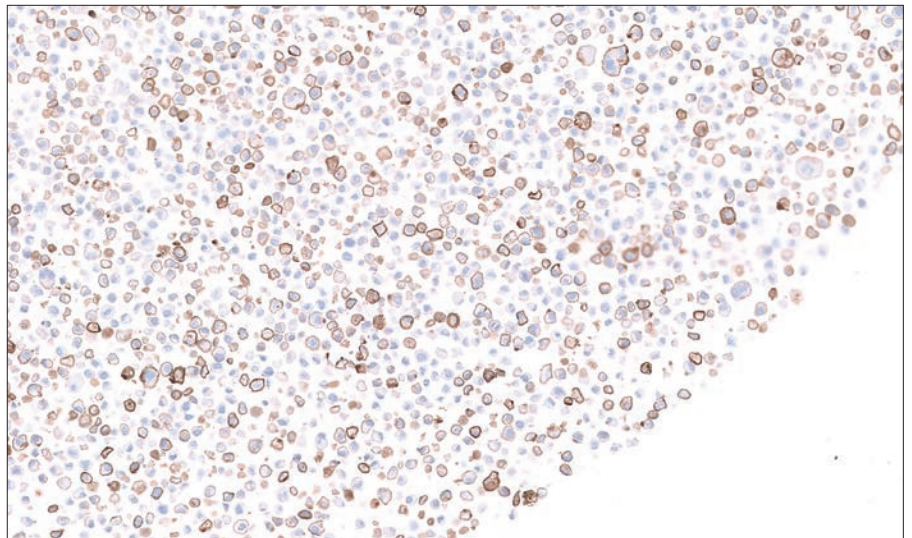

Figure 65: NCI-H226 cell pellet (5× magnification).

Calculation

1. Calculate the average percentage of cells exhibiting membrane staining across all 4 quadrants to estimate the total percentage of cells exhibiting membrane staining across the entire PD-L1 positive CCL pellet

2. Determine whether the average staining intensity across all cells in the pellet is ≥ 2+ intensity

Quadrant 1 = 70%

Quadrant 2 = 75%

Quadrant 3 = 70%

Quadrant 4 = 65%
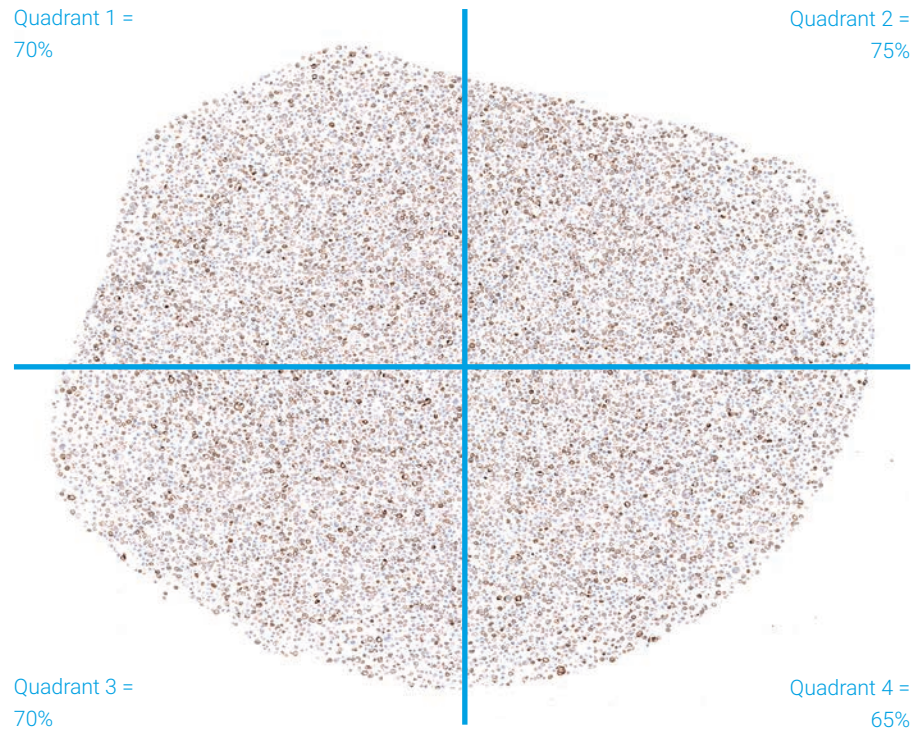
$$\frac{70 + 75 + 70 + 65}{4} = 70$$

– The overall percentage of cells with membrane staining = 70%

– The average staining intensity of all cells in the cell pellet is ≥ 2+

NCI-H226 positive control cell pellet meets acceptance criteria.

Failed CCL

Example 1: Passing PD-L1 Negative CCL with Failed PD-L1 Positive CCL

Passing PD-L1 negative CCL

– No specific staining

– Non-specific staining is < 1+ intensity

– The presence of ≤ 10 total cells with distinct plasma membrane staining, or cytoplasmic staining with ≥ 1+ intensity within the boundaries of the PD-L1 negative cell pellet are acceptable
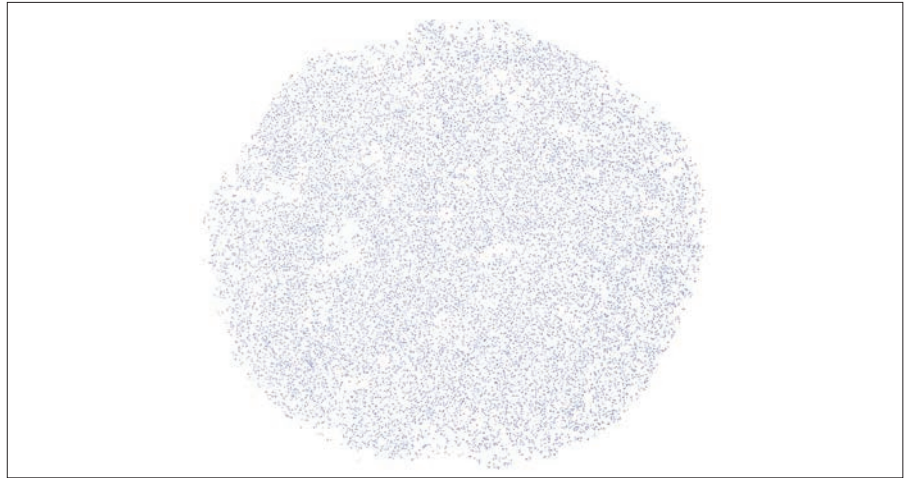
Figure 66: MCF-7 cell pellet (2× magnification).

Failed PD-L1 positive CCL

– Less than 70% of cells exhibit membrane staining, and the average staining intensity across all cells in the pellet is < 2+

Figure 67: NCI-H226 cell pellet (2× magnification).

See following images for higher magnification images depicting details of failure.

Failed PD-L1 positive CCL (10×)

Figure 68: NCI-H226 cell pellet (10× magnification).

Failed PD-L1 positive CCL (20×)

Figure 69: NCI-H226 cell pellet (20× magnification).

Example 2: Passing PD-L1 Negative CCL with Failed PD-L1 Positive CCL

Passing PD-L1 negative CCL

– No specific staining

– Non-specific staining is < 1+ intensity

– The presence of ≤ 10 total cells with distinct plasma membrane staining, or cytoplasmic staining with ≥ 1+ intensity within the boundaries of the PD-L1 negative cell pellet are acceptable
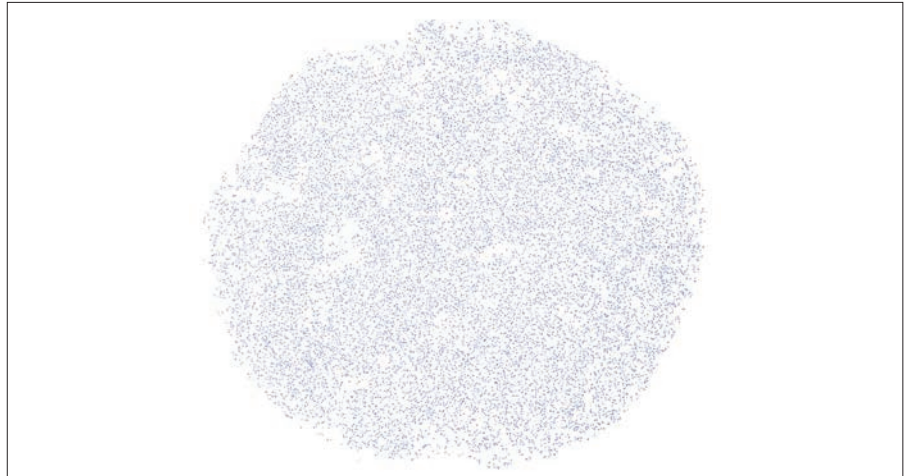
Figure 70: MCF-7 cell pellet (2× magnification).

Failed PD-L1 positive CCL

– Less than 70% of cells exhibit membrane staining, and the average staining intensity across all cells in the pellet is < 2+

Figure 71: NCI-H226 cell pellet (2× magnification).

See following images for higher magnification images depicting details of failure.

Failed PD-L1 positive CCL (10×)
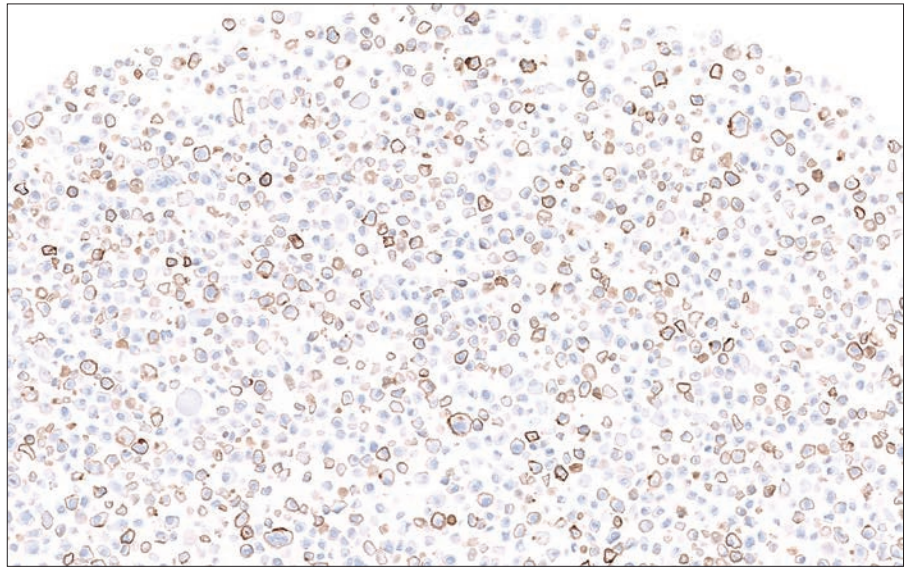
Figure 72: NCI-H226 cell pellet (10× magnification).

Failed PD-L1 positive CCL (20×)
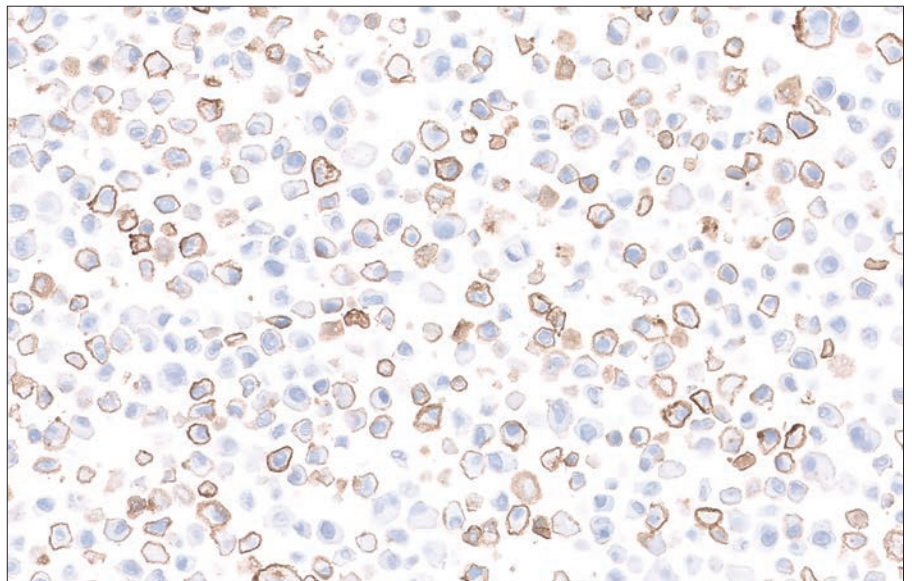
Figure 73: NCI-H226 cell pellet (20× magnification).

Example 3: Passing PD-L1 Positive CCL with Failed PD-L1 Negative CCL

Passing PD-L1 positive CCL

- Cell membrane staining of ≥ 70% of cells

- ≥ 2+ average staining intensity

- Non-specific staining is < 1+

Figure 74: NCI-H226 cell pellet (2× magnification).

Failed PD-L1 negative CCL

- Non-specific (nuclear) staining is ≥ 1+ staining intensity

- There are > 10 total cells with distinct plasma membrane or cytoplasmic staining that is ≥ 1+ intensity
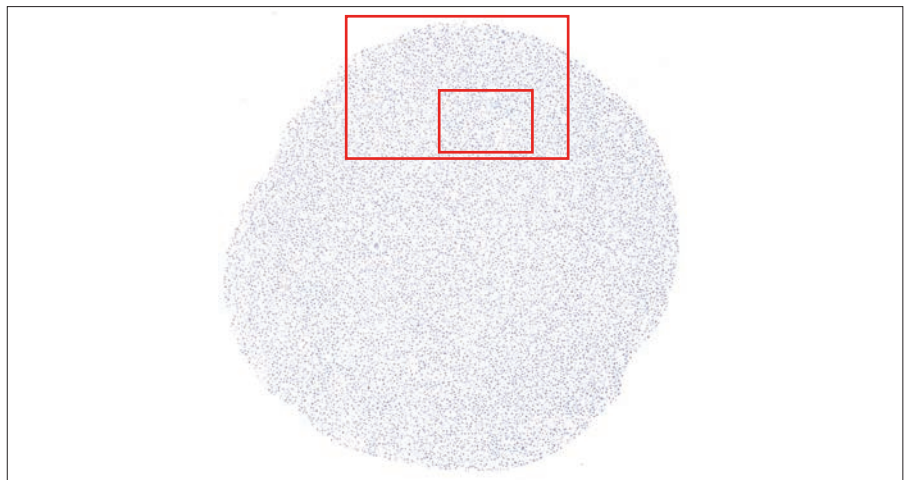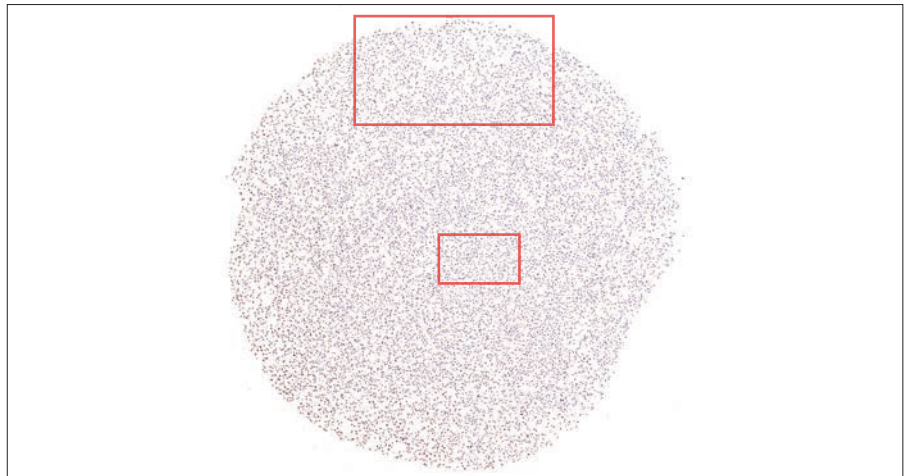

Figure 75: MCF-7 cell pellet (2× magnification).

See following images for higher magnification images depicting details of failure.
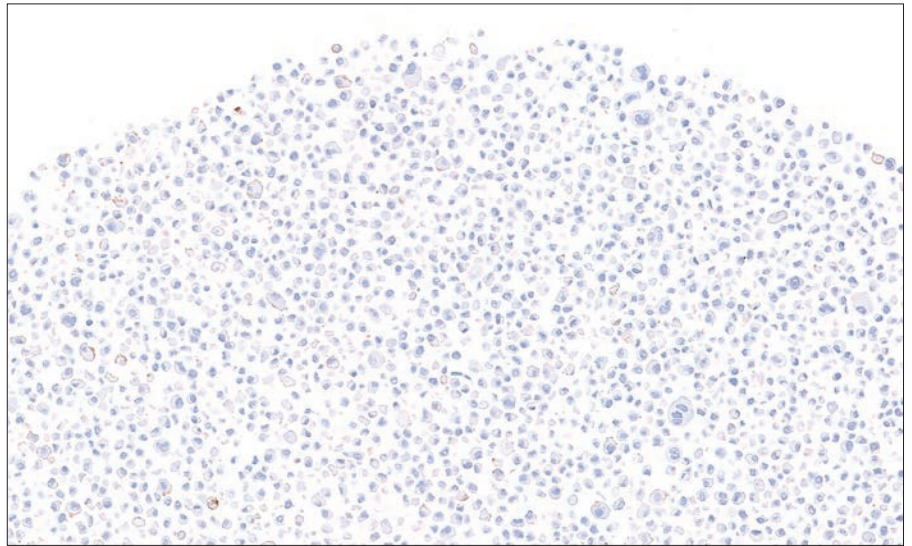
Failed PD-L1 negative CCL (10×)

Figure 76: MCF-7 cell pellet (10× magnification).
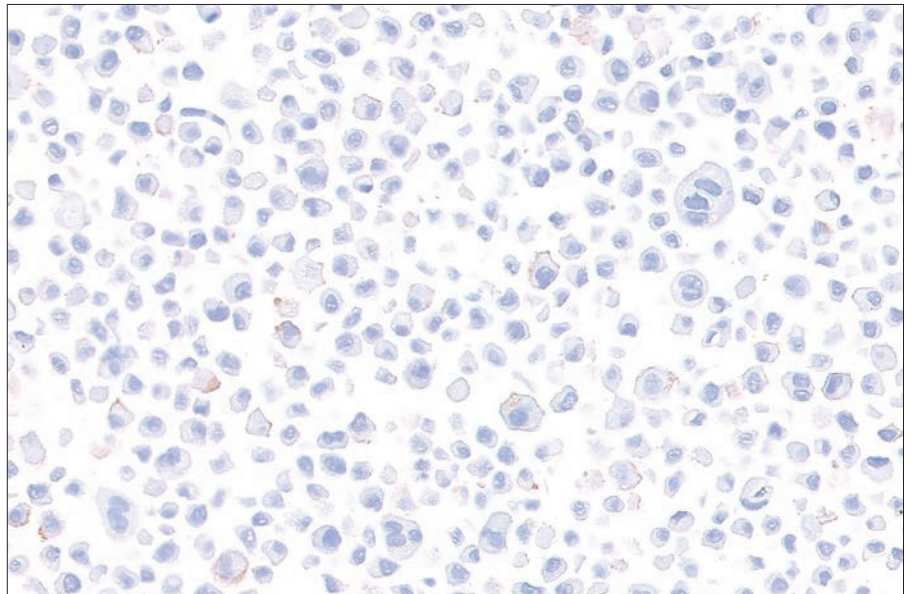
Failed PD-L1 negative CCL (20×)

Figure 77: MCF-7 cell pellet (20× magnification).

Troubleshooting Guide

Troubleshooting Guidelines for PD-L1 IHC 22C3 pharmDx

For further troubleshooting help, contact your local Agilent representative.

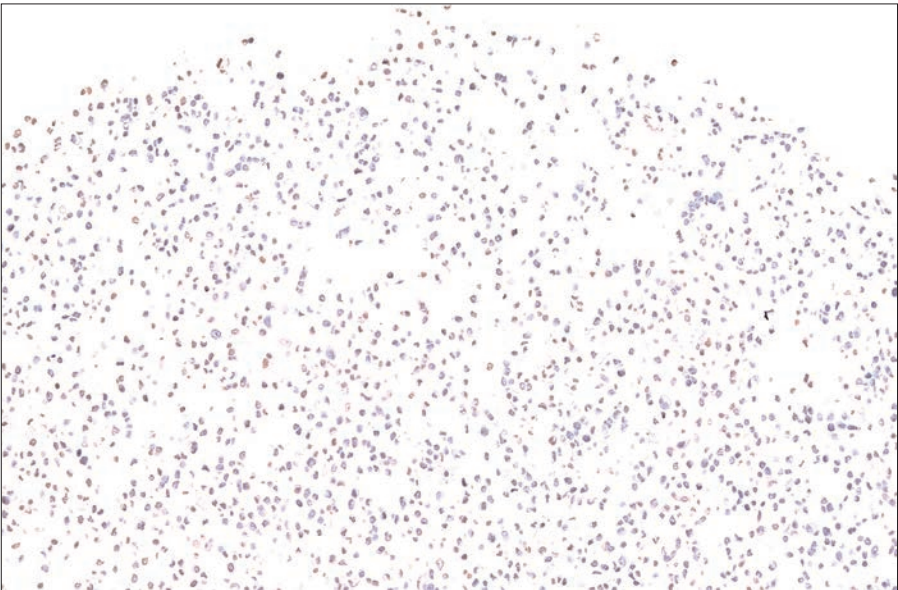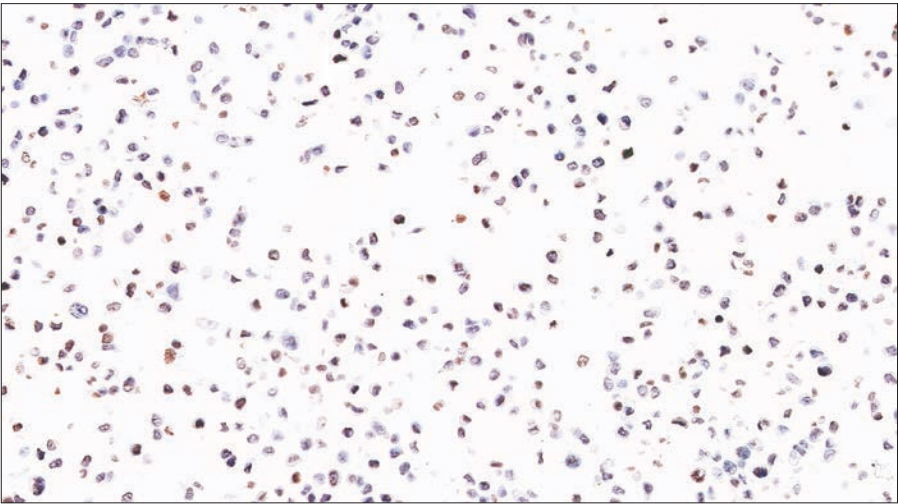| Problem	Probable Cause	Suggested Action
No staining of slides	Programming error	Verify that the PD-L1 IHC 22C3 pharmDx program was selected for programming of slides
	Lack of reaction with DAB+ Substrate-Chromogen Solution (DAB)	Verify that DAB+ Substrate-Chromogen Solution was prepared properly
	Sodium azide in wash buffer	Use only EnVision FLEX Wash Buffer (20×) (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 EnVision FLEX Wash Buffer (20×) (Code K8007)
Weak staining of specimen slides or of the positive cell line on the Agilent-provided Control Slide	Inadequate target retrieval	Verify that the 3-in-1 pre-treatment procedure was correctly performed
	Inappropriate wash buffer used	Use only EnVision FLEX Wash Buffer (20×) (Code K8007)
Excessive background staining of slides	Paraffin incompletely 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 charged slides (such as Superfrost Plus)
	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	Use only EnVision FLEX Wash Buffer (20×) (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

Problem	Probable Cause	Suggested Action
1× Target Retrieval Solution does not meet pH specifications	pH meter is not calibrated correctly	Ensure pH meter is calibrated per manufacturer's recommendations. After re-calibration, re-test the pH of 1× Target Retrieval Solution. Do not modify the pH of 1× Target Retrieval Solution. If the pH is outside the acceptable range (6.1 ± 0.2), discard 1× Target Retrieval Solution. Prepare new 1× Target Retrieval Solution. Check the pH of the new 1× Target Retrieval Solution
	Inferior quality water is used to dilute the Target Retrieval Solution concentrate	Ensure that distilled or deionized water is used to prepare 1× Target Retrieval Solution
	Incorrect Target Retrieval Solution is used	Ensure that the correct Target Retrieval Solution specified in "Materials Required but not Supplied" and/or "Reagent Preparation" section(s) is used

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

Clinical Performance Evaluation

KEYNOTE-590: Controlled Study of Combination Therapy in Esophageal Carcinoma Patients Naïve to Treatment

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

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

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

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

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

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

The primary efficacy outcome measures were OS and PFS as assessed by the investigator according to RECIST 1.1 in squamous cell histology, CPS ≥ 10, and in all patients. The study demonstrated a statistically significant improvement in OS and PFS for all pre-specified study populations. In all patients randomized to pembrolizumab in combination with chemotherapy, compared to chemotherapy the OS HR was 0.73 (95% CI 0.62−0.86) and the PFS HR was 0.65 (95% CI 0.55−0.76). Secondary efficacy outcome measures were ORR and DoR, according to RECIST 1.1 as assessed by the investigator. Table 5 summarizes key efficacy measures from the pre-specified analysis in patients whose tumors expressed PD-L1 with a CPS ≥ 10 in KEYNOTE-590 performed at a median follow-up time of 13.5 months (range: 0.5 to 32.7 months). The Kaplan-Meier curve for OS and PFS are shown in Figures 78 and 79, respectively.

Table 5: Efficacy Results for Pembrolizumab Plus Chemotherapy in KEYNOTE-590 with PD-L1 Expression (CPS ≥ 10)

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

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

A total of 32 patients aged ≥ 75 years for PD-L1 CPS ≥ 10 were enrolled in KEYNOTE-590 (18 in the pembrolizumab combination and 14 in the control). Data about efficacy and safety of pembrolizumab in combination with chemotherapy are too limited in this patient population.
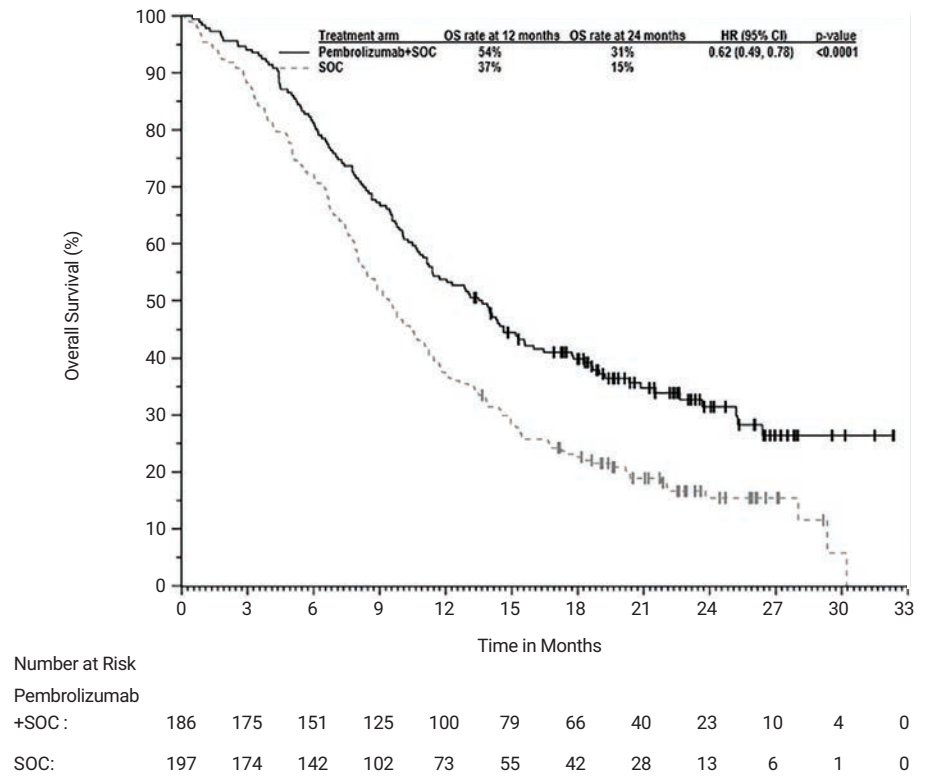
Treatment arm	OS rate at 12 months	OS rate at 24 months	HR (95% CI)	p-value
Pembrolizumab+SOC	54%	31%	0.62 (0.49, 0.78)	<0.0001
SOC	37%	15%		

Number at Risk

Pembrolizumab +SOC :	186	175	151	125	100	79	66	40	23	10	4	0
SOC:	197	174	142	102	73	55	42	28	13	6	1	0

Figure 78: Kaplan-Meier Curve for Overall Survival by Treatment Arm in KEYNOTE-590 with PD-L1 Expression (CPS ≥ 10).

Treatment arm	PFS rate at 12 months	PFS rate at 18 months	HR (95% CI)	p-value
Pembrolizumab+SOC	30%	21%	0.51 (0.41, 0.65)	<0.0001
SOC	9%	5%		

Number at Risk

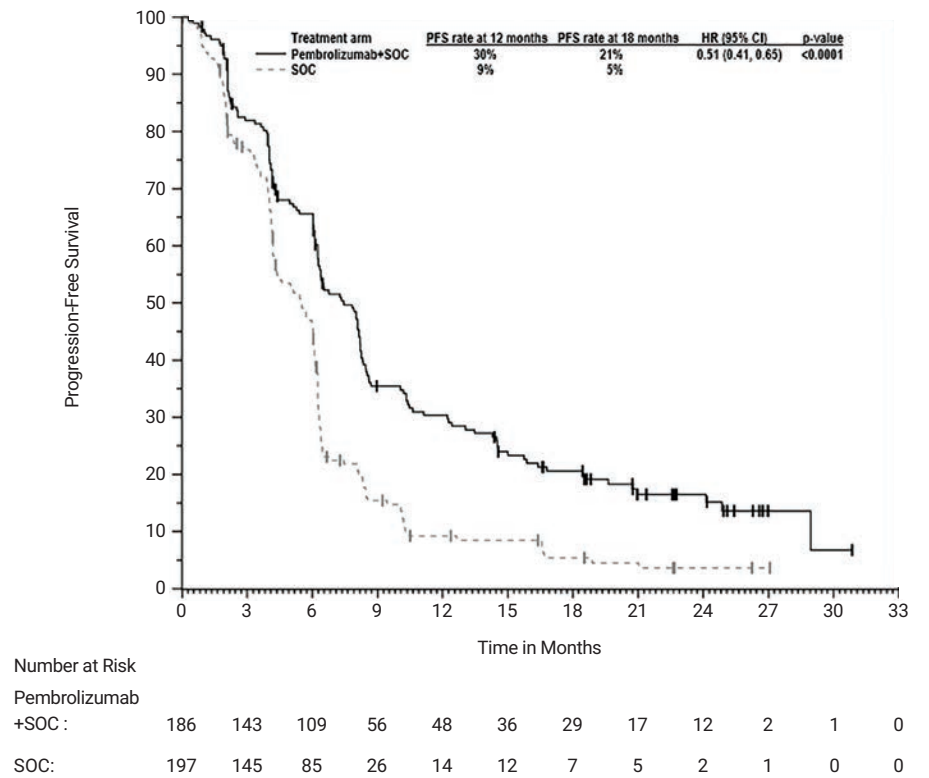|												
Pembrolizumab +SOC :	186	143	109	56	48	36	29	17	12	2	1	0
SOC:	197	145	85	26	14	12	7	5	2	1	0	0

Figure 79: Kaplan-Meier Curve for Progression-Free Survival by Treatment Arm in KEYNOTE-590 with PD-L1 Expression (CPS ≥ 10).

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Notes

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a. PD-L1 IHC 22C3 pharmDx [Instructions for Use]. Santa Clara, CA: Agilent Technologies, Inc.; 2021. **b.** Keytruda [Summary of Product Characteristics]. European Medicines Agency; 2021. **c.** Data on file. Agilent Technologies, Inc.

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