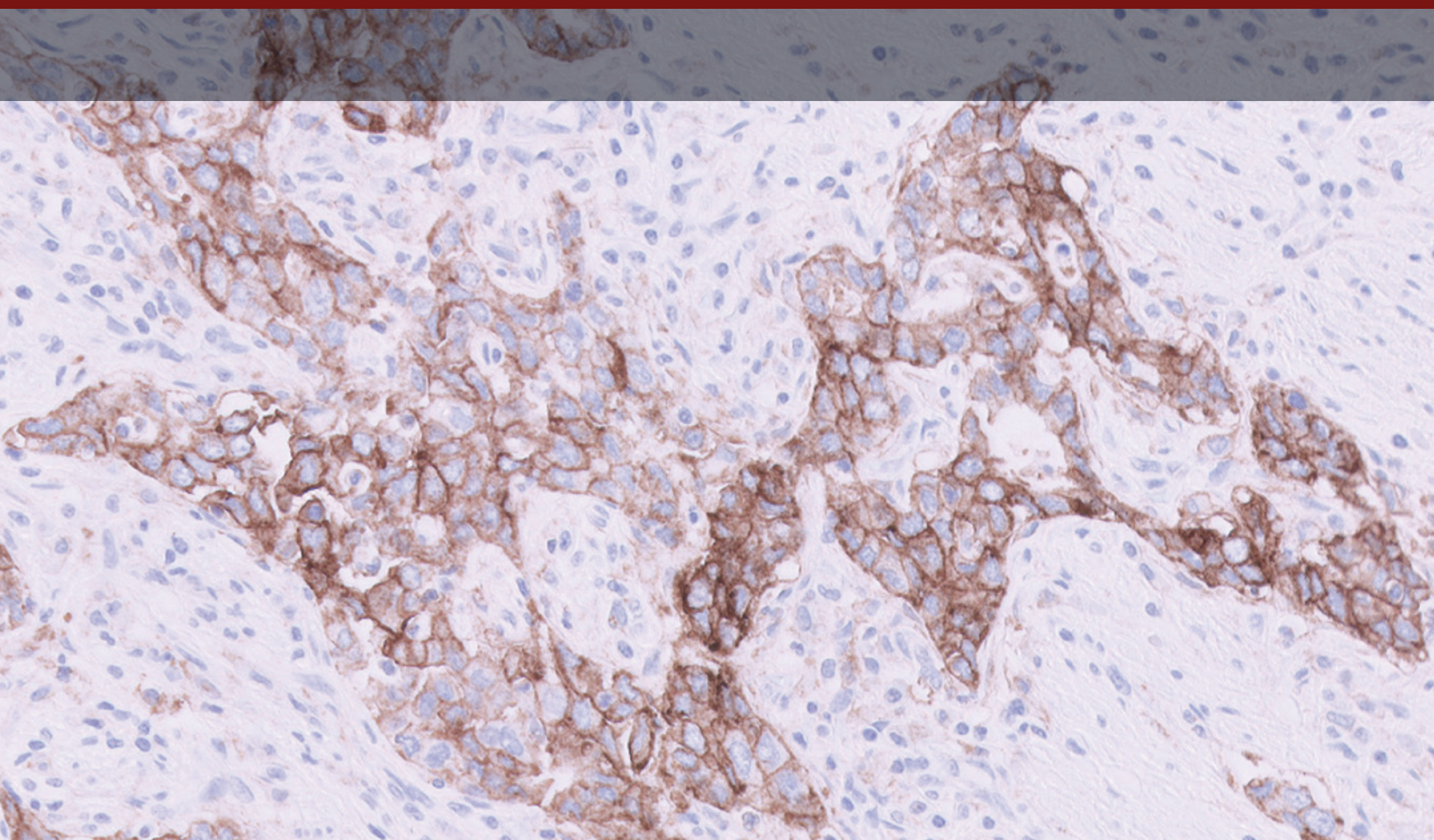


# PD-L1 IHC 22C3 pharmDx Interpretation Manual – Gastric or Gastroesophageal Junction Adenocarcinoma

FDA-approved for in vitro diagnostic use

Rx only





# Table of Contents

<b>Intended Use</b>	<b>04</b>
<b>Introduction</b>	<b>06</b>
<b>PD-L1 Overview</b>	<b>08</b>
<b>PD-L1 IHC 22C3 pharmDx Overview</b>	<b>10</b>
<b>Kit Configuration (SK006)</b>	<b>11</b>
<b>Technical Considerations</b>	<b>12</b>
Specimen Preparation	12
Controls to Assess Staining Quality	12
Lab-supplied Control Tissue	12
Optional Additional Lab-supplied Control: Tonsil Tissue	13
Tissue Processing	13
<b>PD-L1 IHC 22C3 pharmDx Staining Procedure</b>	<b>14</b>
<b>Technical Checklist</b>	<b>17</b>
<b>Slide Evaluation</b>	<b>18</b>
General Considerations	18
Specimen Adequacy	18
Evaluating Controls	19
<b>Slide Evaluation Flowchart</b>	<b>25</b>
<b>Combined Positive Score</b>	<b>26</b>
Definition of Combined Positive Score (CPS)	26
CPS Numerator Inclusion and Exclusion Criteria	26
Determining Combined Positive Score	27
Suggested Methods	29
<b>Interpretation of CPS</b>	<b>32</b>
Identifying Patients with Gastric or GEJ Adenocarcinoma for Treatment	32
<b>Reporting Results</b>	<b>33</b>
<b>Combined Positive Score Summary and Examples</b>	<b>34</b>
Key Considerations in Scoring PD-L1 IHC 22C3 pharmDx Stained Specimens	34
Image Guide for Interpretation of PD-L1 IHC 22C3 pharmDx Staining in Gastric or GEJ Adenocarcinoma	35
<b>Artifacts</b>	<b>62</b>
<b>PD-L1 IHC 22C3 pharmDx CPS Case Examples</b>	<b>72</b>
CPS 0 Case Example	72
Near-Cutoff Negative Case Examples (CPS Range of Greater Than 0 but Less Than 1)	73
Near-Cutoff Positive Case Examples (CPS Range 1–10)	77
CPS ≥ 1 Case Examples	82
<b>Control Cell Line (CCL) Appendix</b>	<b>92</b>
<b>Troubleshooting Guide</b>	<b>104</b>
<b>References</b>	<b>106</b>

## 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) gastric or gastroesophageal junction (GEJ) adenocarcinoma tissue using EnVision FLEX visualization system on Autostainer Link 48.

### **Gastric or Gastroesophageal Junction (GEJ) Adenocarcinoma**

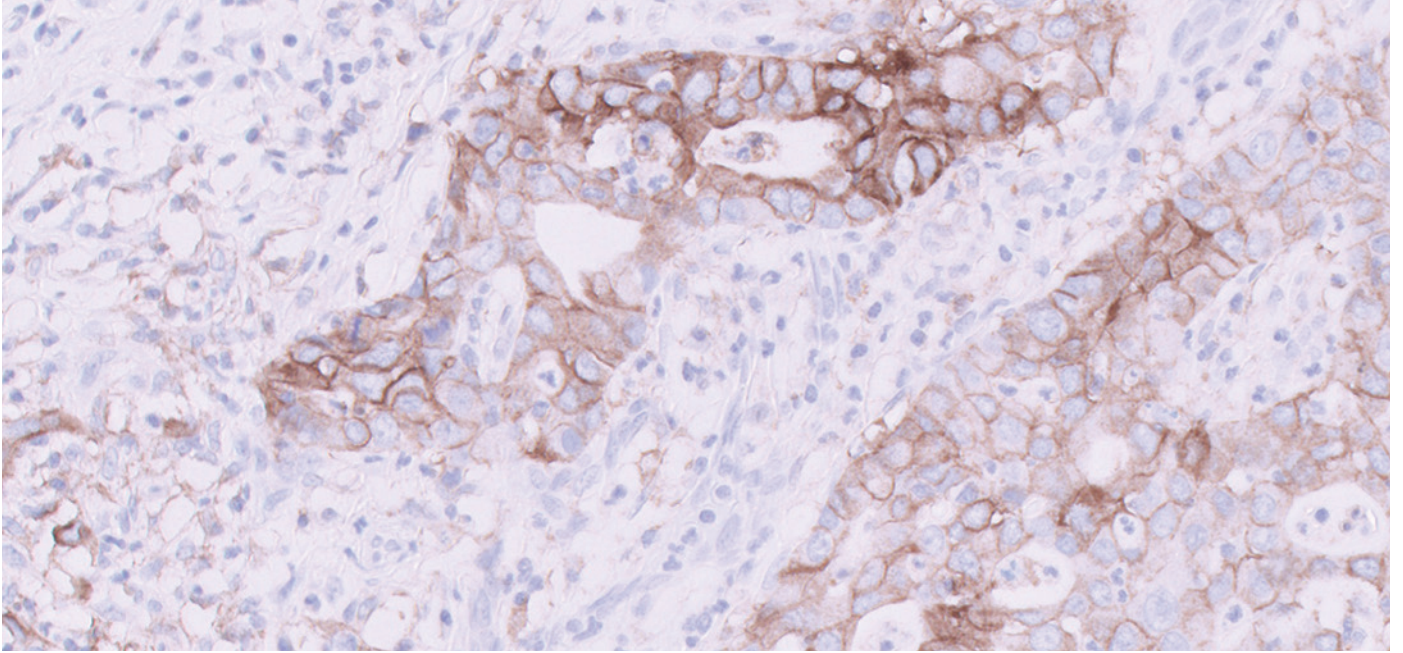
PD-L1 protein expression in gastric or GEJ adenocarcinoma is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. The specimen should be considered to have PD-L1 expression if  $CPS \geq 1$ .

PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying gastric or GEJ adenocarcinoma 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 companion diagnostic FDA-approved as an aid in identifying patients with gastric or GEJ adenocarcinoma for treatment with KEYTRUDA® (pembrolizumab).<sup>2</sup> 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 gastric or GEJ adenocarcinoma 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 gastric or GEJ adenocarcinoma 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 gastric or GEJ adenocarcinoma 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 gastric or GEJ adenocarcinoma 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.

Gastric or GEJ adenocarcinoma 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 < 1
- CPS ≥ 1

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 33.

## Photomicrographs

The included photomicrographs are of gastric or GEJ adenocarcinoma unless otherwise noted.

**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 (Gaithersburg, MD, USA), SageBio LLC (Sharon, MA, USA), IOM Ricera (Viagrande, Italy), and SELARL DIAG (Nice, France) with appropriate ethics approval and through Azenta Life Sciences.*

## 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.

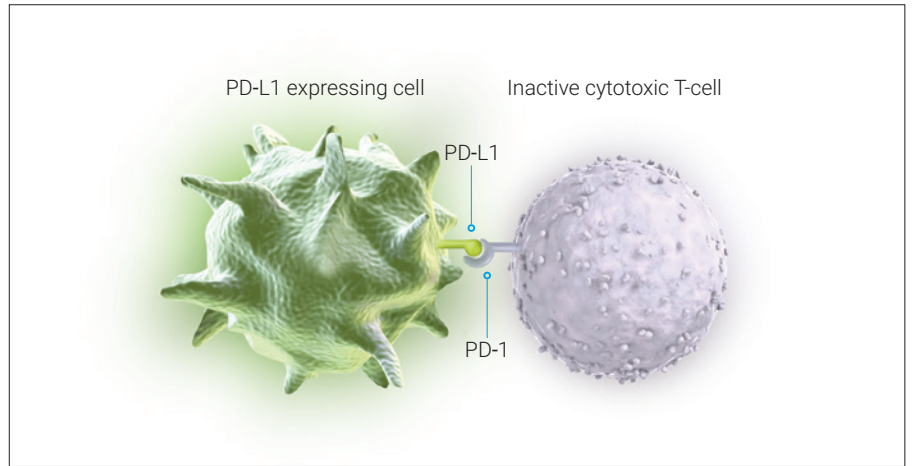
### The Tumor Escapes Detection by Utilizing the PD-1/PD-L1 Pathway

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.

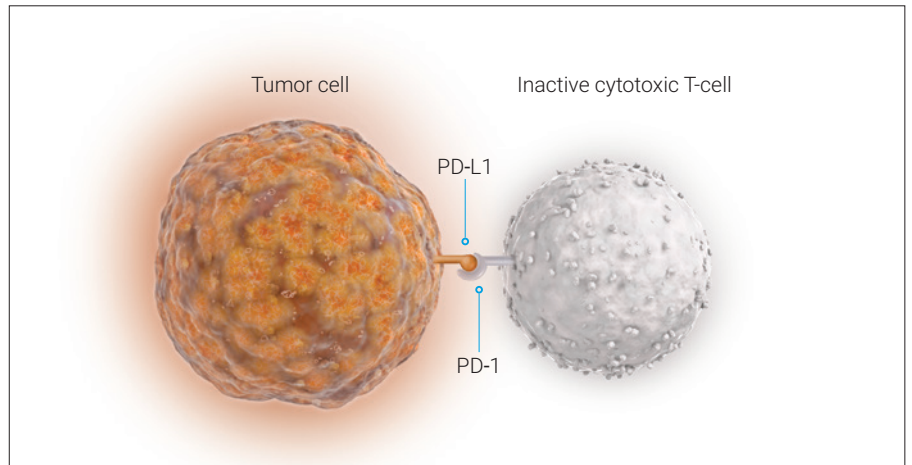
### Anti-PD-1 Therapy Enables the Immune Response Against Tumors

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.

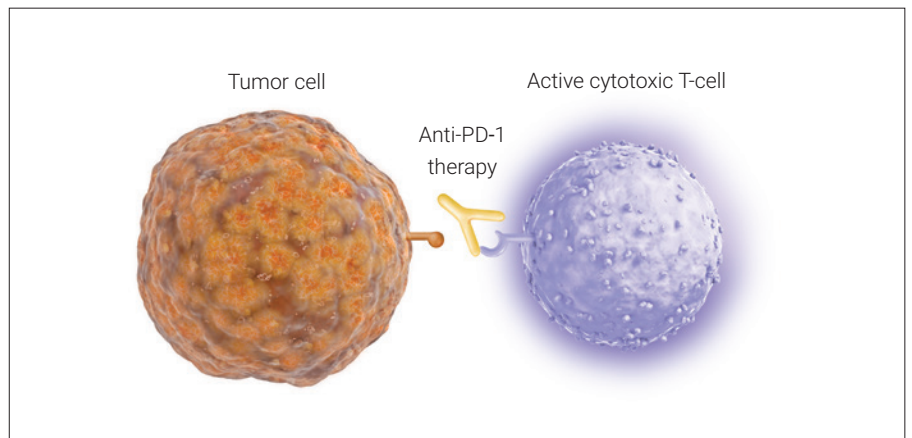
## The PD-1/PD-L1 Pathway



**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 gastric or GEJ adenocarcinoma 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 gastric or GEJ adenocarcinoma 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.

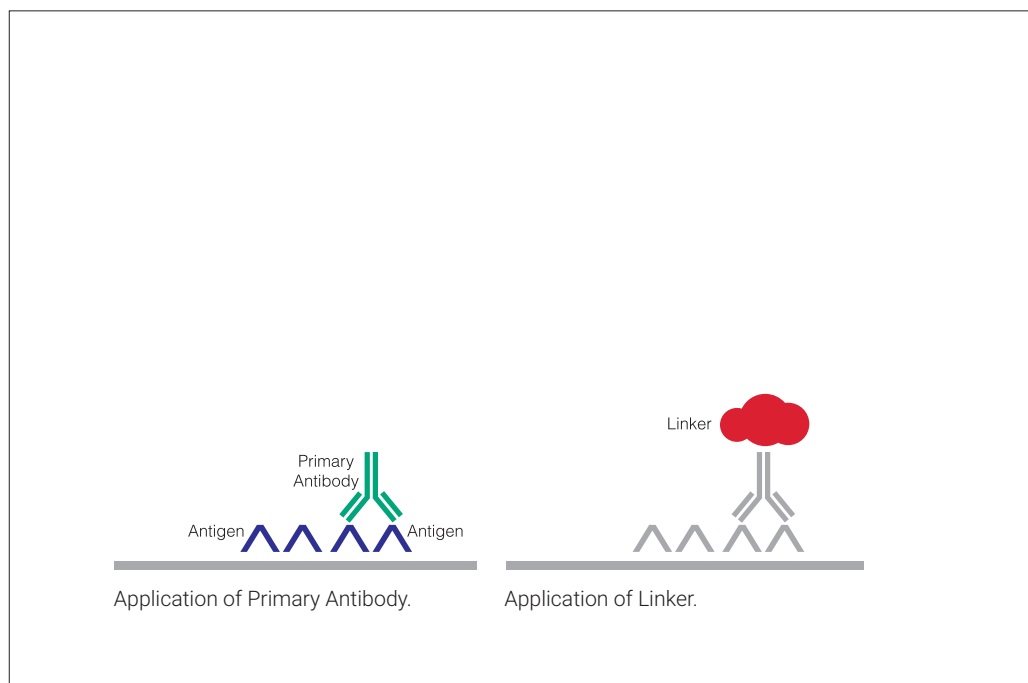


Figure 4: PD-L1 IHC 22C3 pharmDx staining procedure.

## Kit Configuration (SK006)



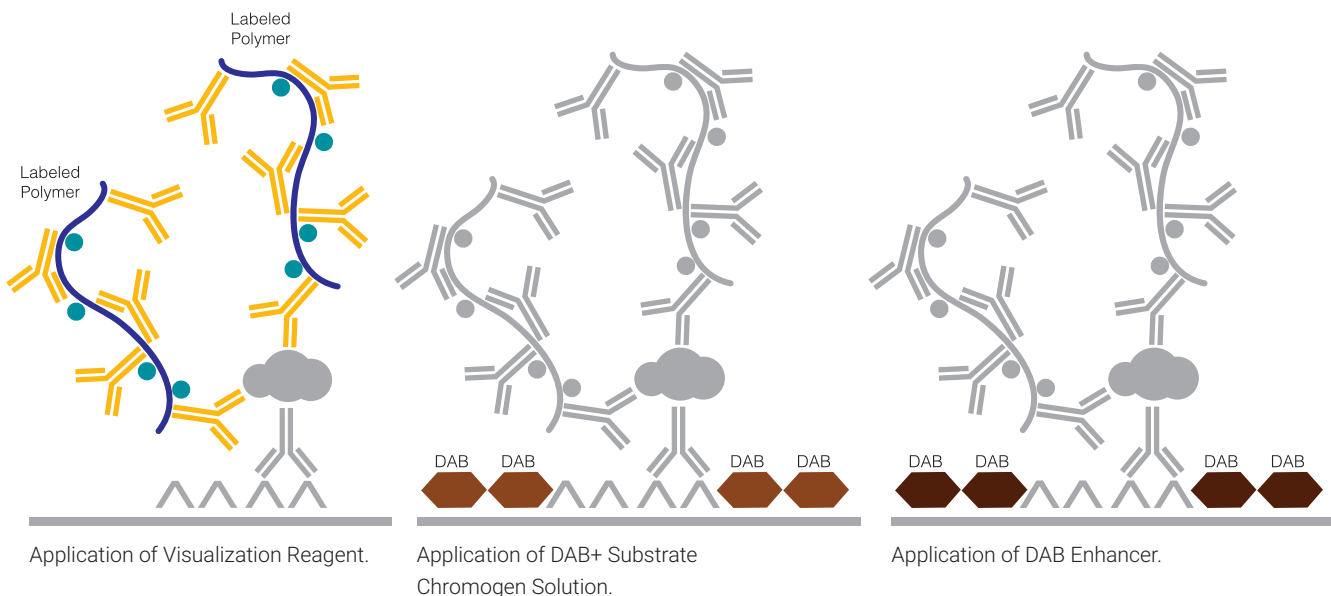
**Figure 5:** PD-L1 IHC 22C3 pharmDx components.

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 (50x)
- 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\*

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

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



# 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. Confirm 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 lab-supplied control tissues stained with the primary antibody and with the Negative Control Reagent
- Serial section of each patient specimen stained with the NCR

## Lab-supplied Control Tissue

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

Refer to the "Recommended Order of Slide Evaluation" table in the PD-L1 IHC 22C3 pharmDx IFU for additional information on appropriate control tissue. 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 Lab-supplied Control: Tonsil Tissue

Tonsil stained with PD-L1 should be prescreened 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) or at room temperature up to 25 °C in the dark and stain within the time period specified in the IFU for each indication and temperature condition.

# 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 preprogrammed 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); the pH of 1× EnVision FLEX Target Retrieval Solution must be  $6.1 \pm 0.2$ . One 30 mL bottle of concentrate provides 1.5 L of 1× 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.

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

## EnVision FLEX Wash Buffer

Dilute EnVision FLEX Wash Buffer (20×) (Code K8007) 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 signal

### **Deparaffinization, Rehydration, and Target Retrieval (3-in-1) Procedure**

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 one rack at a time on the Autostainer Link 48
- Prior to initiating the staining procedure on the Autostainer Link 48, 1× EnVision FLEX Wash Buffer should be manually applied to the slides using a wash bottle for prevention of tissue drying. 1× EnVision FLEX Wash Buffer should not be applied directly on the tissue section but applied sufficiently to the slide so that the tissue section is amply covered. Slides should remain wet prior to the initiation of the staining procedure. Do not allow tissue sections to dry after deparaffinization, rehydration, and target retrieval (3-in-1) procedure (specimen pretreatment) or at any time during the staining procedure. Dried tissue sections may display increased nonspecific staining (including nuclear staining)
- Ensure that Autostainer slide racks are level prior to initiation of the IHC staining procedure. Level Autostainer slide racks are required for staining. Unlevel and/or warped Autostainer slide racks can result in uneven reagent distribution and improper pooling on the glass away from the specimen areas, which increases the risk for tissue drying and may lead to the appearance of nonspecific staining on the Primary Antibody and/or NCR-stained slides. Perform level testing using dry untreated slides for each slide position in all Autostainer slide racks every 3 months or whenever the Autostainer Link 48 is moved or adjusted on the counter. Discard Autostainer slide racks that fail level testing in any slide position and/or have undergone ≥ 175 PT Link pretreatment cycles
- Ensure that the Autostainer Link 48 lid is properly closed to prevent reagent evaporation during the staining procedure
- 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 (Link) (Code K8008)

## Mounting

Use nonaqueous permanent mounting medium. 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 \_\_\_\_\_

	<b>Yes</b>	<b>No</b>
Regular preventive maintenance is performed on the Autostainer Link 48 and PT Link?	<input type="checkbox"/>	<input type="checkbox"/>
PD-L1 IHC 22C3 pharmDx is used before the expiration date printed on the outside of the box?	<input type="checkbox"/>	<input type="checkbox"/>
All PD-L1 IHC 22C3 pharmDx components, including Control Cell Line Slides, are stored in the dark at 2–8 °C?	<input type="checkbox"/>	<input type="checkbox"/>
All PD-L1 IHC 22C3 pharmDx components, including Control Cell Line Slides, are equilibrated to room temperature (20–25 °C) prior to immunostaining?	<input type="checkbox"/>	<input type="checkbox"/>
Appropriate positive and negative control tissues are identified?	<input type="checkbox"/>	<input type="checkbox"/>
Tissues are fixed in neutral buffered formalin?	<input type="checkbox"/>	<input type="checkbox"/>
Tissues are infiltrated with melted paraffin, at or below 60 °C?	<input type="checkbox"/>	<input type="checkbox"/>
Tissue sections of 4–5 µm are mounted on Dako FLEX IHC Microscope Slides or Superfrost Plus slides?	<input type="checkbox"/>	<input type="checkbox"/>
Specimens are oven-dried at 58 ± 2 °C for 1 hour?	<input type="checkbox"/>	<input type="checkbox"/>
Specimens are stained within the indication- and storage condition-specific time period from the date of sectioning given in the IFU?	<input type="checkbox"/>	<input type="checkbox"/>
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.	<input type="checkbox"/>	<input type="checkbox"/>
1× EnVision FLEX Wash Buffer is prepared properly?	<input type="checkbox"/>	<input type="checkbox"/>
DAB+ Substrate-Chromogen Solution is prepared properly?	<input type="checkbox"/>	<input type="checkbox"/>
Slides are counterstained with EnVision FLEX Hematoxylin (Link) (Code K8008)?	<input type="checkbox"/>	<input type="checkbox"/>
The Deparaffinization, Rehydration, and Target Retrieval 3-in-1 procedure is followed using PT Link?	<input type="checkbox"/>	<input type="checkbox"/>
Are the slide racks level?	<input type="checkbox"/>	<input type="checkbox"/>
EnVision FLEX Wash Buffer is manually applied to slides using a wash bottle and slides remain wet with buffer while loading and prior to initiating run on Autostainer Link 48?	<input type="checkbox"/>	<input type="checkbox"/>
The PD-L1 IHC 22C3 pharmDx protocol is selected on Autostainer Link 48?	<input type="checkbox"/>	<input type="checkbox"/>
Autostainer Link 48 lid is properly closed during staining procedure?	<input type="checkbox"/>	<input type="checkbox"/>
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.	<input type="checkbox"/>	<input type="checkbox"/>

Additional observations or comments:

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# 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 32. Before examining the patient specimen for PD-L1 staining, it is important to examine the controls to assess staining quality.

PD-L1 expression is best evaluated 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. Lab-supplied 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.

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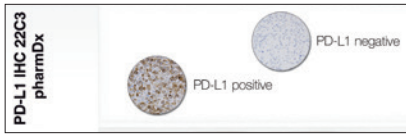
A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide for the specimen to be considered adequate for PD-L1 evaluation.

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### **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 nonspecific 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.

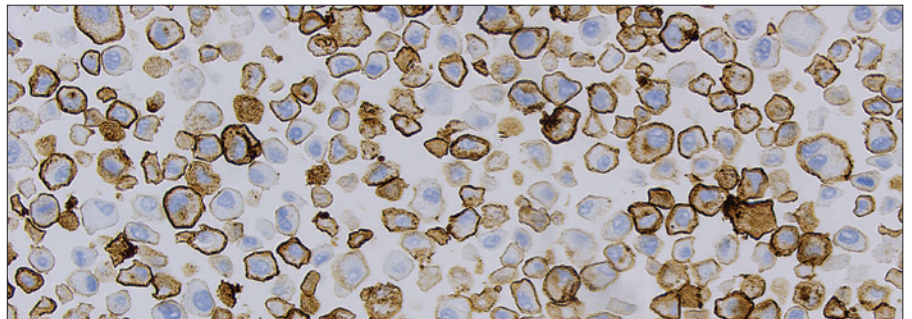
Evaluate staining intensity using the following guide:

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  $\geq 70\%$  of cells
- $\geq 2+$  average staining intensity of cells with membrane staining
- Nonspecific staining  $< 1+$  intensity



**Figure 7:** PD-L1 IHC 22C3 pharmDx Control Cell Line Slide positive cell pellet with acceptable staining (20 $\times$  magnification).

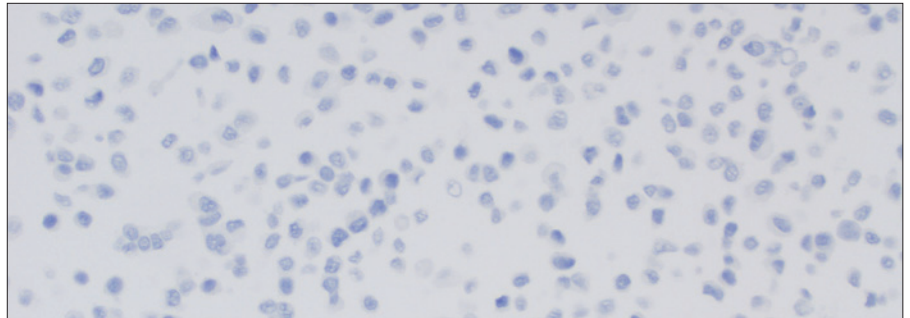


### Negative Control Cell Pellet

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

- No cells with membrane staining\*
- Nonspecific staining < 1+ intensity\*

\* Note that staining of a few cells in the MCF-7 cell pellet may occasionally be observed. The following acceptance criteria are applicable: the presence of  $\leq 10$  total cells with distinct cell membrane staining, and/or nonspecific staining with  $\geq 1+$  intensity within the boundaries of the MCF-7 cell pellet are acceptable



**Figure 8:** PD-L1 IHC 22C3 pharmDx Control Cell Line Slide negative cell pellet with no staining (20 $\times$  magnification).

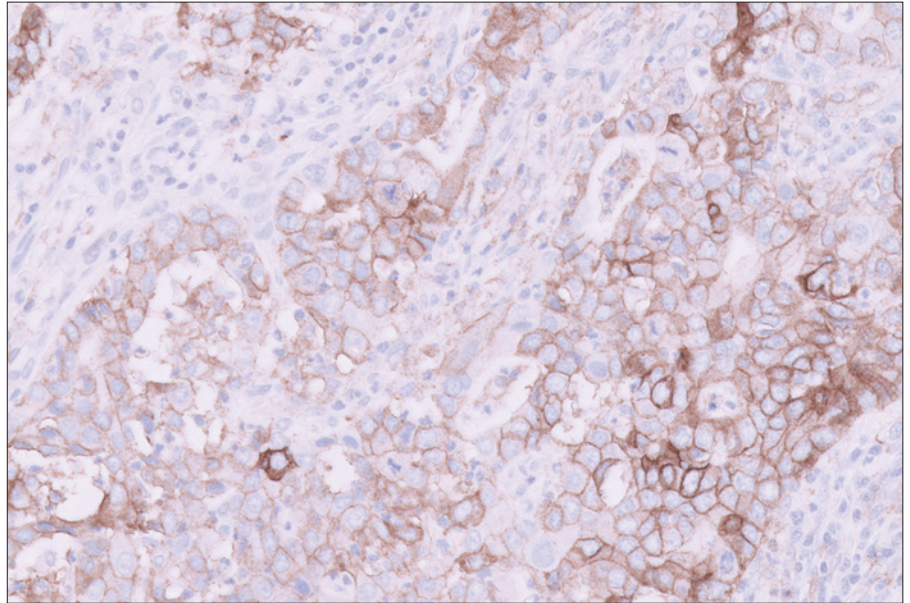
See the Control Cell Line (CCL) Appendix on page 92 for images of passing, borderline, and failing control cell line staining.

## Positive and Negative Lab-supplied Control Tissues

Examine the 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.

- Requirements for slide stained with PD-L1: Presence of brown membrane staining should be observed. Nonspecific staining, including nuclear staining, should be  $\leq 1+$
- Requirements for slide stained with Negative Control Reagent: No membrane staining. Nonspecific staining, including nuclear staining, should be  $\leq 1+$

If staining of positive lab-supplied control tissue is not satisfactory, all results with the patient specimen should be considered invalid.



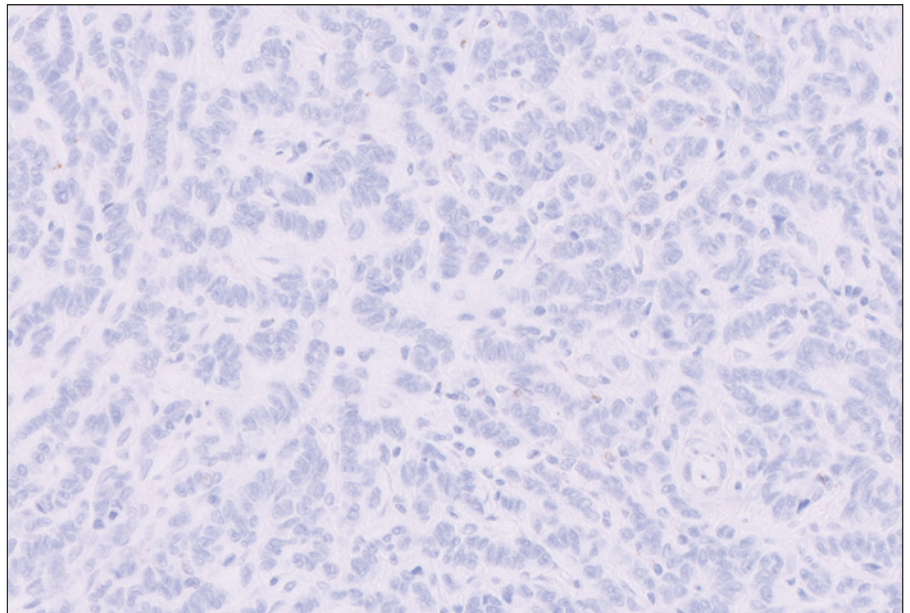
**Figure 9:** Ideal positive lab-supplied gastric or GEJ adenocarcinoma control tissue stained with the PD-L1 primary antibody (20× magnification).

Examine the 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 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.

- Requirements for slide stained with PD-L1: No membrane staining in tumor cells. Nonspecific staining, including nuclear staining, should be  $\leq 1+$
- Requirements for slide stained with Negative Control Reagent: No membrane staining. Nonspecific staining, including nuclear staining, should be  $\leq 1+$

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

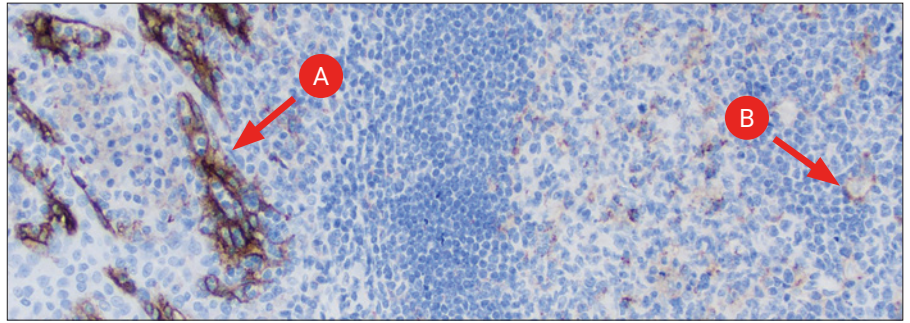


**Figure 10:** Ideal negative lab-supplied gastric or GEJ adenocarcinoma control tissue stained with the PD-L1 primary antibody demonstrating lack of staining except for a few staining immune cells (20x magnification).

### Optional Additional Control Tissue

In addition to the Control Cell Line Slide and lab-supplied 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).

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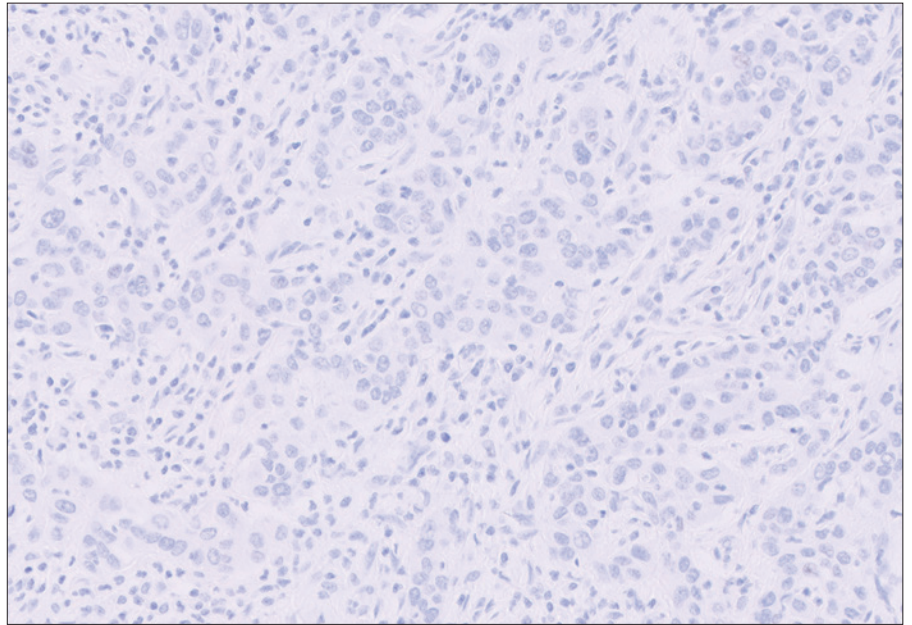
Do not use lab-supplied control tissue as an aid in interpretation of patient results.

---

### Negative Control Reagent (NCR)

Examine the slides stained with the NCR to identify nonspecific staining, including nuclear staining, that may interfere with PD-L1 staining interpretation, making the specimen non-evaluable. Satisfactory performance is indicated by 0 specific staining and  $\leq 1+$  nonspecific staining, including nuclear staining (Figure 12).

Examine the patient specimens stained with the NCR to determine if there is any nonspecific staining, including nuclear staining, that may interfere with interpreting the PD-L1 stained slide.



**Figure 12:** Gastric or GEJ adenocarcinoma tissue specimen stained with NCR (20× magnification).

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

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# Slide Evaluation Flowchart

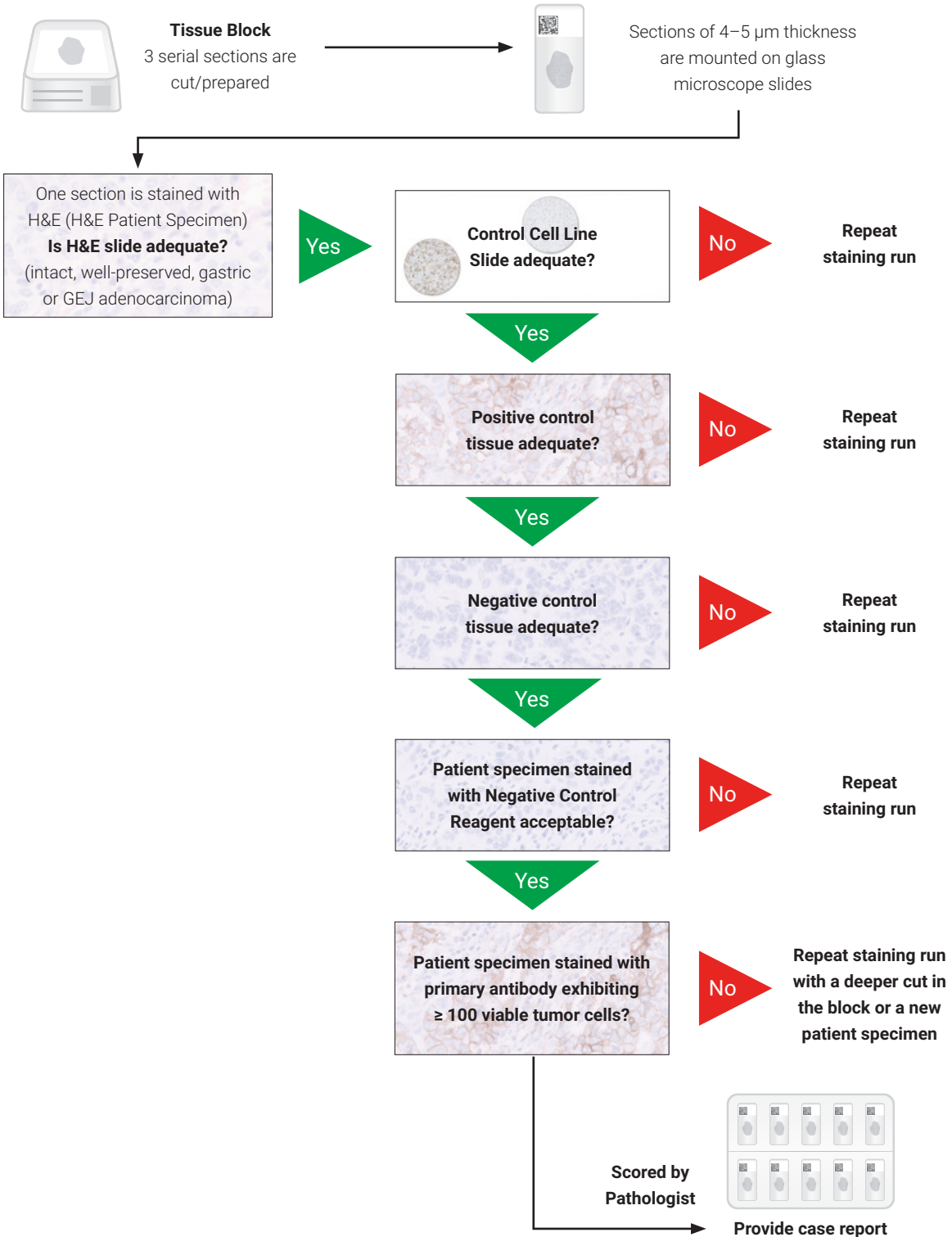


Figure 13: Recommended order of slide evaluation.

# Combined Positive Score

## Definition of Combined Positive Score (CPS)

PD-L1 expression in gastric or GEJ adenocarcinoma 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 ( $\geq 1+$ ) 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 ( $\geq 1+$ ) 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 28 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 nonstaining 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 / surgical specimens) 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
- 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 nonstaining (CPS denominator)
  - Determine the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) (CPS numerator; see Tables 1 and 2 on page 28 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 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	<ul style="list-style-type: none"> <li>– Nonstaining tumor cells</li> <li>– Tumor cells with only cytoplasmic staining</li> <li>– Adenoma, dysplasia, and carcinoma in situ</li> </ul>
Immune Cells	Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma <sup>†</sup> : <ul style="list-style-type: none"> <li>– Lymphocytes (including lymphocyte aggregates)</li> <li>– Macrophages<sup>‡</sup></li> </ul> Only MICs directly associated with the response to the tumor are scored	<ul style="list-style-type: none"> <li>– Nonstaining MICs</li> <li>– MICs associated with adenoma, dysplasia, and carcinoma in situ</li> <li>– MICs (including lymphoid aggregates) associated with ulcers, chronic gastritis, and other processes not associated with the tumor</li> <li>– MICs associated with normal structures</li> <li>– Neutrophils, eosinophils, and plasma cells</li> </ul>
Other Cells	Not included	<ul style="list-style-type: none"> <li>– Normal cells (including ganglion cells)</li> <li>– Stromal cells (including fibroblasts)</li> <li>– Necrotic cells and/or cellular debris</li> </ul>

\* 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

<sup>†</sup> 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

<sup>‡</sup> Macrophages and histiocytes are considered the same cells

**Table 2:** CPS Denominator Inclusion/Exclusion Criteria for Gastric or GEJ Adenocarcinoma

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	All viable invasive tumor cells (PD-L1 staining or nonstaining)	<ul style="list-style-type: none"> <li>– Nonviable tumor cells</li> <li>– Adenoma, dysplasia, and carcinoma in situ</li> </ul>
Immune Cells	Not included	All immune cells
Other Cells	Not included	<ul style="list-style-type: none"> <li>– Normal cells (including ganglion cells)</li> <li>– Stromal cells (including fibroblasts)</li> <li>– Necrotic cells and/or cellular debris</li> </ul>

## 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.**

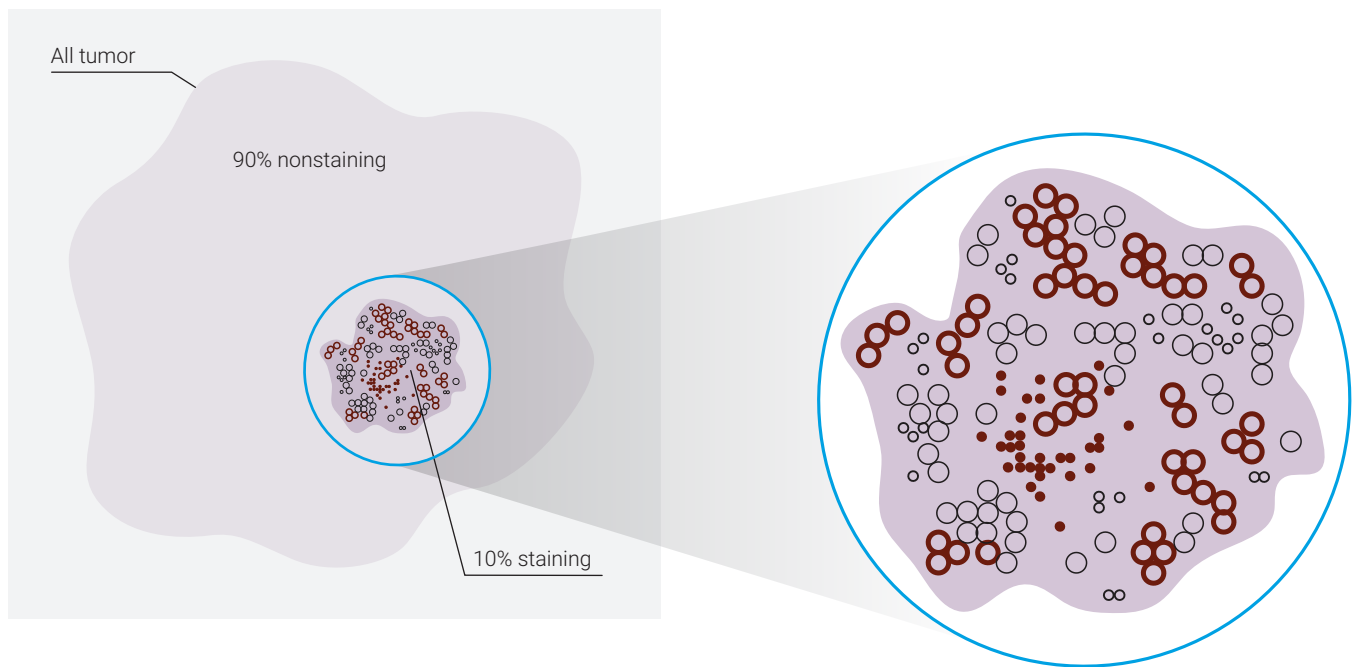
### Example 1: Calculation of Combined Positive Score Based on a Small PD-L1 Staining Area

First: Evaluate the tumor area for perceptible and convincing staining as described in “Determining Combined Positive Score” on page 27.

**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:

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

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

- PD-L1 staining tumor cell
- Nonstaining tumor cell
- PD-L1 staining mononuclear inflammatory cell
- Nonstaining mononuclear inflammatory cell

### Clinical Interpretation: CPS ≥ 1

<sup>§</sup> Including tumor cells, lymphocytes, macrophages

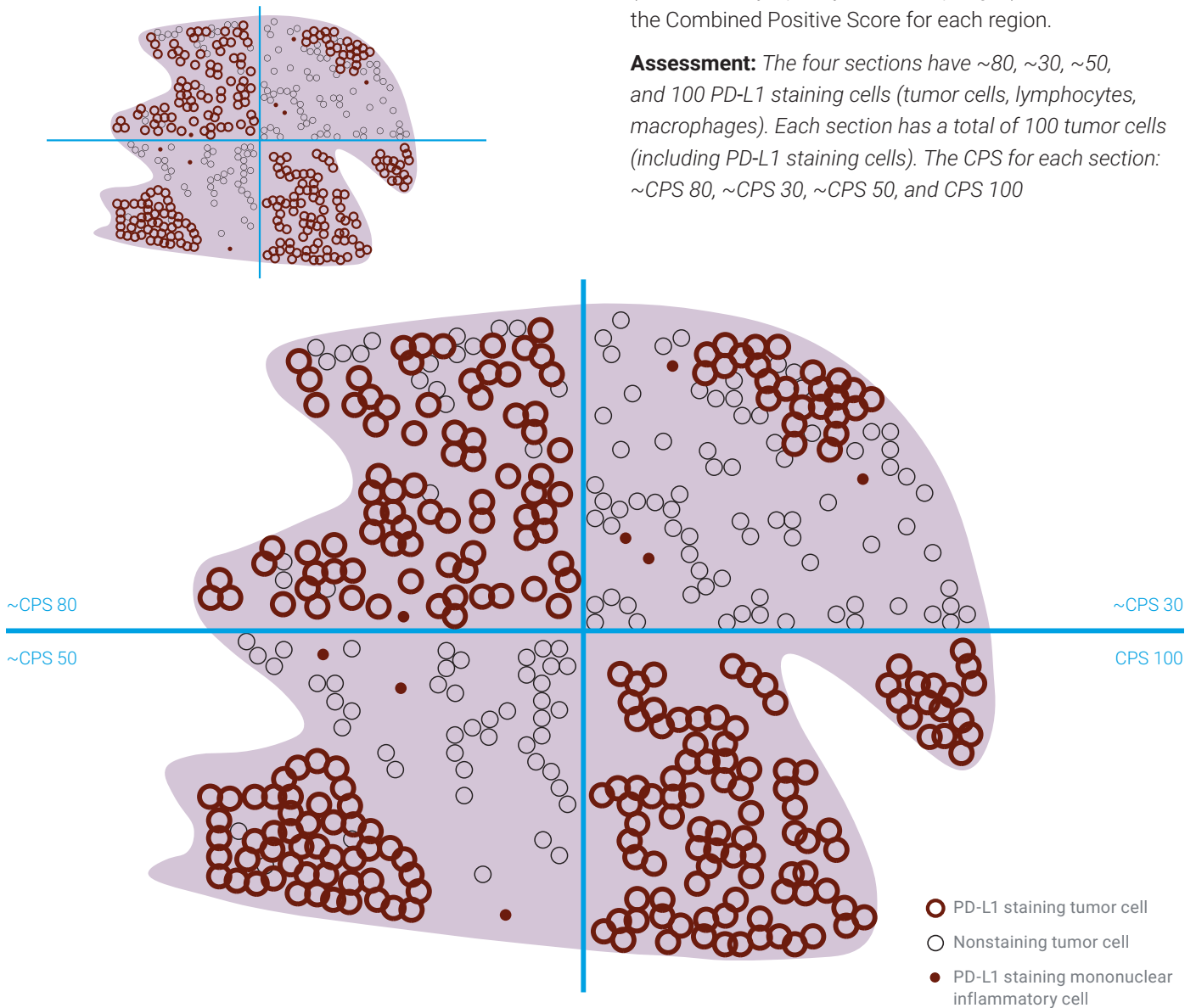
**Figure 14:** Example of tumor with small PD-L1 staining area.

### 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:  
 $(80 + 30 + 50 + 100) / 4 \approx \text{CPS } 65$

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

Clinical Interpretation: CPS ≥ 1

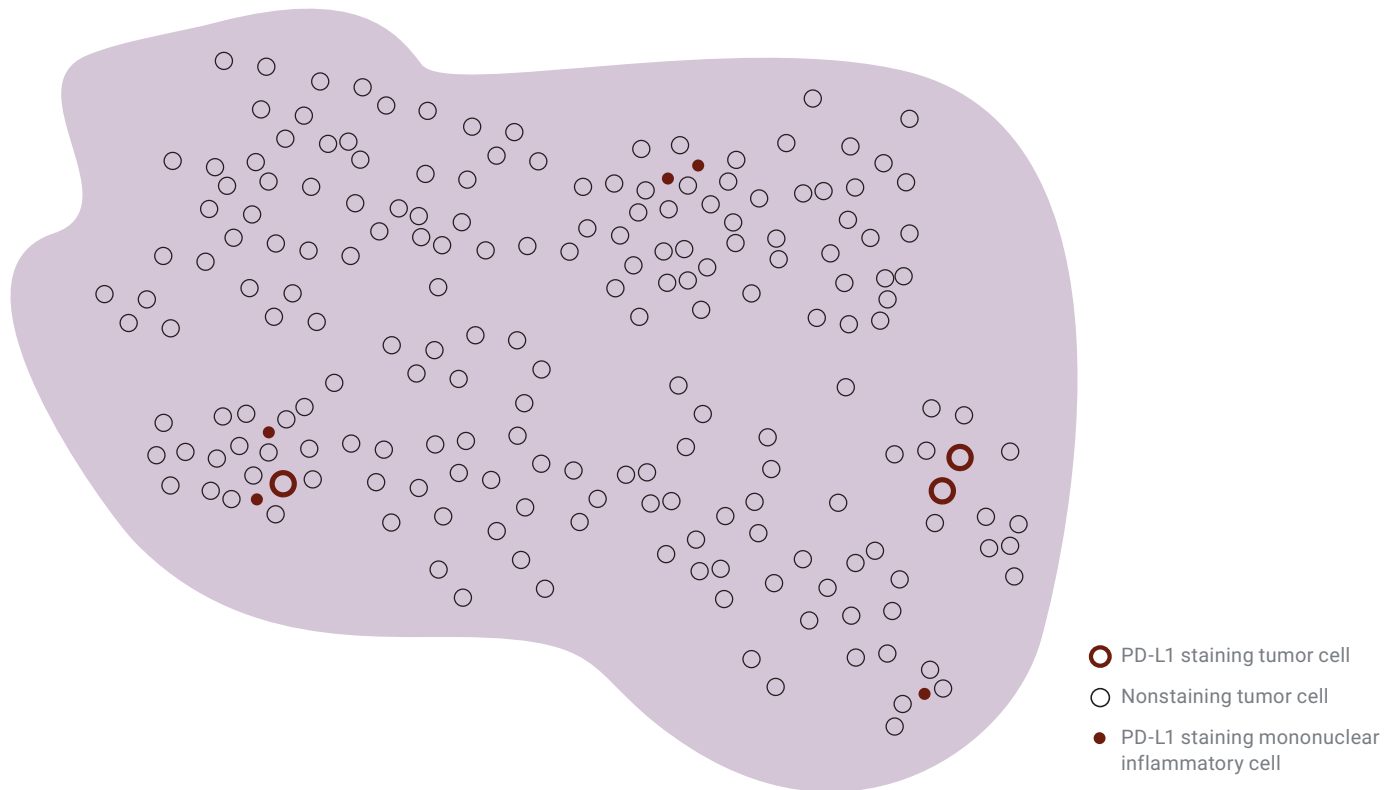
Figure 15: Example with heterogeneous PD-L1 staining area.

### Example 3: Calculation of Combined Positive Score for a Near-Cutoff Specimen

First: Evaluate the specimen for perceptible and convincing staining as described in “Determining Combined Positive Score” on page 27.

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 nonstaining areas) and estimate the total number of viable tumor cells (PD-L1 staining and nonstaining tumor cells). Calculate the Combined Positive Score.

**Assessment:** Four areas of the tumor specimen have convincing staining. There are 8 PD-L1 staining cells (tumor cells, lymphocytes, macrophages) in the four staining areas. 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:

$$\text{CPS} = \frac{\# \text{ PD-L1 staining cells}^*}{\text{Total \# of viable tumor cells}} \times 100 = \frac{8 \text{ PD-L1 staining cells}}{200 \text{ tumor cells}} \times 100 = 4$$

Clinical Interpretation: CPS ≥ 1

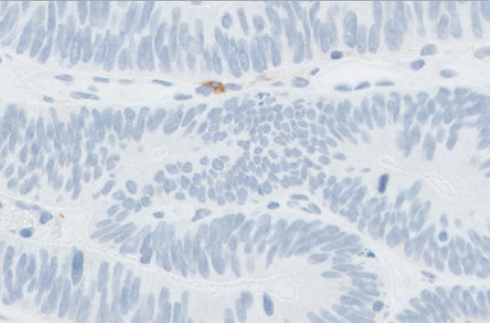
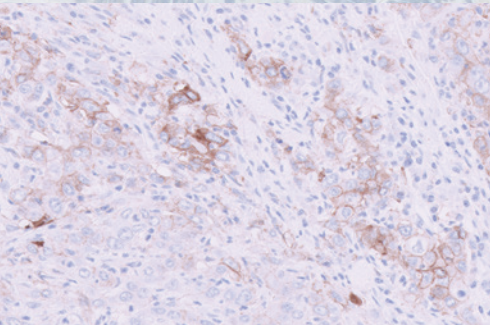
\* Including tumor cells, lymphocytes, macrophages

Figure 16: Example of near-cutoff specimen (CPS range of greater than 0 but less than or equal to 10).

# 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)
< 1	CPS is less than 1	
≥ 1	CPS is greater than or equal to 1	

For more information, please refer to the current version of the Instructions for Use (IFU) for PD-L1 IHC 22C3 pharmDx, Code SK006.

## Identifying Patients with Gastric or GEJ Adenocarcinoma for Treatment

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



# 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 ( $\geq 100$  cells): Yes:  No:

## PD-L1 IHC 22C3 pharmDx Result to Treating Physician

Combined Positive Score: \_\_\_\_\_

CPS < 1:  CPS  $\geq$  1:

Comments to Treating Physician:

- See the 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 gastric or GEJ adenocarcinoma are:

- Viable tumor cells with perceptible and convincing partial or complete linear membrane staining (at any intensity) that is perceived as 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 level in gastric or GEJ adenocarcinoma 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 gastric or GEJ adenocarcinoma unless otherwise noted in the figure caption.

## Image Guide for Interpretation of PD-L1 IHC 22C3 pharmDx Staining in Gastric or GEJ Adenocarcinoma

### Cells Included in the Combined Positive Score (CPS)

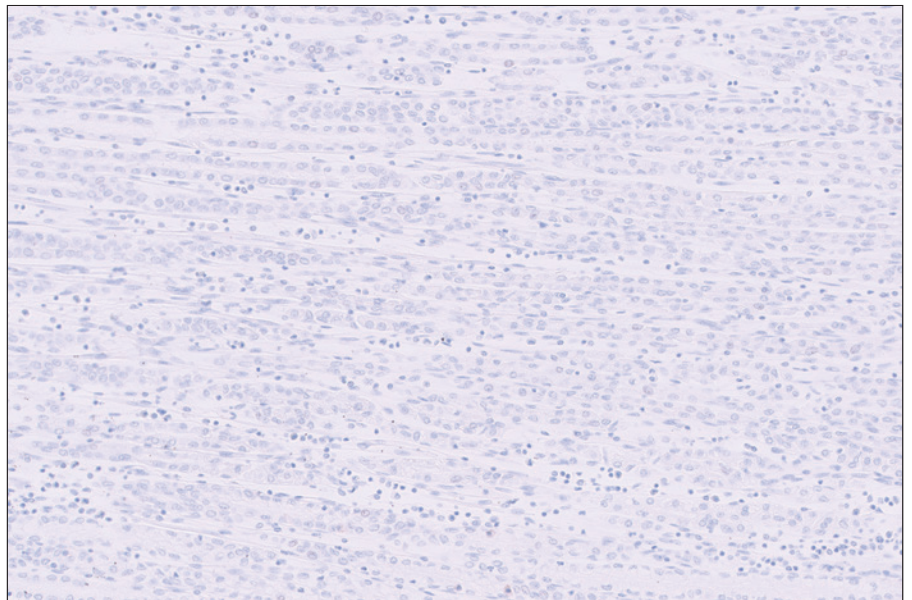
Tumor cells, lymphocytes, and macrophages exhibiting appropriate PD-L1 expression are defined as PD-L1 staining cells. All PD-L1 staining cells are included in the CPS numerator for determination of the Combined Positive Score (see Tables 1 and 2 on page 28 for additional CPS inclusion/exclusion criteria). Below is an image guide to aid in accurately determining CPS in gastric or GEJ adenocarcinoma specimens. All images are gastric or GEJ adenocarcinoma unless otherwise noted in the figure caption.

### Tumor Cells

#### Tumor Cell Size

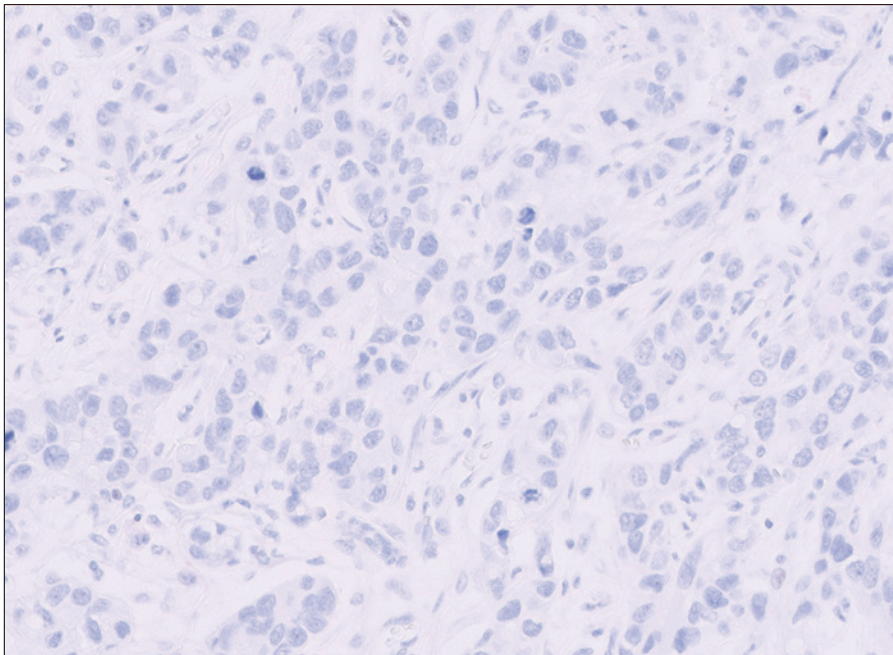
Gastric or GEJ adenocarcinoma includes different morphologies and tumor cell sizes that can impact the Combined Positive Score (CPS) by increasing or decreasing the total number of tumor cells that are included in the CPS denominator. Well-differentiated adenocarcinoma may exhibit larger tumor cells 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 CPS denominator, the greater the number of PD-L1 staining tumor cells, lymphocytes, and macrophages that are needed in the CPS numerator to bring the overall score to CPS 1 or above. As a guideline, if tumor cells are 20 μm in diameter and completely fill a 20× field, there would be approximately 2500 tumor cells in that field.

#### Small Cell Size



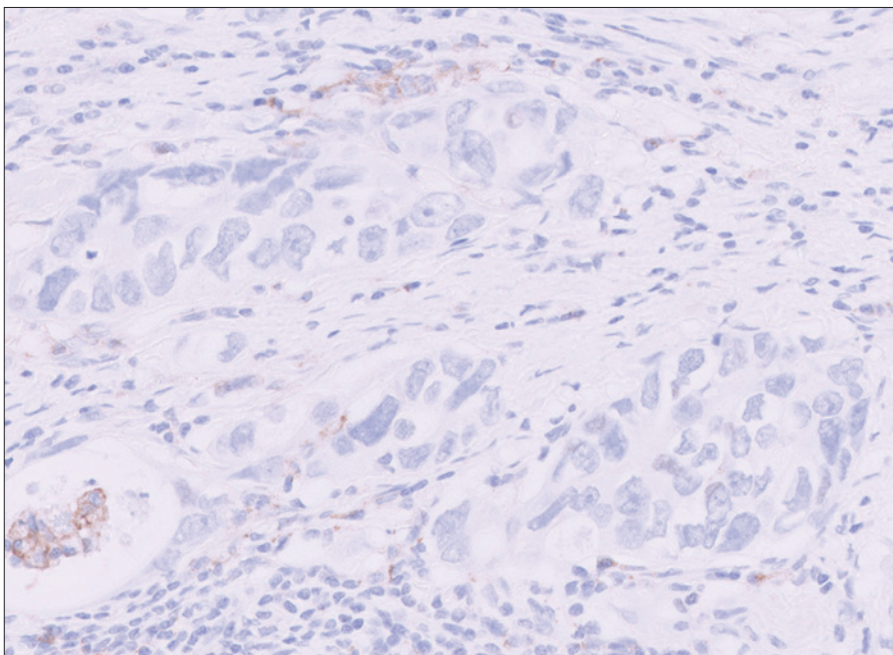
**Figure 17a:** Gastric or GEJ adenocarcinoma specimen with small sized tumor cells (20× magnification).

### Medium Cell Size



**Figure 17b:** Gastric or GEJ adenocarcinoma specimen with medium sized tumor cells (20× magnification).

### Large Cell Size



**Figure 17c:** Gastric or GEJ adenocarcinoma specimen with large sized tumor cells (20× magnification).

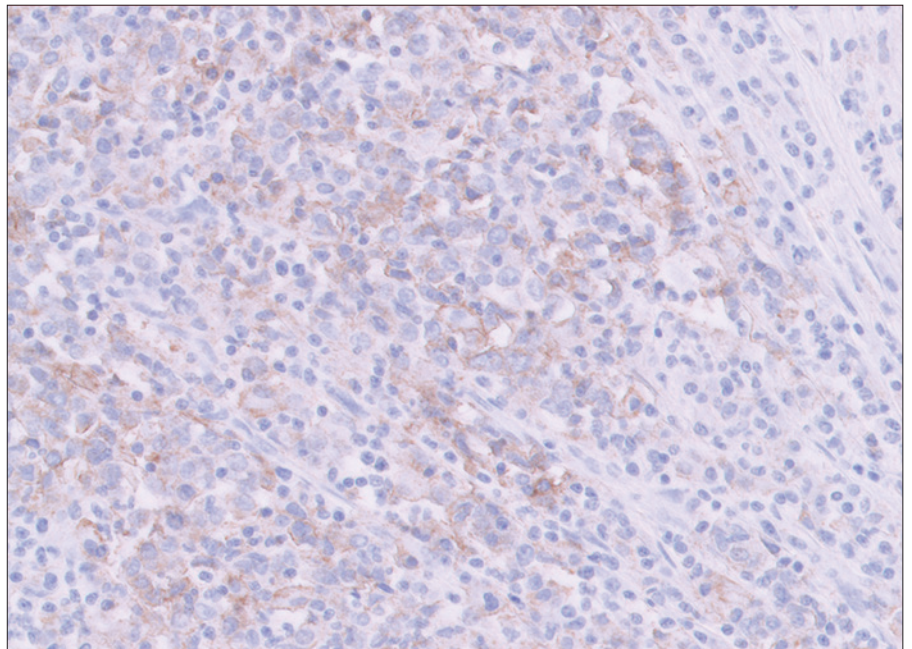
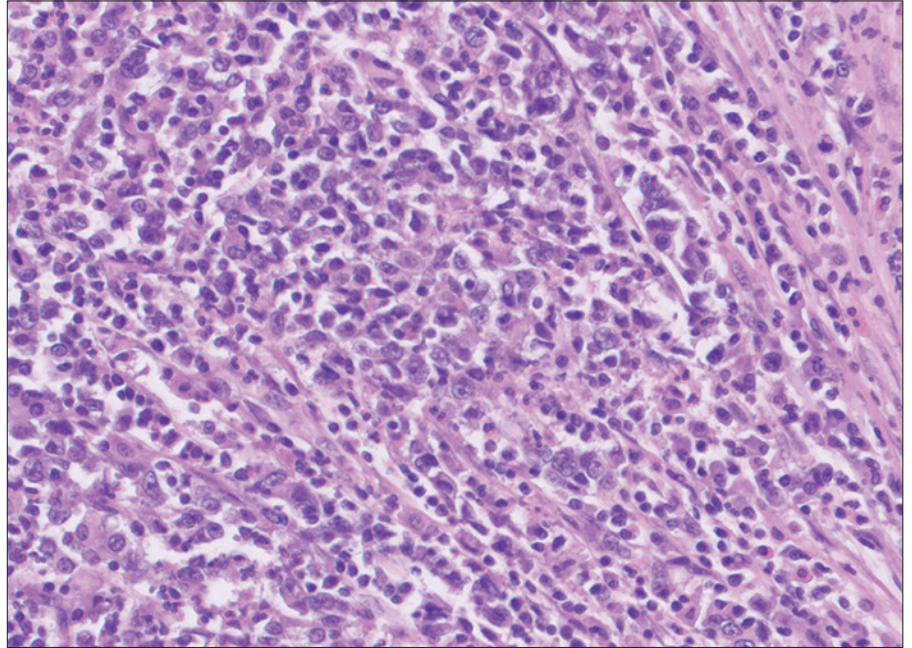
### Key Point

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



### Tumor and Immune Cells That Are Difficult to Distinguish

Tumor cells and immune cells may be difficult to distinguish 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 types. This is especially important when determining the CPS denominator.



**Figure 18:** Gastric or GEJ adenocarcinoma specimen exhibiting an area of admixed tumor and immune cells that are difficult to distinguish from each other – H&E (top) and corresponding PD-L1 field (bottom) (20× magnification).

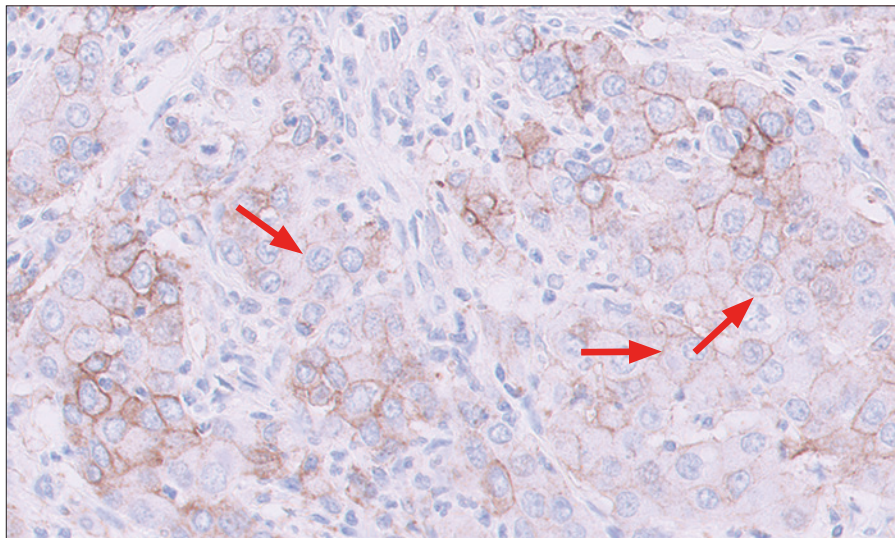
#### Key Point

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

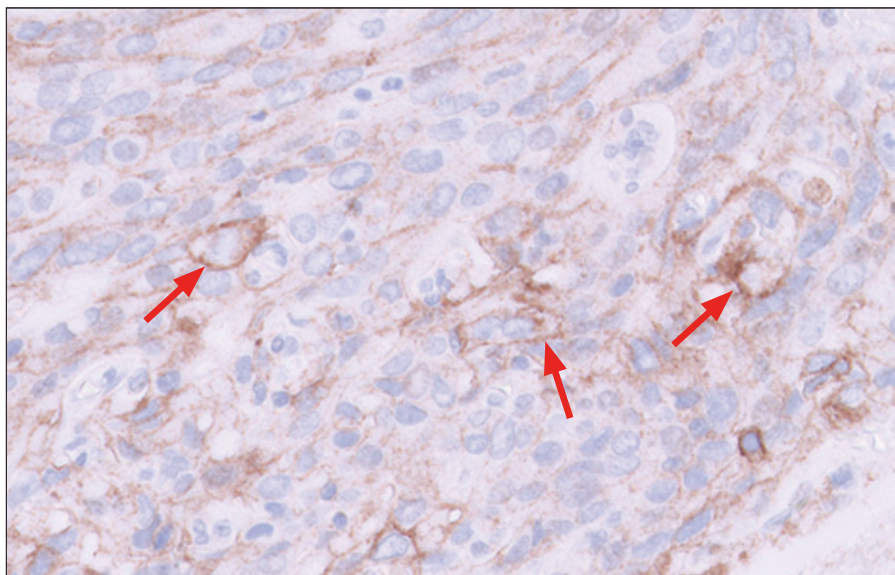
### Linear Membrane Staining

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

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

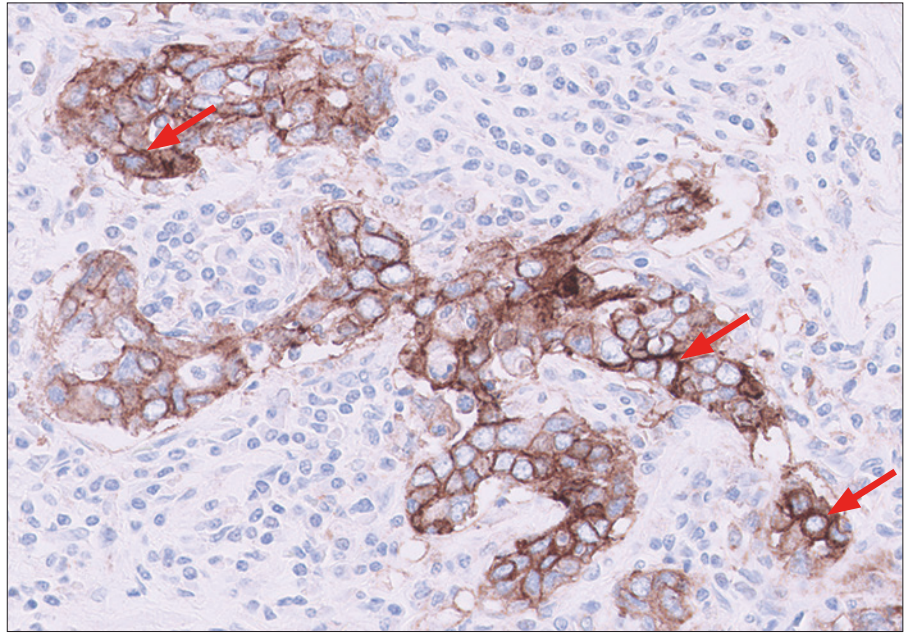


**Figure 19a:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting 1+ linear membrane staining of tumor cells (arrows) (20× magnification).



**Figure 19b:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting 2+ linear membrane staining of tumor cells (arrows) (20× magnification).





**Figure 19c:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting 3+ linear membrane staining of tumor cells (arrows) (20× magnification).

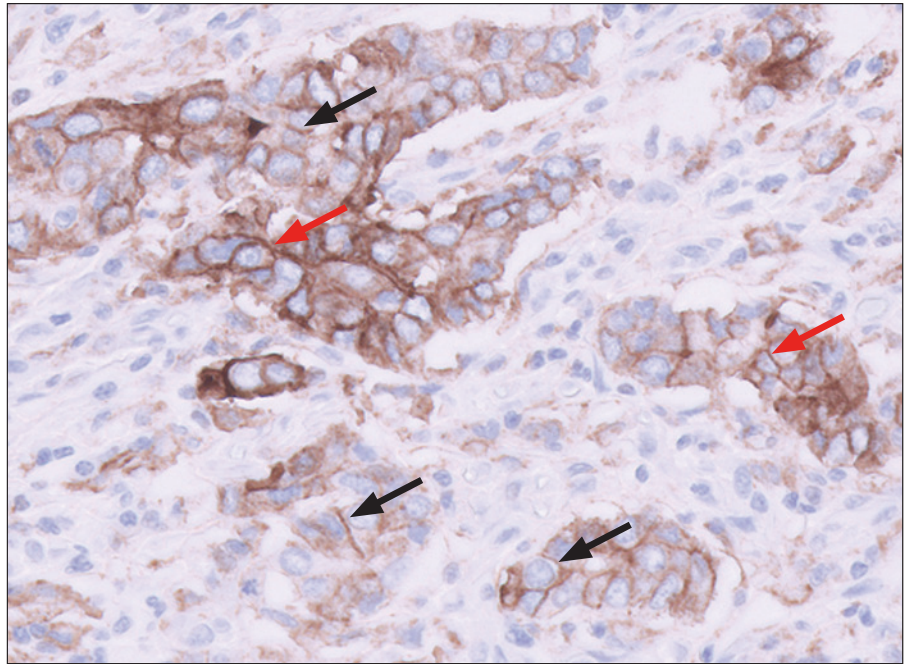
**Key point**

Convincing linear membrane staining of tumor cells at any intensity should be included in the score



### Partial and Complete Linear Membrane Staining

Tumor cells can exhibit partial or complete linear membrane staining. Any tumor cells with partial or complete linear membrane staining that is perceptible and convincing at 20× magnification should be included in the CPS numerator.



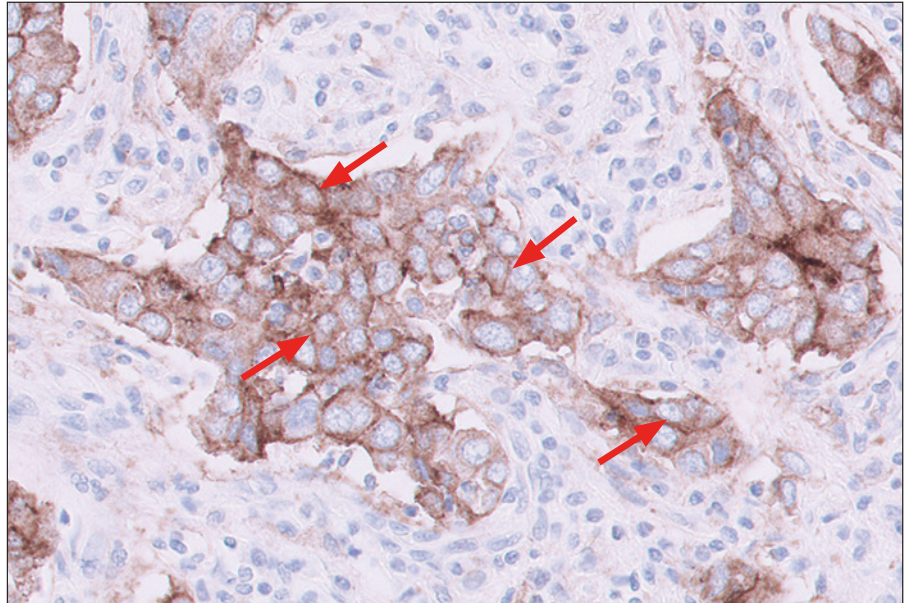
**Figure 20:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting partial (black arrows) and complete (red arrows) linear membrane staining of tumor cells (20× magnification).

#### Key point

Convincing partial or complete linear membrane staining of tumor cells should be included in the score

### Linear Membrane and Cytoplasmic Staining

Tumor cells with both convincing linear membrane staining ( $\geq 1+$  intensity) and cytoplasmic staining at 20 $\times$  magnification should be included in the CPS numerator. Tumor cells exhibiting only cytoplasmic staining are excluded from the CPS numerator. If linear membrane staining is distinct from cytoplasmic staining, then the cell should be included in the CPS numerator.



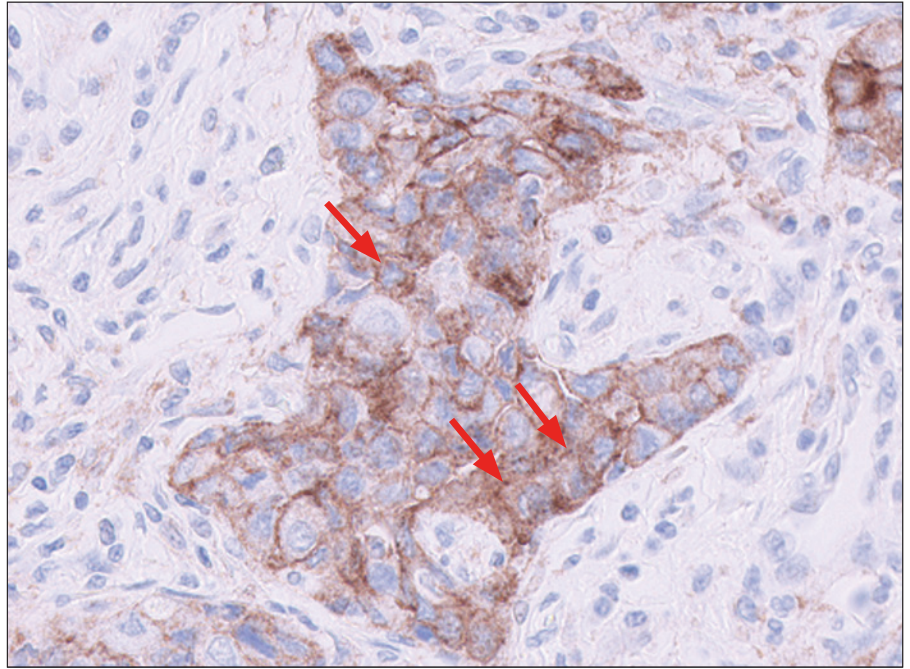
**Figure 21:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting granular but convincingly linear membrane staining distinct from cytoplasmic staining (arrows) (20 $\times$  magnification).

#### Key point

Tumor cells exhibiting convincing linear membrane staining that is distinct from cytoplasmic staining are included in the score

### Granular Staining

Linear membrane staining of tumor cells can be smooth or granular. Tumor cells can exhibit a granular membrane staining pattern where membrane and cytoplasmic staining are difficult to distinguish. Only perceptible and convincing linear membrane staining of tumor cells ( $\geq 1+$  intensity) observed at no higher than 20 $\times$  magnification should be included in the CPS numerator.



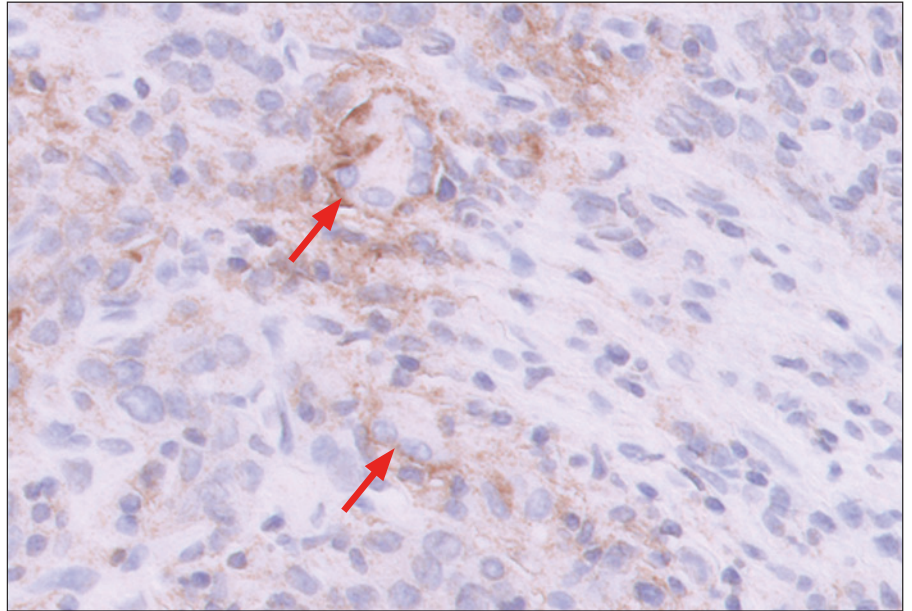
**Figure 22:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting granular membrane staining of tumor cells (arrows) (20 $\times$  magnification).

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



**Figure 23:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting staining of multinucleate tumor cells (arrows) (20× magnification).

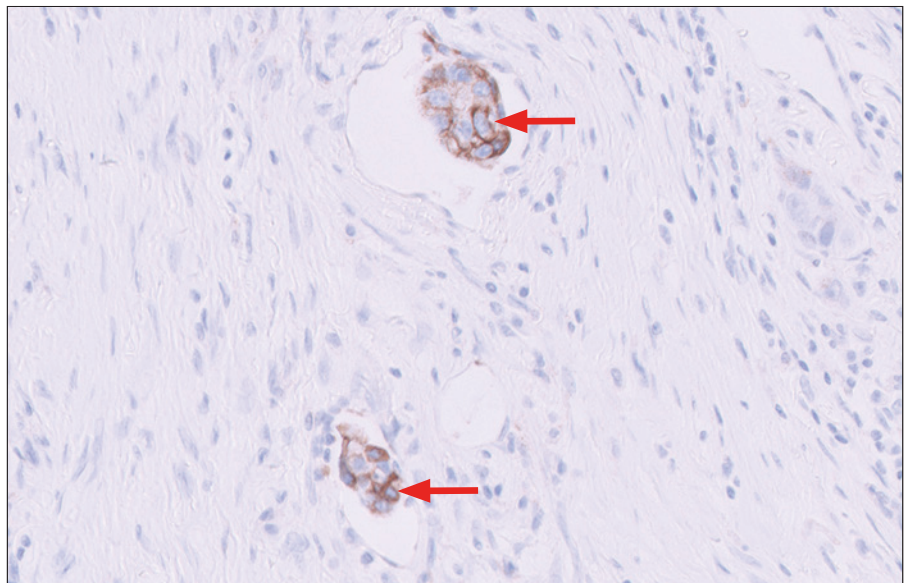
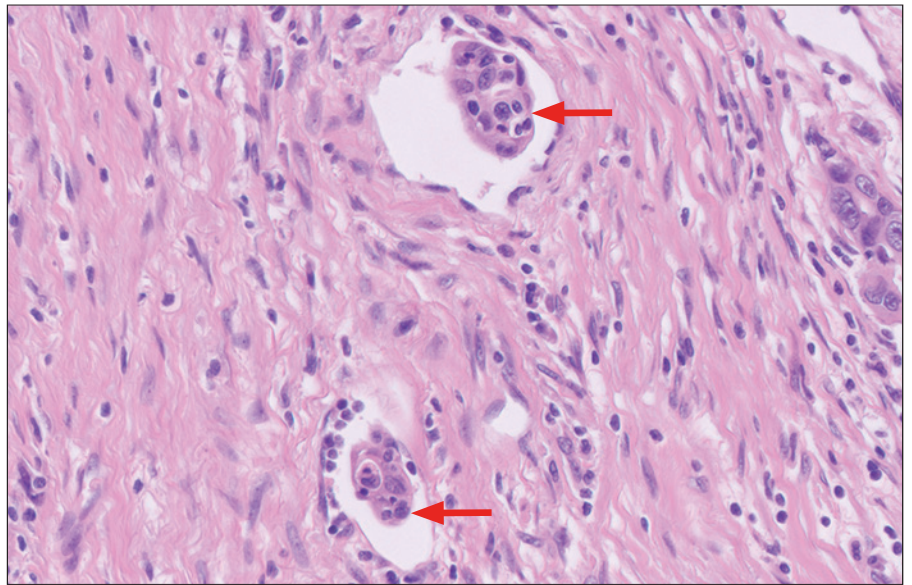
#### Key Point

Multinucleate tumor cells can be seen in gastric or GEJ adenocarcinoma and follow the same criteria for inclusion/exclusion as mononucleate tumor cells



### Intravascular Tumor Cells

Viable intravascular tumor cells should be included in the CPS denominator, and if exhibiting convincing linear membrane staining, should be included in the CPS numerator.



**Figure 24:** Intravascular tumor cells (arrows) should be included in the CPS numerator and denominator – H&E (top) and corresponding PD-L1 field (bottom) (20× magnification).

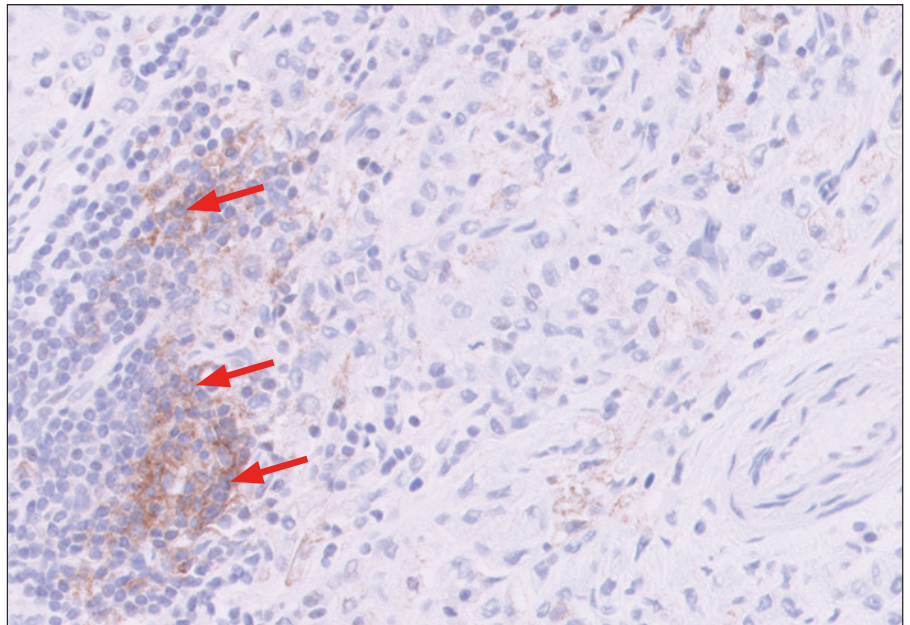
## Immune Cells

### Tumor-associated Mononuclear Inflammatory Cells (MICs)

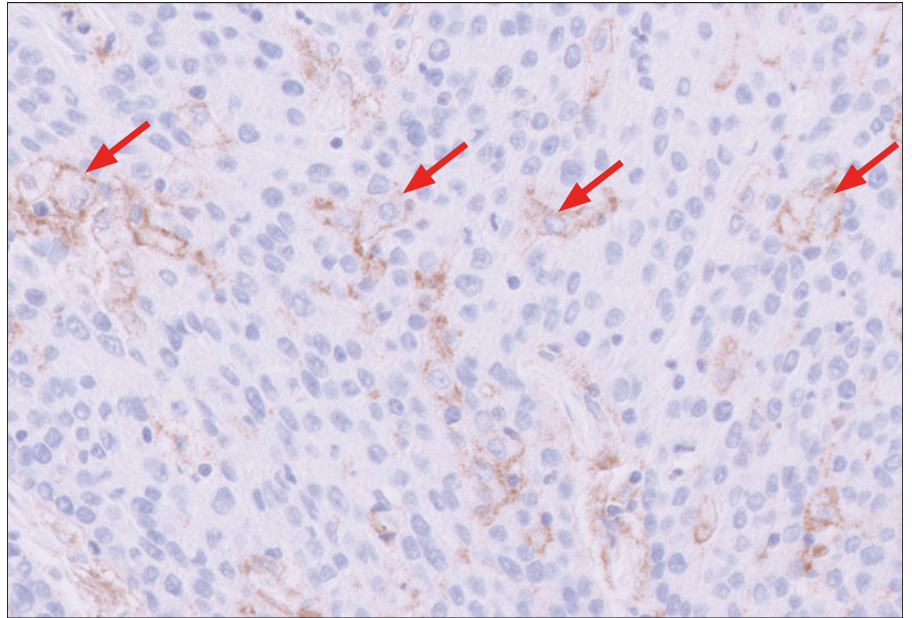
Tumor-associated lymphocytes and macrophages (mononuclear inflammatory cells, MICs) exhibiting membrane and/or cytoplasmic staining at a 20× magnification ( $\geq 1+$  intensity) are considered PD-L1 staining cells and should be included in the CPS numerator. Tumor-associated MICs are present within 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.

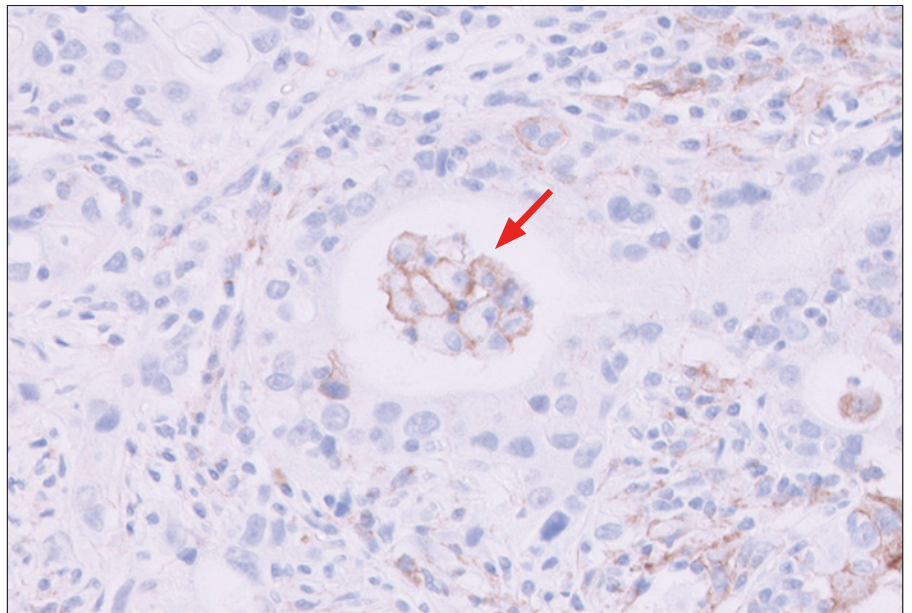
**Note:** PD-L1 staining lymphocytes often have indistinguishable membrane and cytoplasmic staining due to a high nuclear to cytoplasmic ratio; PD-L1 staining macrophages often have distinct membrane staining and low cytoplasmic staining. All PD-L1 staining tumor-associated MICs should be included in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



**Figure 25a:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting staining of tumor-associated lymphocytes (arrows) (20× magnification).

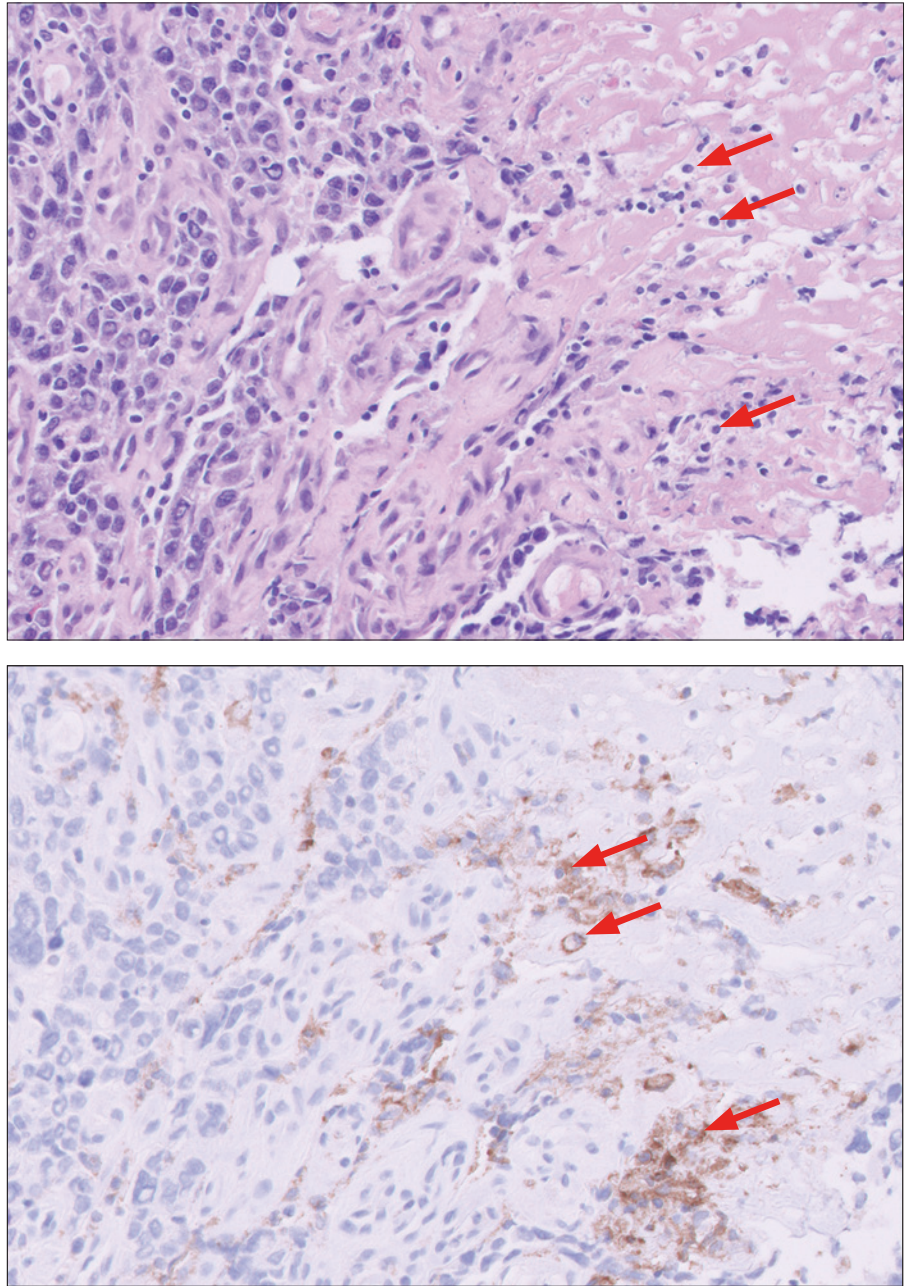


**Figure 25b:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting staining of tumor-associated intratumoral macrophages (arrows) (20× magnification).



**Figure 25c:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting PD-L1 staining intraluminal macrophages within tumor that should be included in the CPS numerator (arrow) (20× magnification).

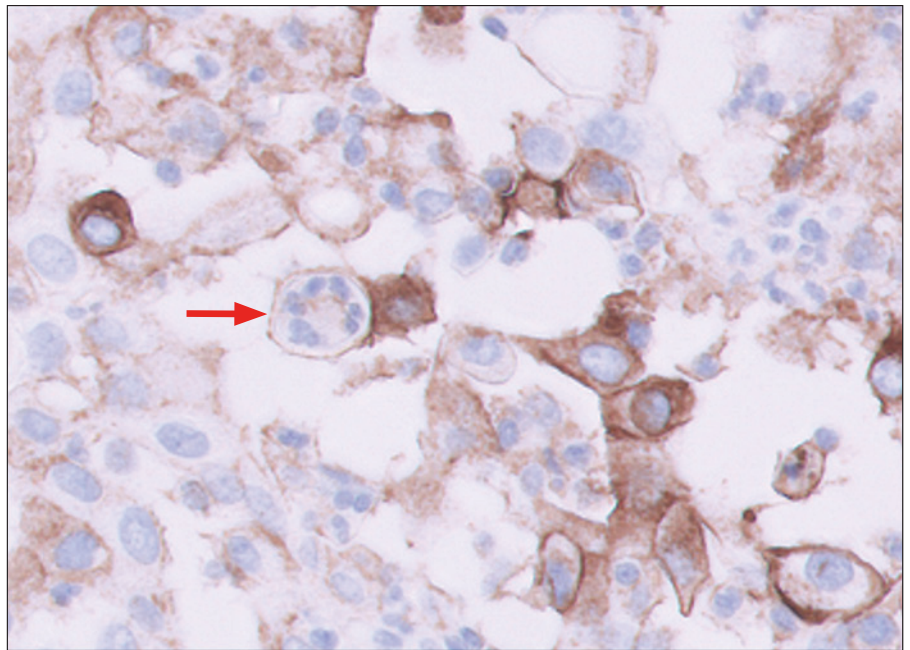




**Figure 25d:** Gastric or GEJ adenocarcinoma specimen exhibiting staining of tumor-associated mononuclear inflammatory cells (MICs) within necrosis but adjacent to and associated with viable tumor cells that should be included in the CPS numerator (arrows) – H&E (top) and corresponding PD-L1 field (bottom) (20× magnification).

### Multinucleated Giant Cells

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



**Figure 26:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting staining of tumor-associated multinucleated giant cells (arrow) (20× magnification).

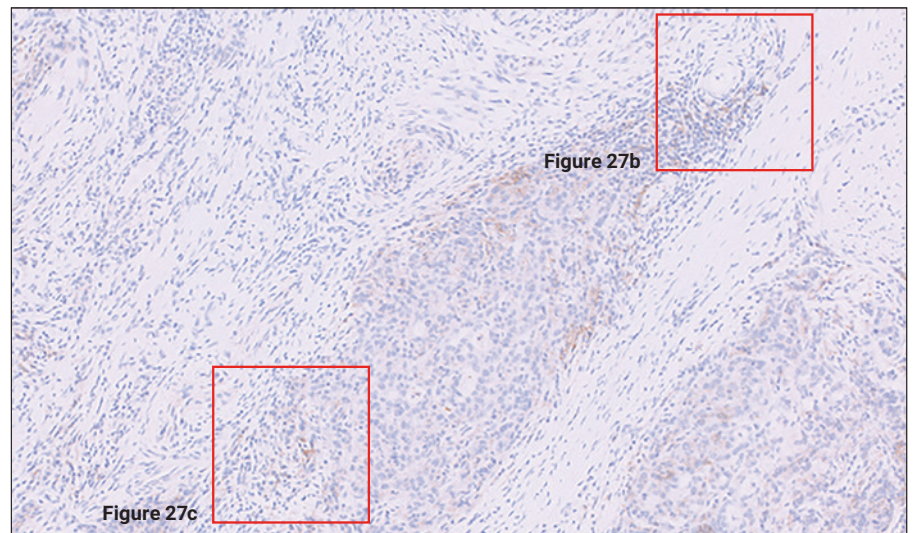
### Key point

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

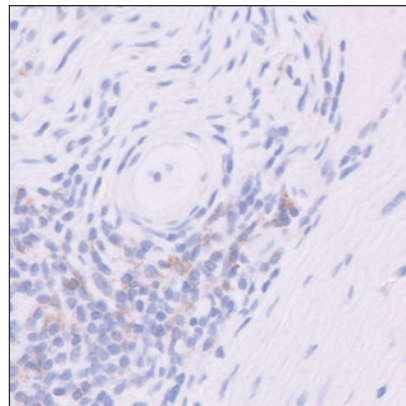
### Immune Cell Inclusion/Exclusion: 20× Rule

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:

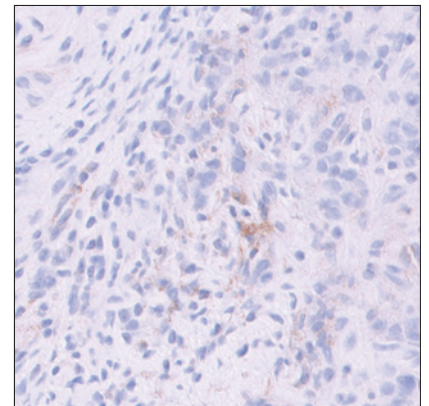
Move the slide so that the small tumor nest is in the approximate center of a 20× field or such that the large tumor mass fills approximately half of a 20× field. MICs that lie within this field should be included in scoring. MICs outside of this field should be excluded from scoring as long as they do not surround neighboring tumor. See Figures 27a–27c for an example of determining which PD-L1 staining MICs are included in the CPS numerator.



**Figure 27a:** At 5× magnification, two areas of PD-L1 staining MICs are visible. Following the instructions above, zoom in to 20× magnification on each field to determine which PD-L1 staining MICs to include in the CPS numerator (5× magnification).



**Figure 27b:** Tumor cells are absent from this 20× field containing PD-L1 staining MICs, thus none of these MICs should be included in the CPS numerator (20× magnification).



**Figure 27c:** When positioning the tumor such that it fills approximately half of a 20× field, PD-L1 staining MICs that are present within the same field should be included in the CPS numerator as long as they are directly related to the response against the tumor (20× magnification).



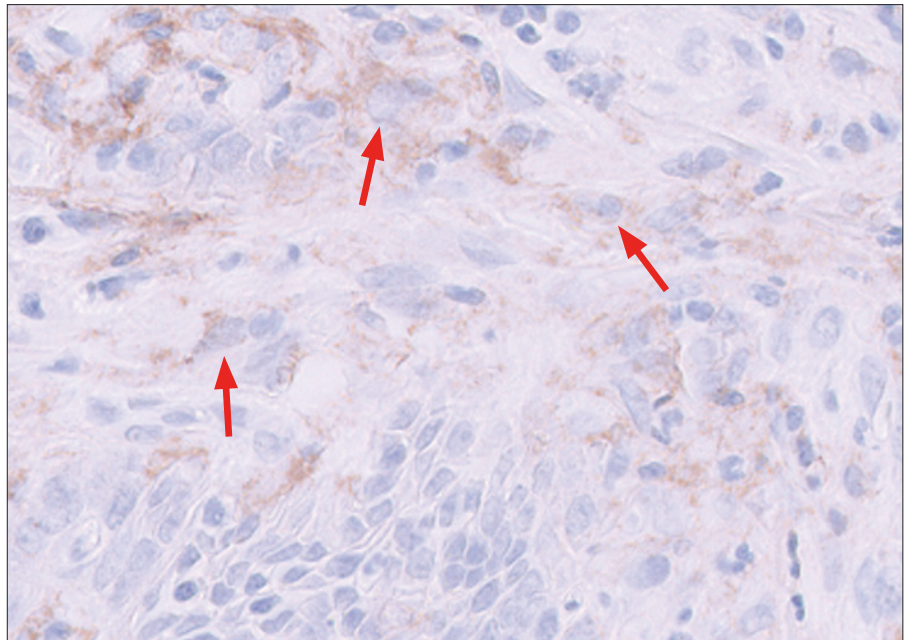
## Cells Excluded from CPS

Only tumor cells exhibiting PD-L1 membrane staining and tumor-associated MICs exhibiting PD-L1 membrane and/or cytoplasmic staining should be included in the CPS numerator. On the following pages are other cells that can exhibit PD-L1 expression but should be excluded from the CPS calculation (CPS numerator 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 28 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.



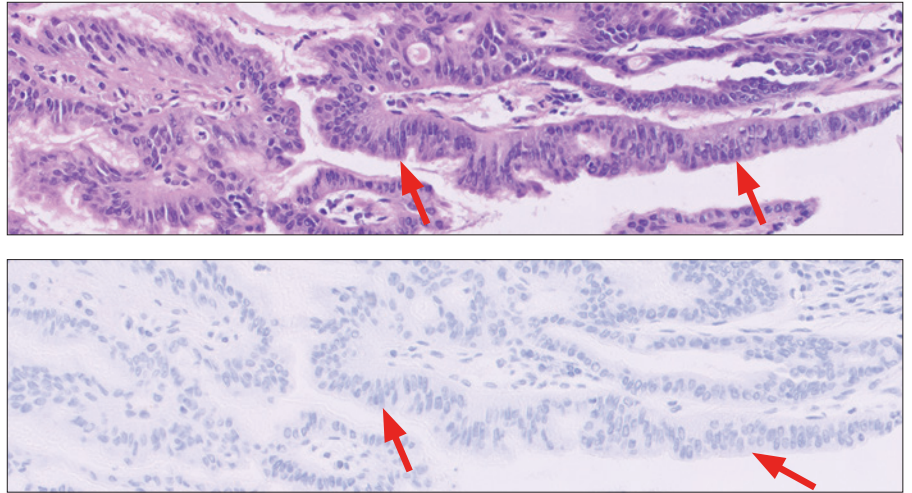
**Figure 28:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting only cytoplasmic staining of tumor cells (arrows) (20× magnification).

### Key point

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

### Low-grade Dysplasia

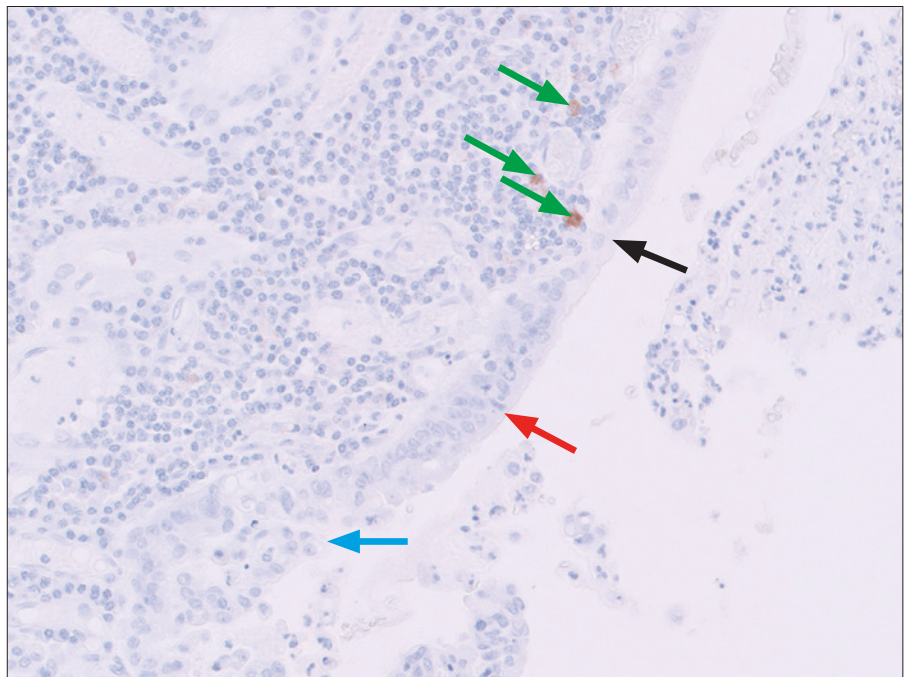
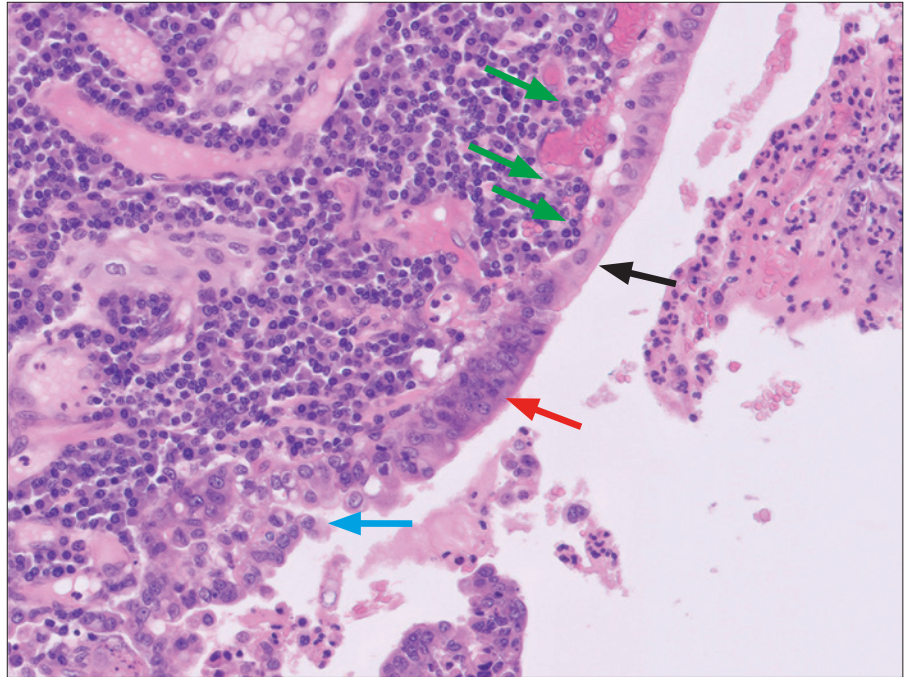
Both PD-L1 staining and nonstaining low-grade dysplasia as well as PD-L1 staining MICs associated with low-grade dysplasia should be excluded from the CPS calculation.



**Figure 29:** Low-grade dysplasia (arrows) should be excluded from the CPS calculation – H&E (top) and corresponding PD-L1 field (bottom) (20× magnification).

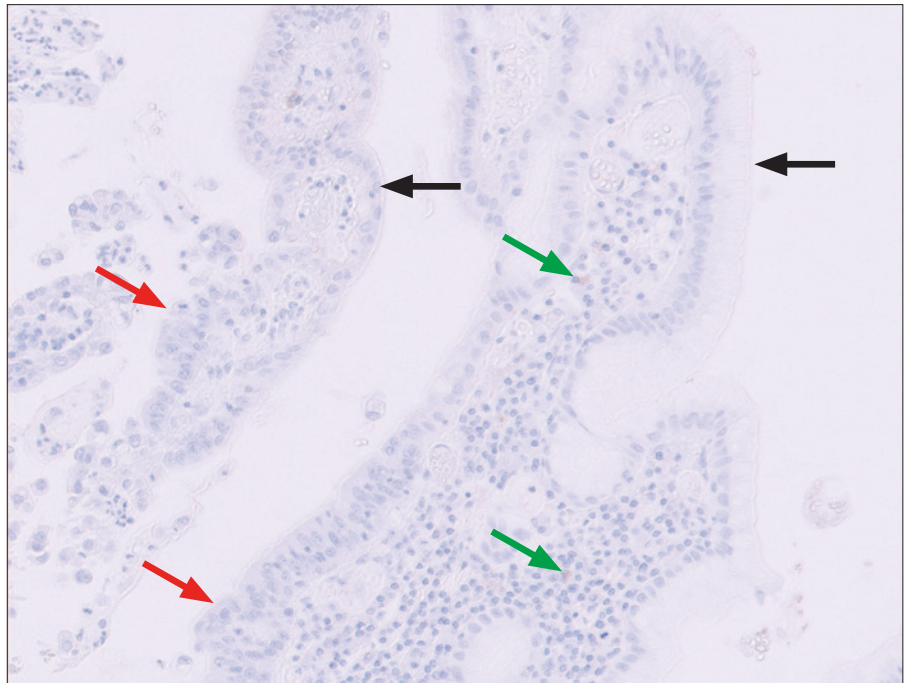
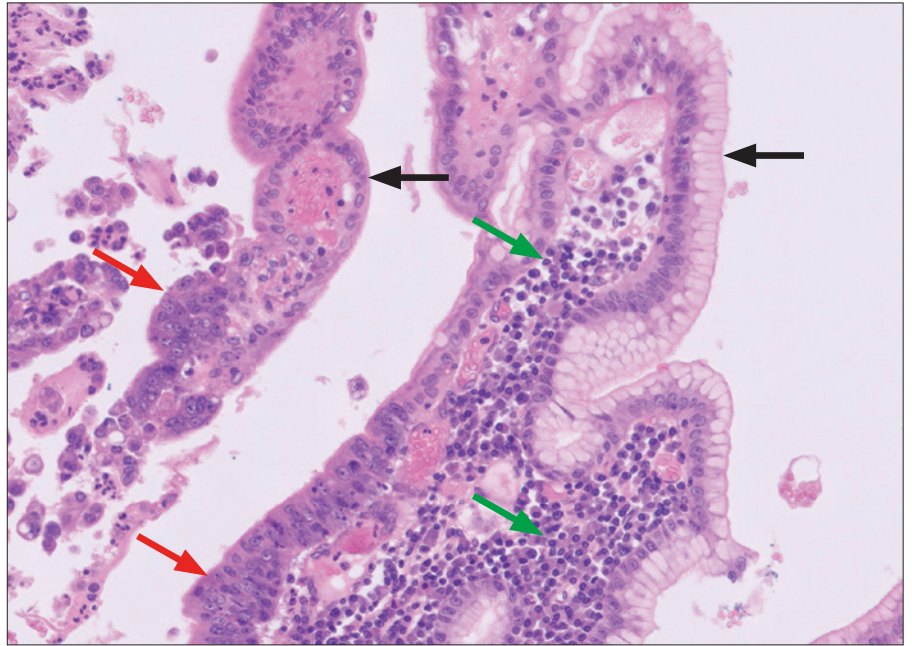
### High-grade Dysplasia (Carcinoma in Situ, or CIS)

Both PD-L1 staining and nonstaining high-grade dysplasia or carcinoma in situ (CIS) as well as PD-L1 staining MICs associated with high-grade dysplasia/CIS should be excluded from the CPS calculation.



**Figure 30a:** Benign epithelium (black arrow), high-grade dysplasia/CIS (red arrow), and PD-L1 staining MICs associated with these benign features (green arrows) should be excluded from the CPS calculation. Invasive adenocarcinoma (blue arrow) should be included in the CPS calculation – H&E (top) and corresponding PD-L1 field (bottom) (20× magnification).





**Figure 30b:** Benign epithelium (black arrows), PD-L1 staining MICs associated with benign epithelium (green arrows), and high-grade dysplasia/CIS (red arrows) should be excluded from the CPS calculation – H&E (top) and corresponding PD-L1 field (bottom) (20× magnification).

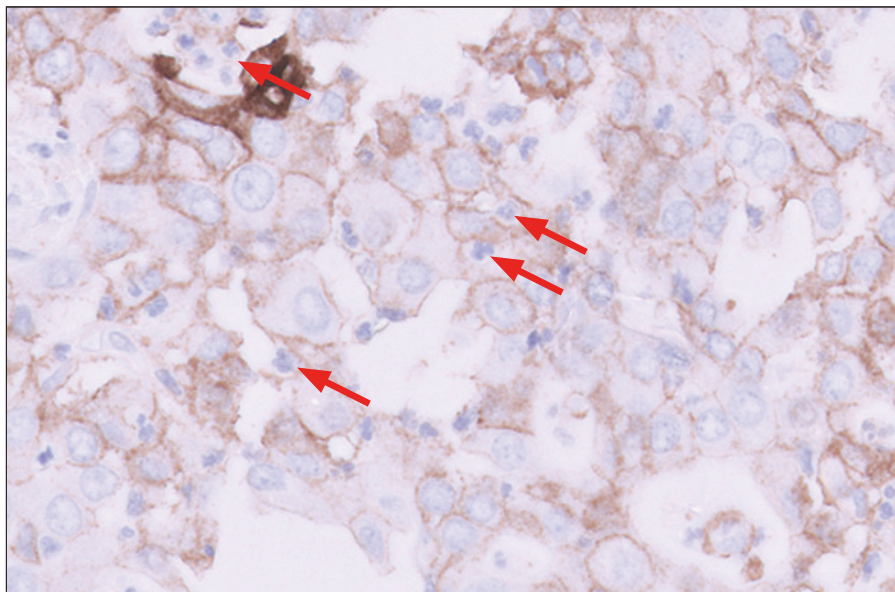
**Key point**

Gastric or GEJ low-grade dysplasia and high-grade dysplasia/carcinoma in situ (CIS) can exhibit PD-L1 staining and should be excluded from the score

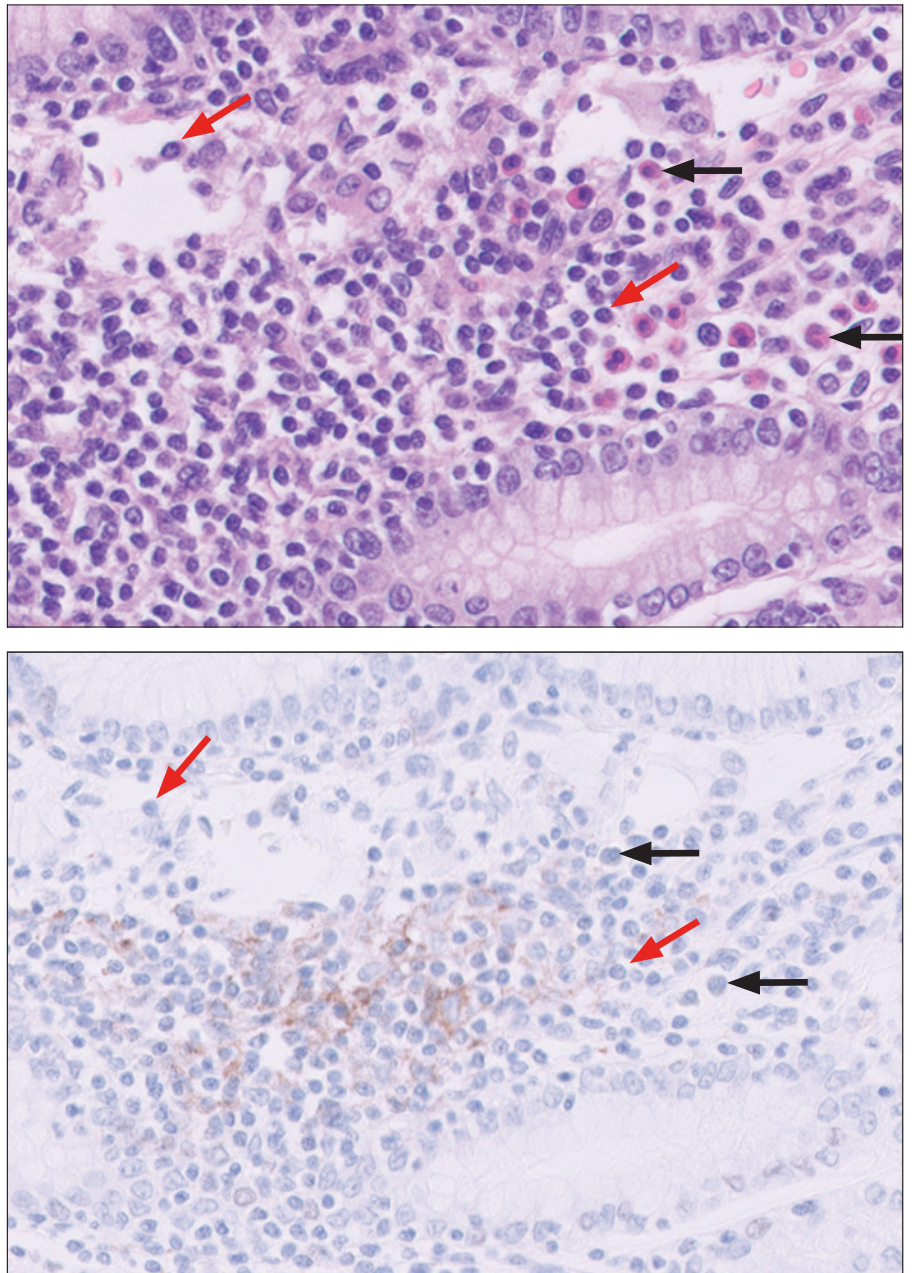


### Other Immune Cells Excluded from CPS

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 49 for the immune cell inclusion/exclusion 20× rule. Neutrophils, eosinophils, and plasma cells should be excluded from the CPS calculation.



**Figure 31a:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting staining of neutrophils (arrows) (20× magnification).

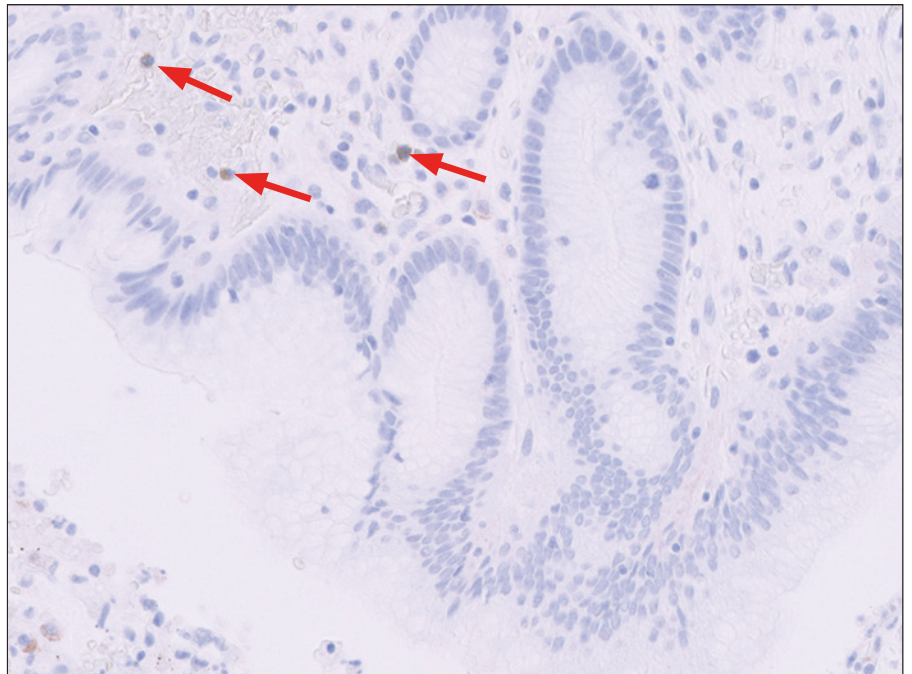
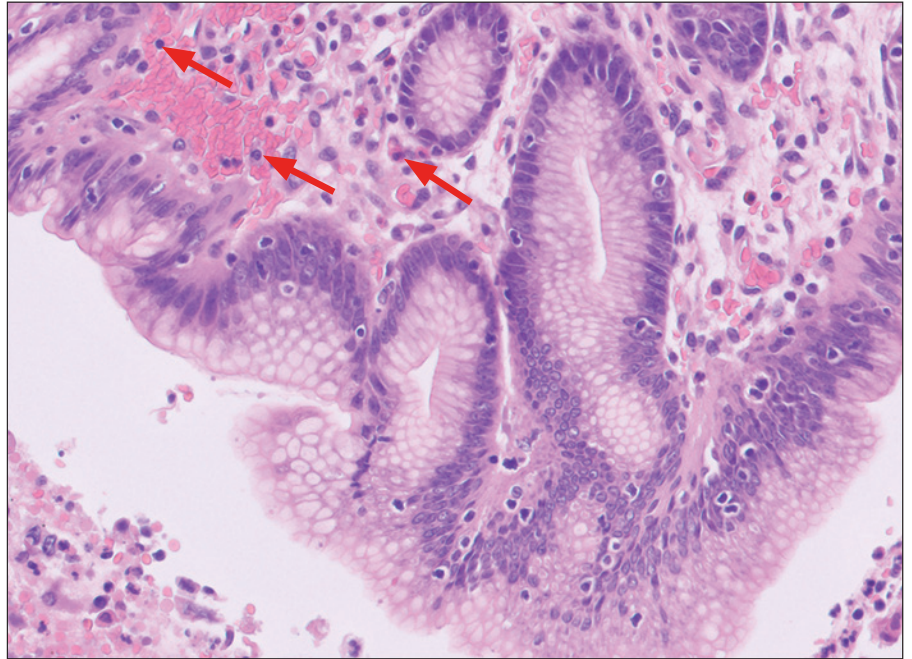


**Figure 31b:** Gastric or GEJ adenocarcinoma specimen with focus containing plasma cells (red arrows) and eosinophils (black arrows) which should be excluded from the CPS calculation – H&E (top) and corresponding PD-L1 field (bottom) (20× magnification).

**Key point**

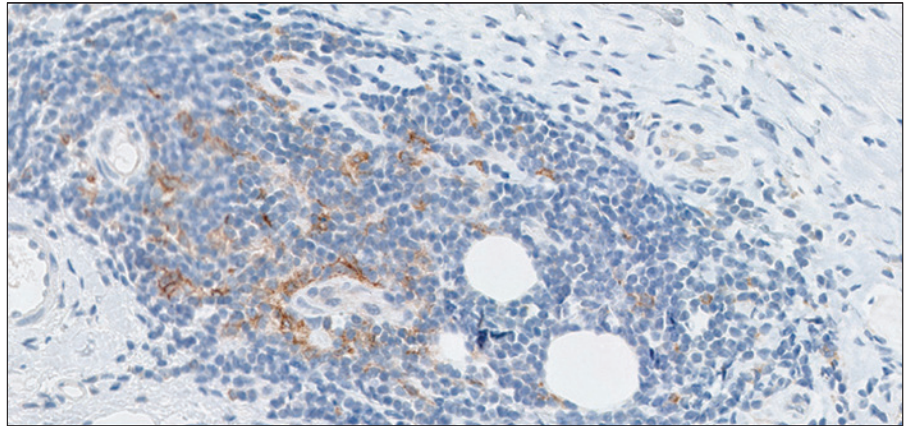
Neutrophils, eosinophils, and plasma cells should be excluded from the score

### MICs Associated with Benign Cells



**Figure 32a:** Gastric or GEJ adenocarcinoma specimen exhibiting staining of immune cells associated with benign epithelium which should be excluded from the score (arrows) – H&E (top) and corresponding PD-L1 field (bottom) (20× magnification).



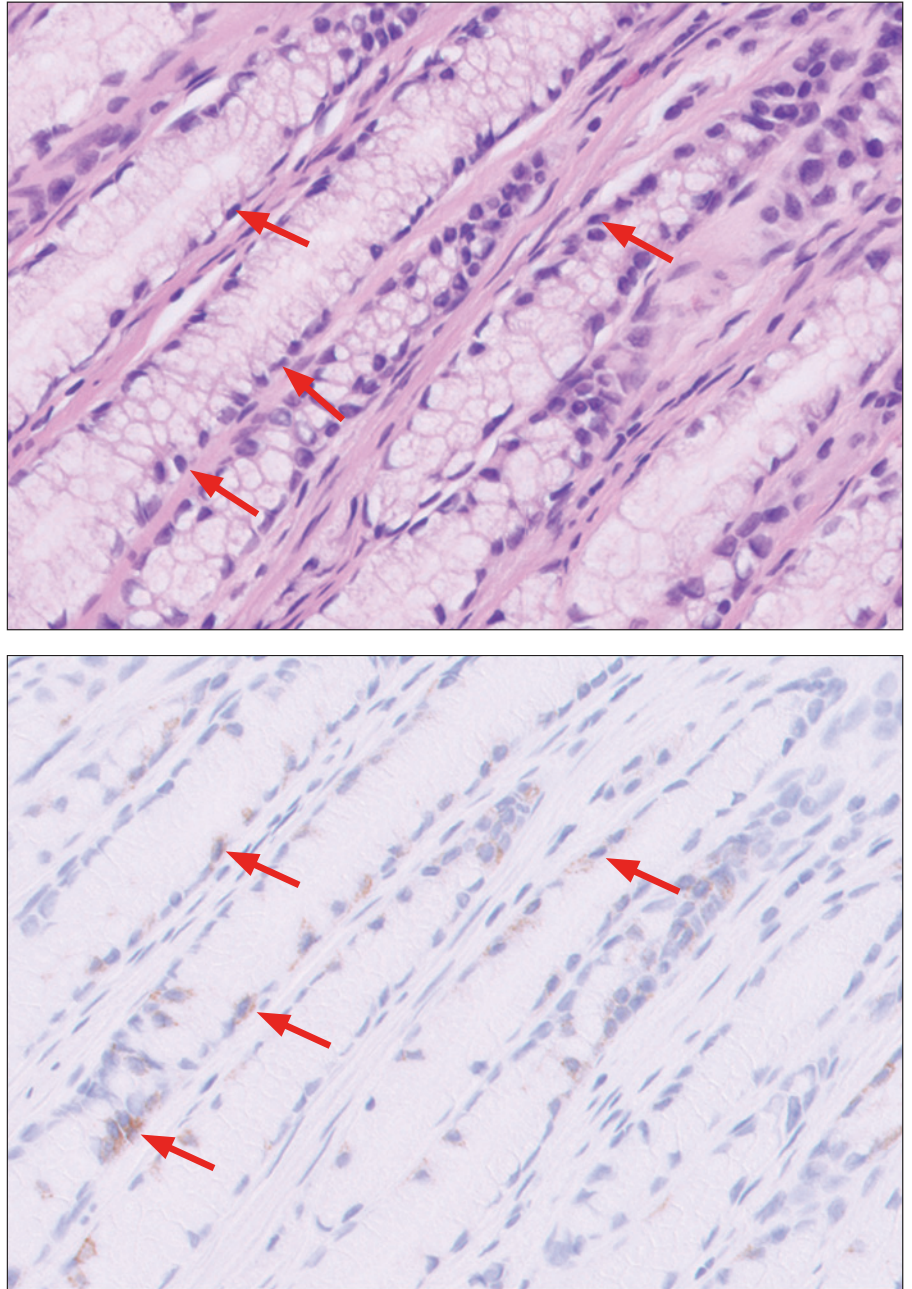


**Figure 32b:** PD-L1 staining MICs within a lymphoid aggregate that are not associated with the tumor response (10× magnification).

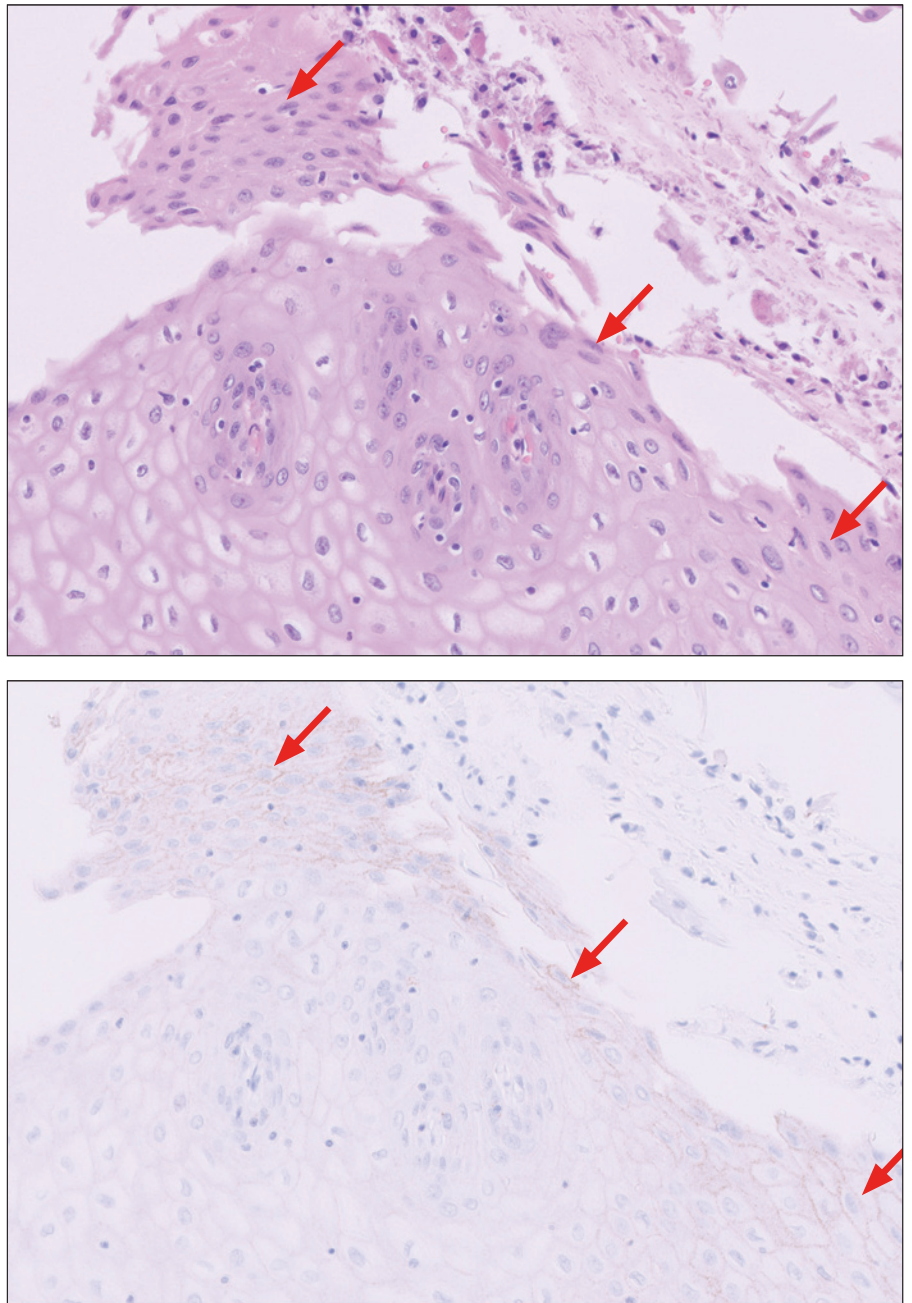
**Key point**

Both benign cells and immune cells associated with benign cells may exhibit PD-L1 staining and both should be excluded from the score

### Other Cells Excluded from CPS



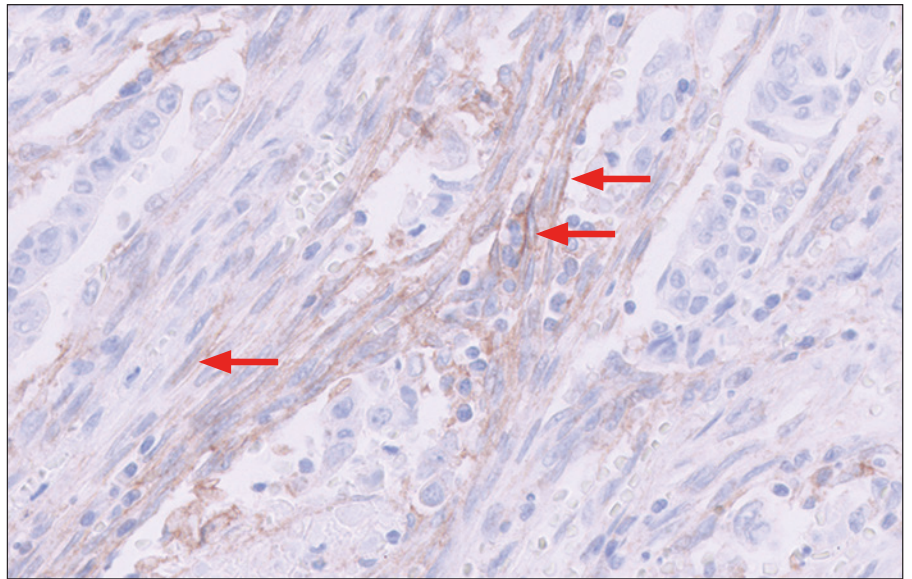
**Figure 33a:** Gastric or GEJ adenocarcinoma specimen exhibiting staining of benign glands (arrows) – H&E (top) and corresponding PD-L1 field (bottom) (20× magnification).



**Figure 33b:** Gastric or GEJ adenocarcinoma specimen exhibiting staining of benign surface epithelium (arrows) – H&E (top) and corresponding PD-L1 field (bottom) (20× magnification).



## Stromal Cells



**Figure 34:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting staining of stromal cells, including fibroblasts (arrows) (20× magnification).

### Key point

PD-L1 staining stromal cells should be excluded from the score



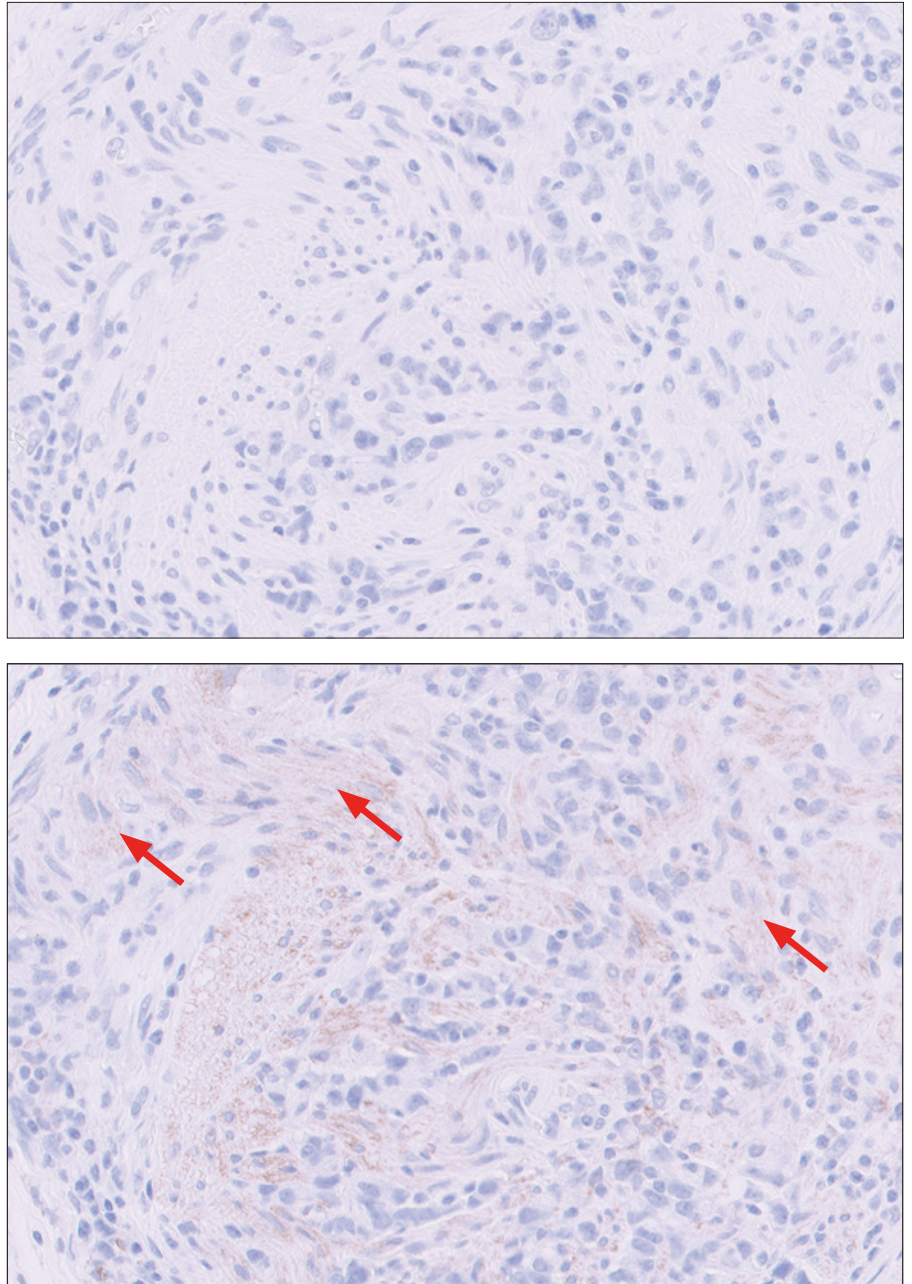


# Artifacts

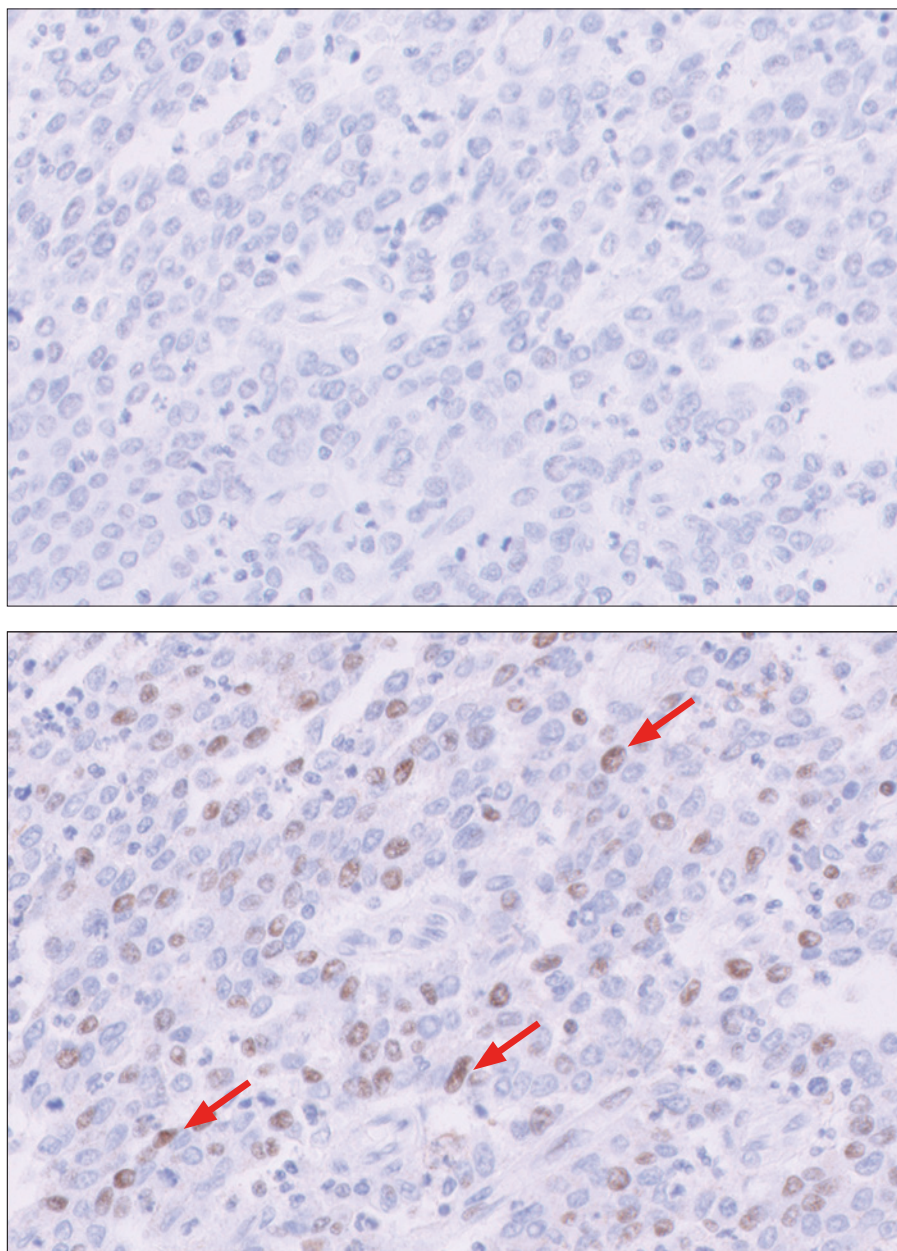
The following pages provide examples of artifacts you may see in PD-L1 IHC 22C3 pharmDx stained specimens.

## Specific Nonscorable Staining

Specific nonscorable staining is defined as chromogen-related staining due to the anti-PD-L1 antibody/PD-L1 antigen interaction that is present on cells or cellular compartments that are not included when scoring with CPS. It is specific staining because it is present on the PD-L1 slide and absent from the NCR slide; however, it is nonscorable because it is present on either cells (e.g., stromal cells, peripheral nerve, plasma cells, etc.) or cellular compartments (e.g., cytoplasm or nucleus of tumor cells) that are not included in the CPS algorithm. It is recommended that pathologists use their best clinical judgment to determine whether specific nonscorable staining present in the PD-L1 slide interferes with their evaluation of specific scorable PD-L1 expression to such an extent that a confident CPS score cannot be rendered. In cases where interference from specific nonscorable staining prevents confidence in scoring, the PD-L1 slide is considered non-evaluable. Note that even cases with high (> 1+ intensity) specific staining of nonscorable tissue components in the PD-L1 slide are considered evaluable if the pathologist can still provide a confident CPS score from the tissue.



**Figure 35a:** Gastric or GEJ adenocarcinoma specimen exhibiting  $\leq 1+$  intensity specific nonscorable cytoplasmic staining of peripheral nerve cells (arrows). Absence of similar staining in the NCR-stained slide (top) indicates that this cytoplasmic staining in the PD-L1 slide (bottom) is specific, but normal cells such as peripheral nerve cells should be excluded from scoring (20 $\times$  magnification).



**Figure 35b:** Gastric or GEJ adenocarcinoma specimen with nuclear staining of tumor cells absent from the NCR-stained slide (top) and present in the PD-L1 stained slide (bottom), indicating that this nuclear staining is specific staining. Since the nucleus is a cell compartment that is not scorable, and the nuclear reactivity does not interfere with evaluation of membrane staining on tumor cells, the PD-L1 stained slide should be considered evaluable (20× magnification).

## Nonspecific Staining

Nonspecific staining, including nonspecific nuclear staining, is defined as chromogen-related staining that is not related to the anti-PD-L1 antibody/PD-L1 antigen interaction and can be visible either on both the NCR- and PD-L1 stained slides, or only on the NCR slide. It is caused by several factors. These factors include, but are not limited to:

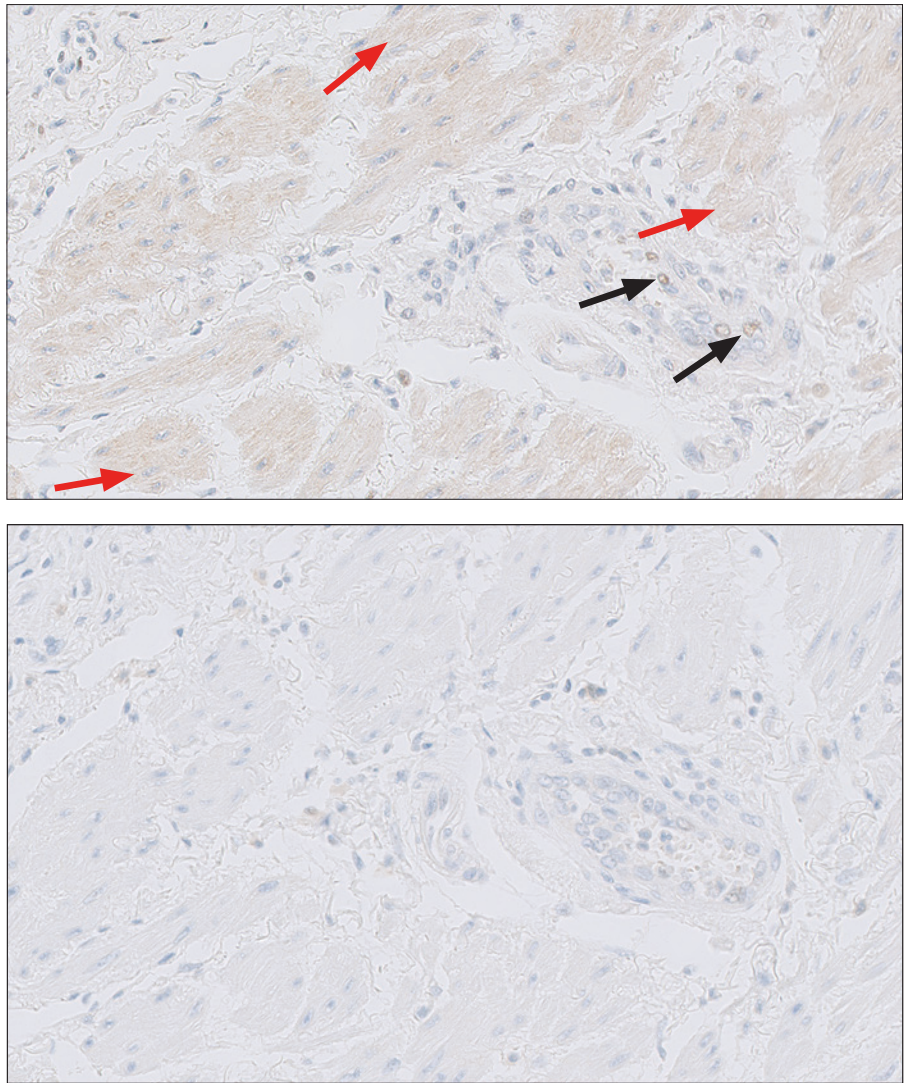
- Pre-analytic fixation and processing of the specimen including use of fixatives other than neutral buffered formalin (not recommended)
- Incomplete removal of paraffin from the section
- Incomplete rinsing of reagents from slides during staining
- Drying of slides; ensure slides remain wet with buffer while loading onto Autostainer Link 48 and prior to initiating run. Ensure that slide drying does not occur during the staining procedure
- Cross-reactivity of the secondary antibody in the detection system
- Reagent trapping (tissue folding; tissue drying; hydrophobic or ionic interactions with "sticky" tissues or substances such as cartilage, muscle fibers, dense fibrosis, mucin, necrotic debris)

The nonspecific staining of the NCR-stained test section is useful in determining the level of nonspecific staining in the PD-L1 stained test section. All specimens must have  $\leq 1+$  nonspecific staining to be considered acceptable for PD-L1 expression evaluation.

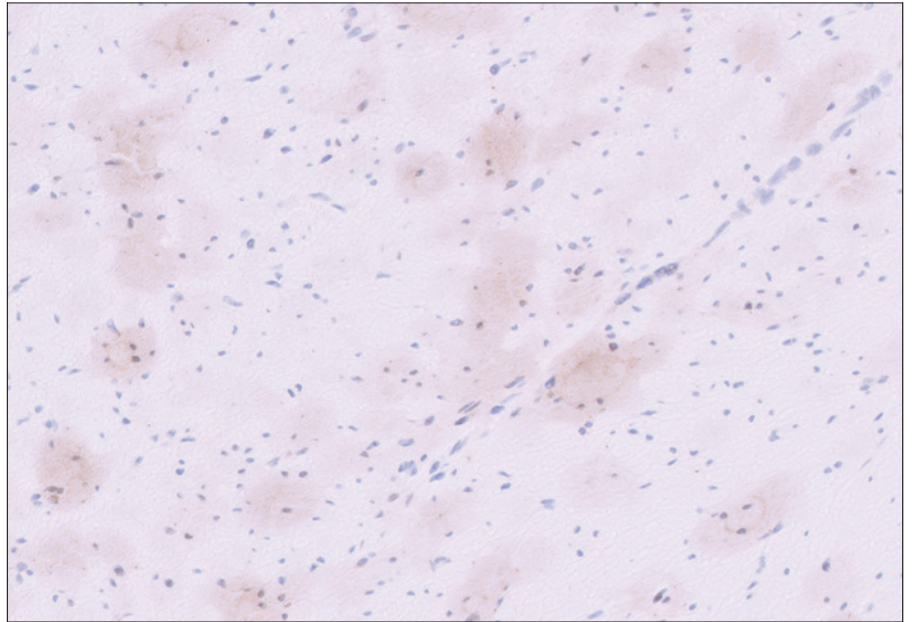
When staining of cell nuclei is present in both the specimen slide stained with the PD-L1 IHC 22C3 pharmDx primary antibody as well as in the NCR-stained slide or only in the NCR-stained slide, it is considered nonspecific nuclear staining and, if present, should be  $\leq 1+$  intensity within the scorable tumor region(s) of both the PD-L1 and NCR-stained slides and excluded from scoring. If the intensity of the nonspecific nuclear staining within scorable tumor region(s) is  $> 1+$  in the NCR-stained slide, or both the NCR- and PD-L1 stained slide, the PD-L1 slide should be marked as non-evaluable and a retest of the specimen should be performed.

Nonspecific staining with PD-L1 IHC 22C3 pharmDx is rare.

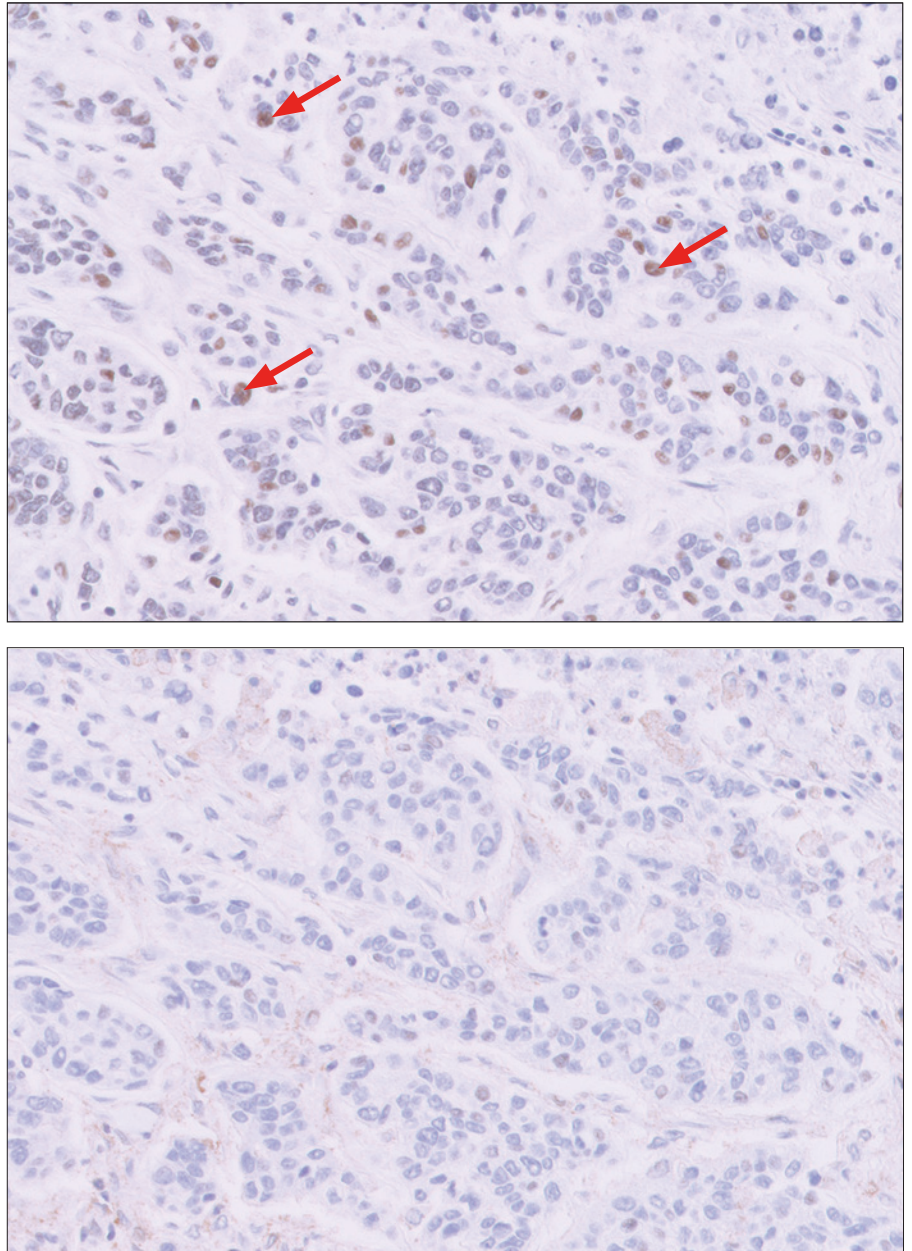




**Figure 36a:** Esophageal adenocarcinoma specimen stained with NCR (top) exhibiting acceptable ( $\leq 1+$  intensity) nonspecific staining on smooth muscle cells (red arrows) and occasional endothelial cell nuclei (black arrows). Note that in this case, the nonspecific staining is not also present in the PD-L1 slide (bottom) (20x magnification).



**Figure 36b:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting acceptable  $\leq 1+$  intensity nonspecific DAB droplets staining (20 $\times$  magnification).



**Figure 36c:** Gastric or GEJ adenocarcinoma specimen exhibiting nonspecific nuclear staining (arrows) that is present in both the NCR- (top) and PD-L1 stained (bottom) slides, but is stronger ( $\geq 1+$  intensity) in the NCR slide. As the acceptance criteria for nonspecific staining, including nuclear staining, in the NCR and PD-L1 slides or NCR slide alone is  $\leq 1+$  intensity, the PD-L1 stained slide in this case should be considered non-evaluable (20 $\times$  magnification).

#### Key point

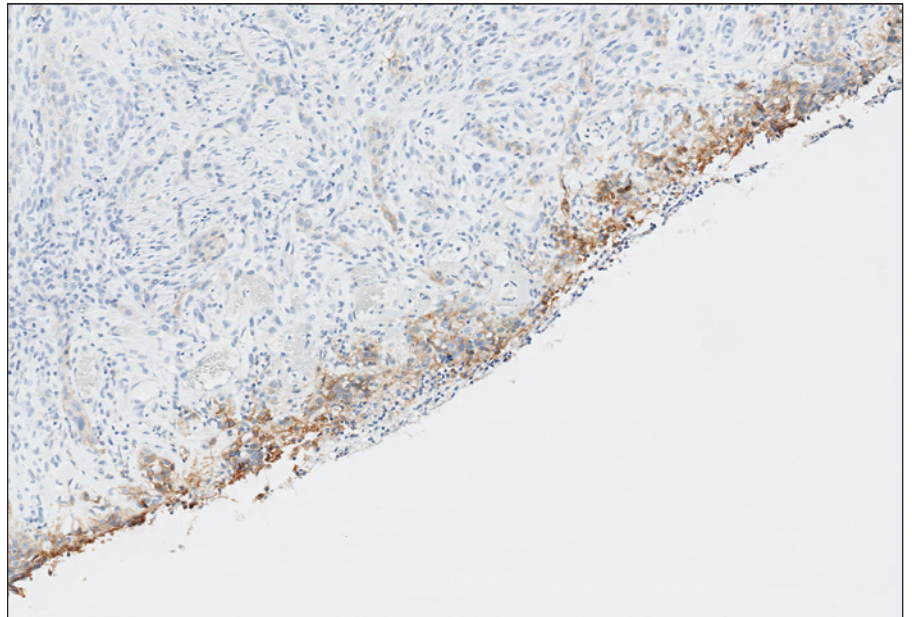
All specimens must have  $\leq 1+$  nonspecific staining, including nuclear staining



## Edge Artifact

Increased nonspecific staining that is observed around the periphery of the tissue specimen is known as the “edge artifact”.

- Edge artifact is commonly due to drying of the tissue section after the 3-in-1 pretreatment procedure is performed (i.e., after loading on the Autostainer Link 48 and/or at any point during the staining procedure)
- If staining is only at the edge of the tissue section (i.e., a few cell layers of staining at the periphery and ending abruptly with penetration into the centrally located tumor), scoring at the edge of the tissue specimen should be avoided
- Inadequate processing of thick tissue samples may mimic edge artifact by rendering the central portion of the tissue suboptimally fixed relative to the peripheral areas. In these circumstances, the immunoreactivity based on the suboptimal central portion may be mistakenly interpreted as nonstaining because optimal fixation is only present at the periphery



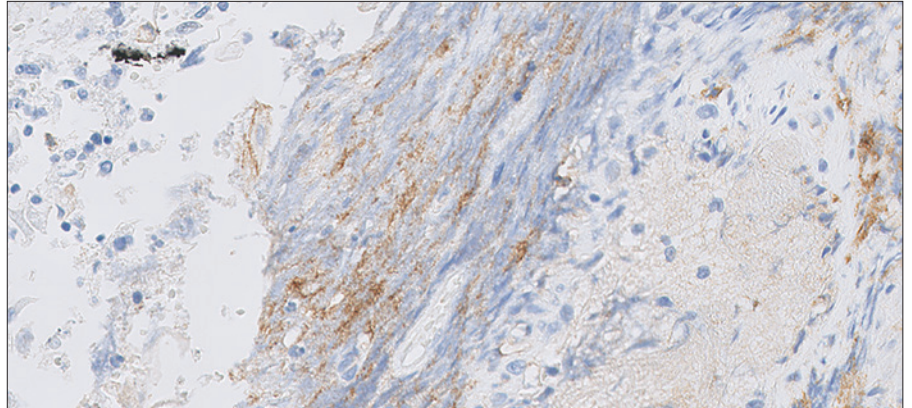
**Figure 37:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting edge artifact staining which should be excluded from the score (5× magnification).

### Key Point

Scoring of the edge of a specimen should be avoided if staining is inconsistent with the rest of the specimen and if it appears to be nonspecific in character

## 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.



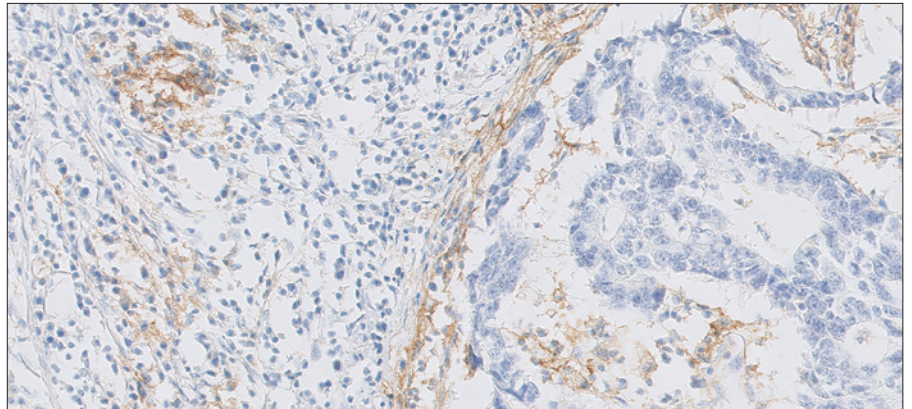
**Figure 38:** Esophageal cancer specimen stained with PD-L1 primary antibody exhibiting crush artifact; crush artifact should be excluded from the score (20× magnification).

### 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 stained with PD-L1 primary antibody exhibiting poor tissue fixation (20× magnification).

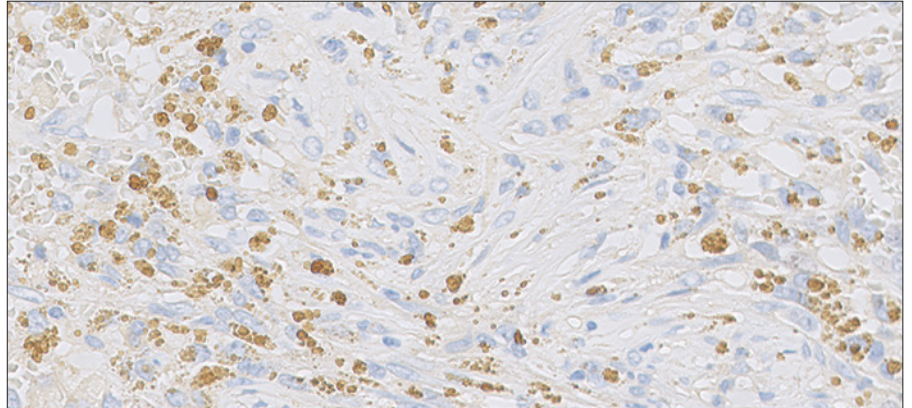
### Key Point

Proper fixation is important for accurate PD-L1 assessment. Poorly fixed foci should be excluded from scoring



## Hemosiderin

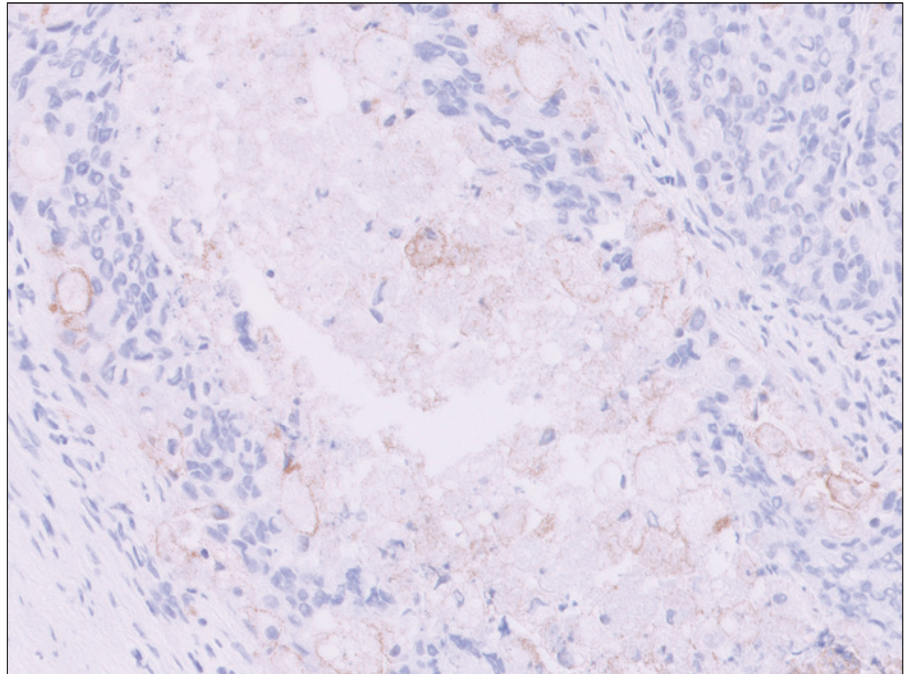
Hemosiderin pigment may be present in gastric or GEJ adenocarcinoma tissue and will often exhibit a golden-brown color which may be confused with DAB staining. This pigment should be ignored and excluded from the score. Utilization of the NCR slide can be useful to help distinguish hemosiderin pigment from DAB staining.



**Figure 40:** Triple-negative breast cancer (TNBC) specimen stained with PD-L1 primary antibody exhibiting golden-brown hemosiderin pigment (20× magnification).

## Necrosis

Necrosis can be described as morphological changes indicative of cell death with undefined cellular detail. PD-L1 staining necrosis may be present in gastric or GEJ adenocarcinoma specimens and should be excluded from the score.



**Figure 41:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting staining of necrosis which should be excluded from the score (20× magnification).

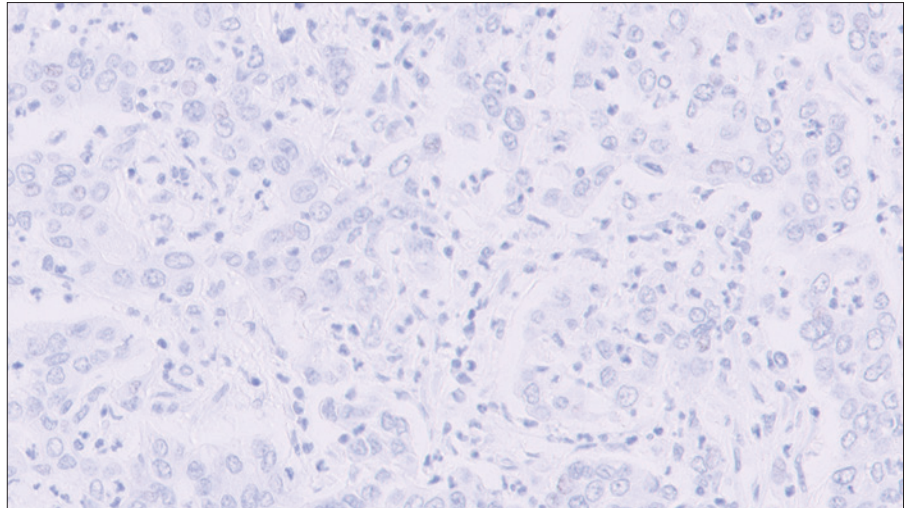
### Key Point

PD-L1 staining necrosis should not be scored

# PD-L1 IHC 22C3 pharmDx CPS Case Examples

## CPS 0 Case Example

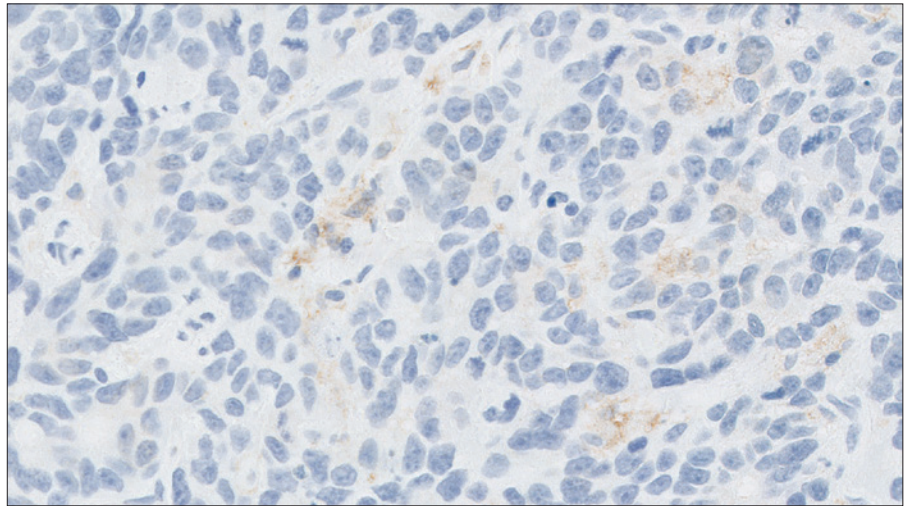
### Case 1: CPS 0



**Figure 42:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 0 (20× magnification).

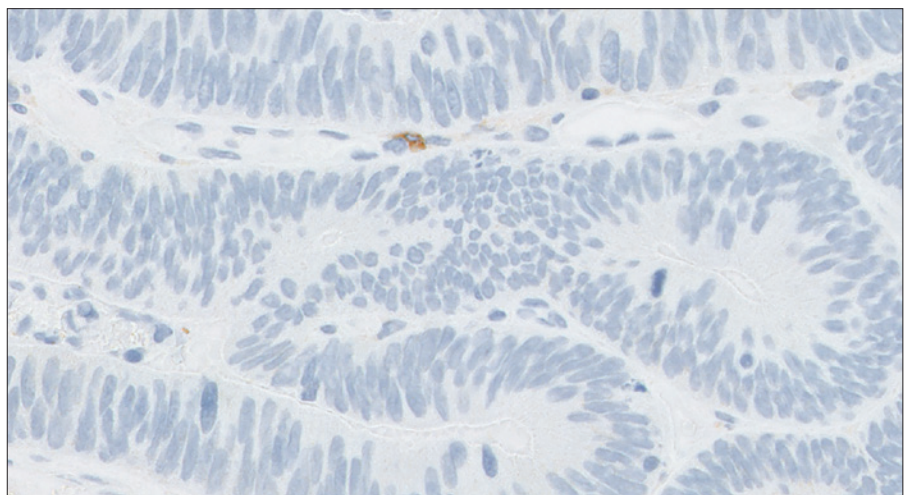
### Near-Cutoff Negative Case Examples (CPS Range of Greater Than 0 but Less Than 1)

#### Case 2: Near-Cutoff Negative (CPS Range of Greater Than 0 but Less Than 1)



**Figure 43:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a PD-L1 expression level of CPS < 1 (20× magnification). Note: Although this field contains PD-L1 staining cells, their numbers are not high enough to bring the score to CPS 1. The CPS should always be reported as a whole number (no fractions). Therefore, the score for this case should be reported as CPS 0.

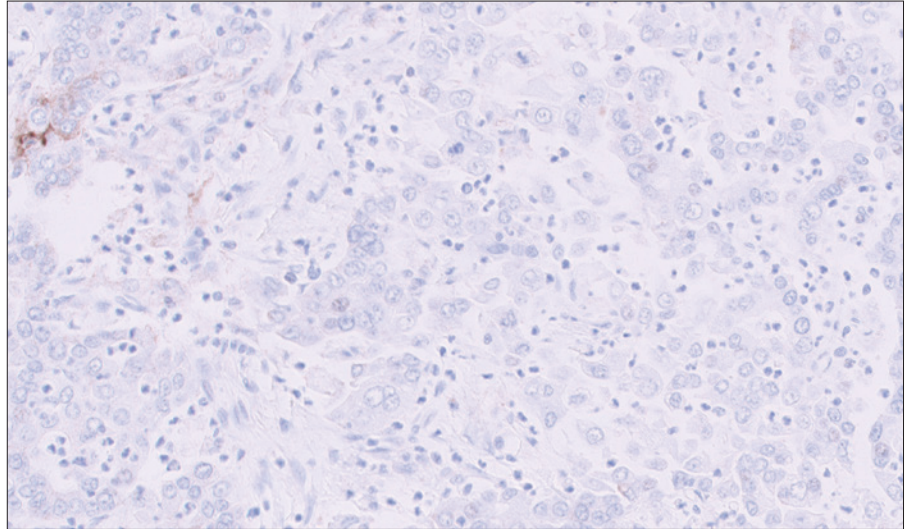
#### Case 3: Near-Cutoff Negative (CPS Range of Greater Than 0 but Less Than 1)



**Figure 44:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a PD-L1 expression level of CPS < 1 (20× magnification). Note: Although this field contains PD-L1 staining cells, their numbers are not high enough to bring the score to CPS 1. The CPS should always be reported as a whole number (no fractions). Therefore, the score for this case should be reported as CPS 0.

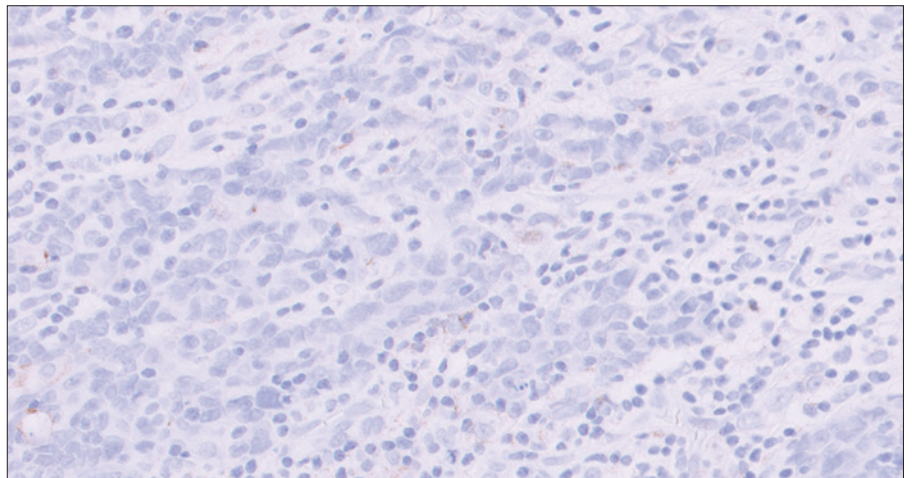


### Case 4: Near-Cutoff Negative (CPS Range of Greater Than 0 but Less Than 1)



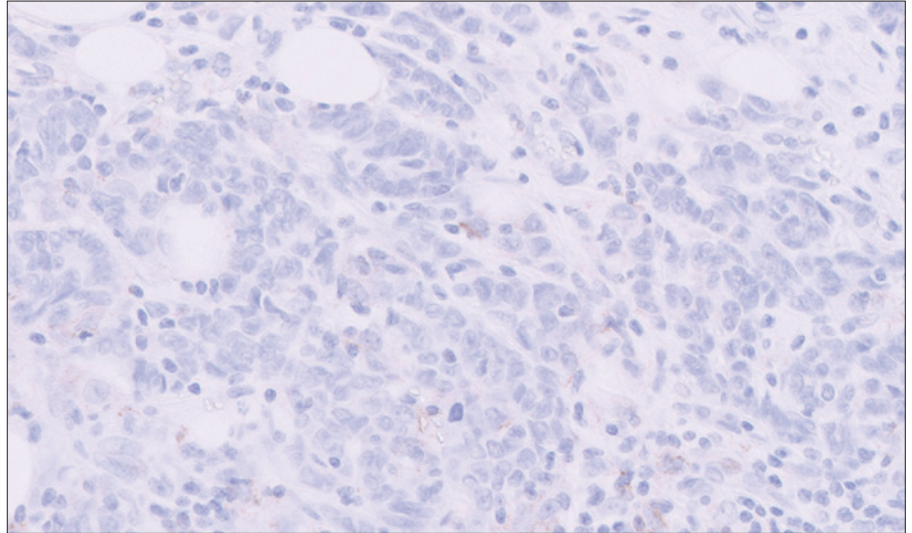
**Figure 45:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a PD-L1 expression level of CPS < 1 (20× magnification). Note: Although this field contains PD-L1 staining cells, their numbers are not high enough to bring the score to CPS 1. The CPS should always be reported as a whole number (no fractions). Therefore, the score for this case should be reported as CPS 0.

### Case 5: Near-Cutoff Negative (CPS Range of Greater Than 0 but Less Than 1)



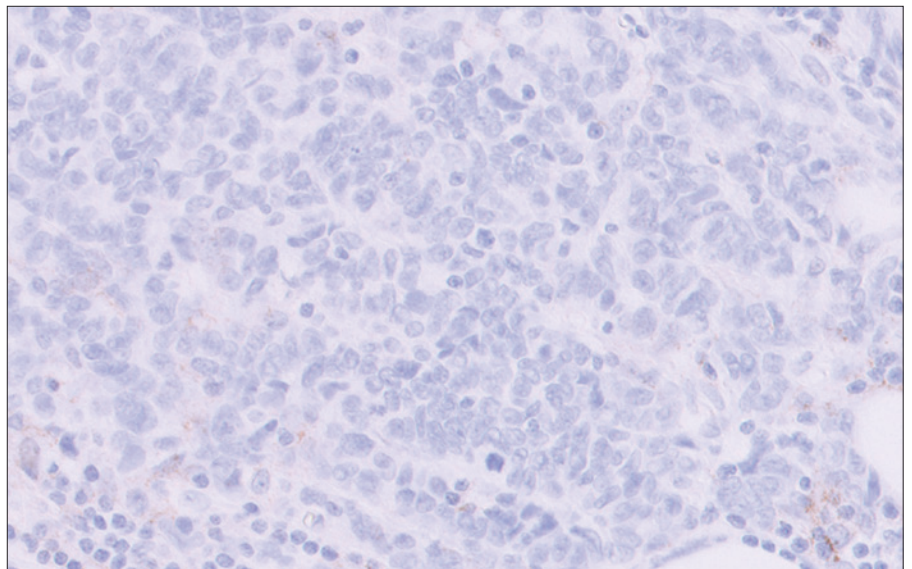
**Figure 46:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a PD-L1 expression level of CPS < 1 (20× magnification). Note: Although this field contains PD-L1 staining cells, their numbers are not high enough to bring the score to CPS 1. The CPS should always be reported as a whole number (no fractions). Therefore, the score for this case should be reported as CPS 0.

### Case 6: Near-Cutoff Negative (CPS Range of Greater Than 0 but Less Than 1)



**Figure 47:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a PD-L1 expression level of CPS < 1 (20× magnification). Note: Although this field contains PD-L1 staining cells, their numbers are not high enough to bring the score to CPS 1. The CPS should always be reported as a whole number (no fractions). Therefore, the score for this case should be reported as CPS 0.

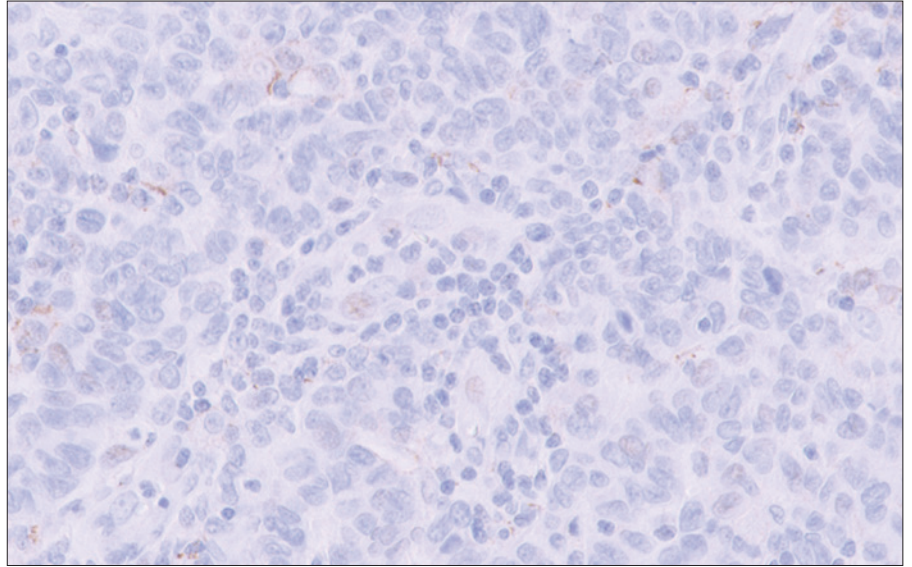
### Case 7: Near-Cutoff Negative (CPS Range of Greater Than 0 but Less Than 1)



**Figure 48:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a PD-L1 expression level of CPS < 1 (20× magnification). Note: Although this field contains PD-L1 staining cells, their numbers are not high enough to bring the score to CPS 1. The CPS should always be reported as a whole number (no fractions). Therefore, the score for this case should be reported as CPS 0.



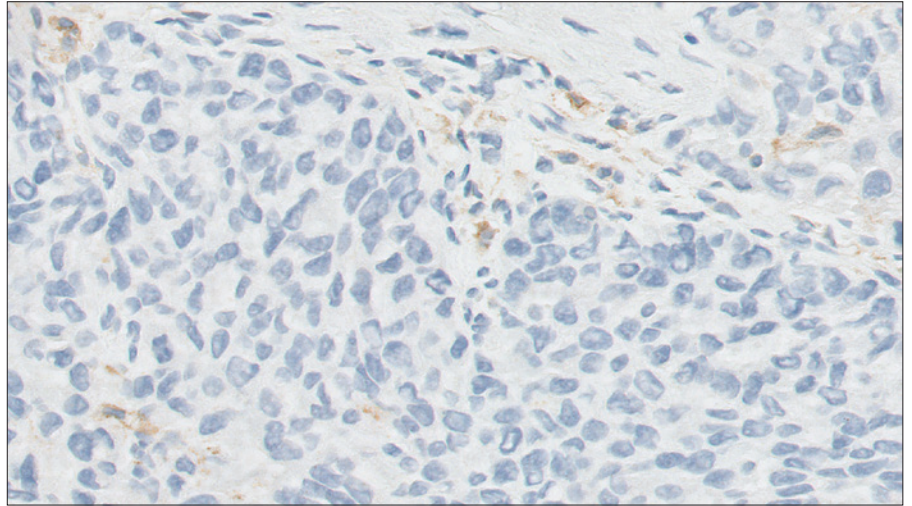
### Case 8: Near-Cutoff Negative (CPS Range of Greater Than 0 but Less Than 1)



**Figure 49:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a PD-L1 expression level of CPS < 1 (20× magnification). Note: Although this field contains PD-L1 staining cells, their numbers are not high enough to bring the score to CPS 1. The CPS should always be reported as a whole number (no fractions). Therefore, the score for this case should be reported as CPS 0.

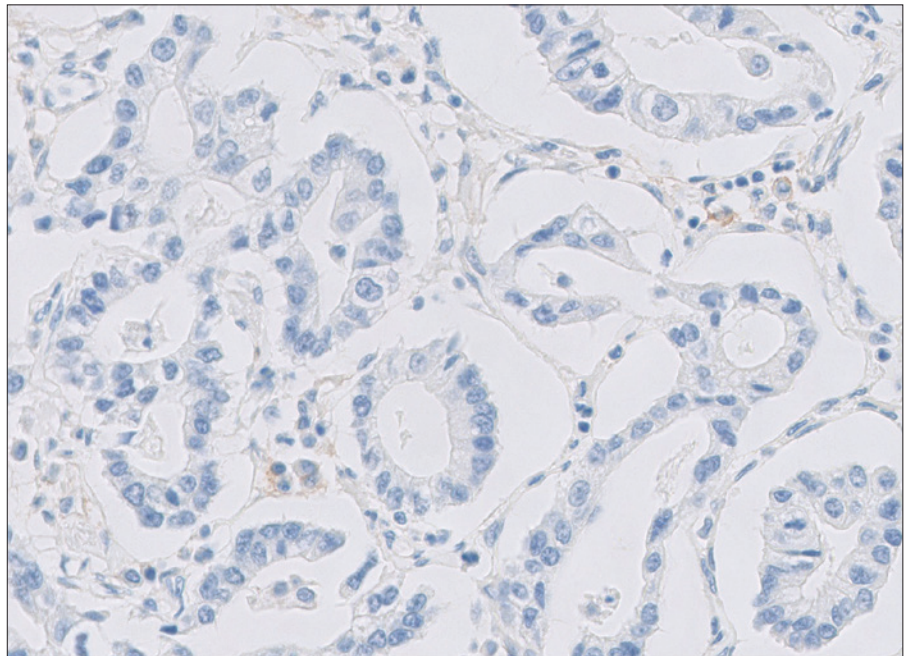
## Near-Cutoff Positive Case Examples (CPS Range 1–10)

### Case 9: Near-Cutoff Positive (CPS Range 1–10)



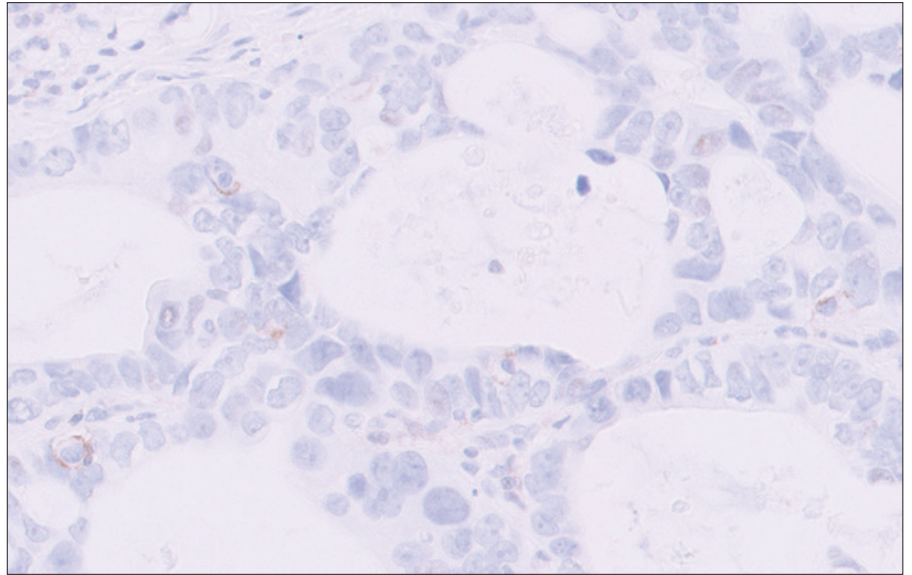
**Figure 50:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 2, however any numerical CPS between 1–5 could be assigned to this image (20× magnification).

### Case 10: Near-Cutoff Positive (CPS Range 1–10)



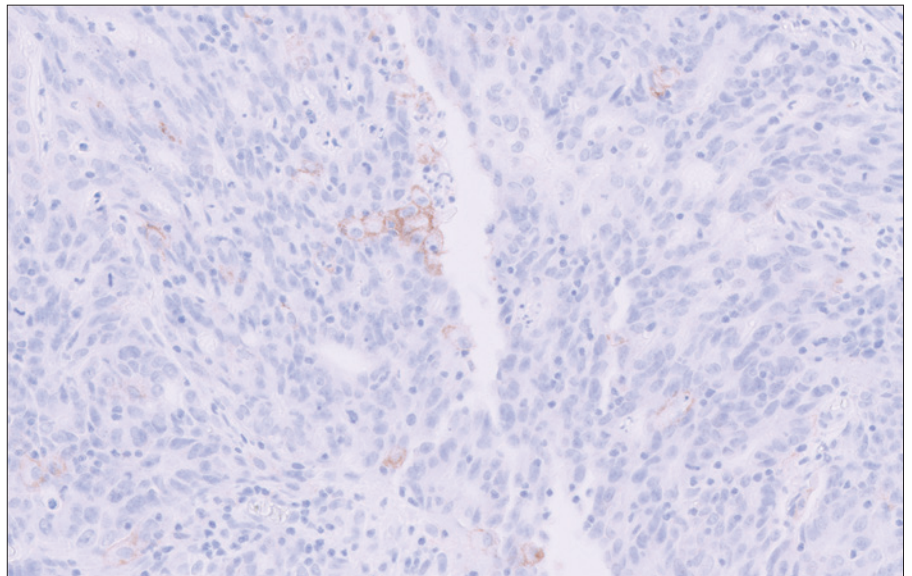
**Figure 51:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 2, however any numerical CPS between 1–3 could be assigned to this image (20× magnification).

### Case 11: Near-Cutoff Positive (CPS Range 1–10)



**Figure 52:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 2, however any numerical CPS between 1–4 could be assigned to this image (20× magnification).

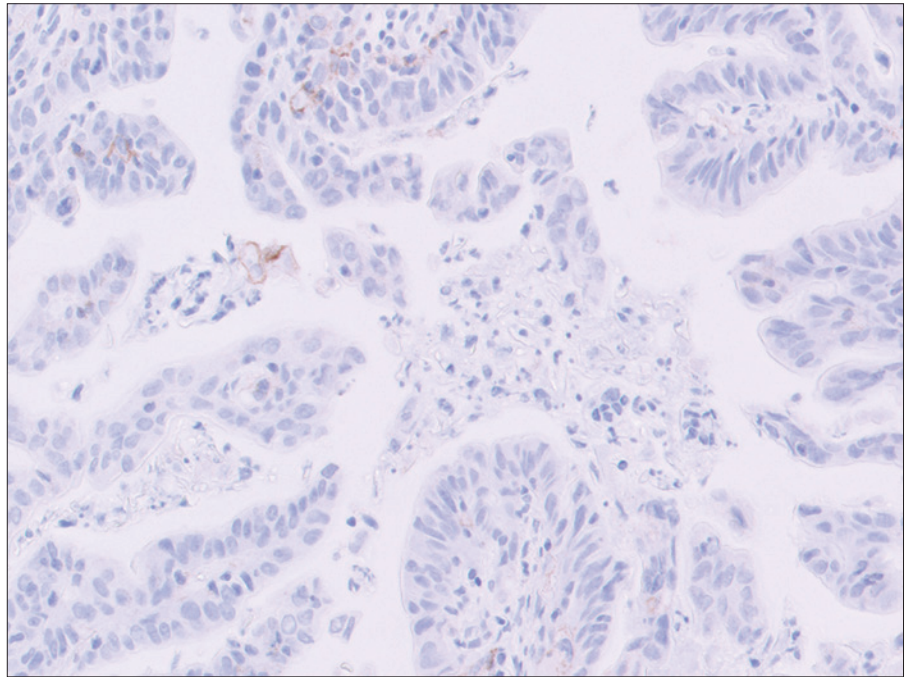
### Case 12: Near-Cutoff Positive (CPS Range 1–10)



**Figure 53:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 3, however any numerical CPS between 1–5 could be assigned to this image (20× magnification).

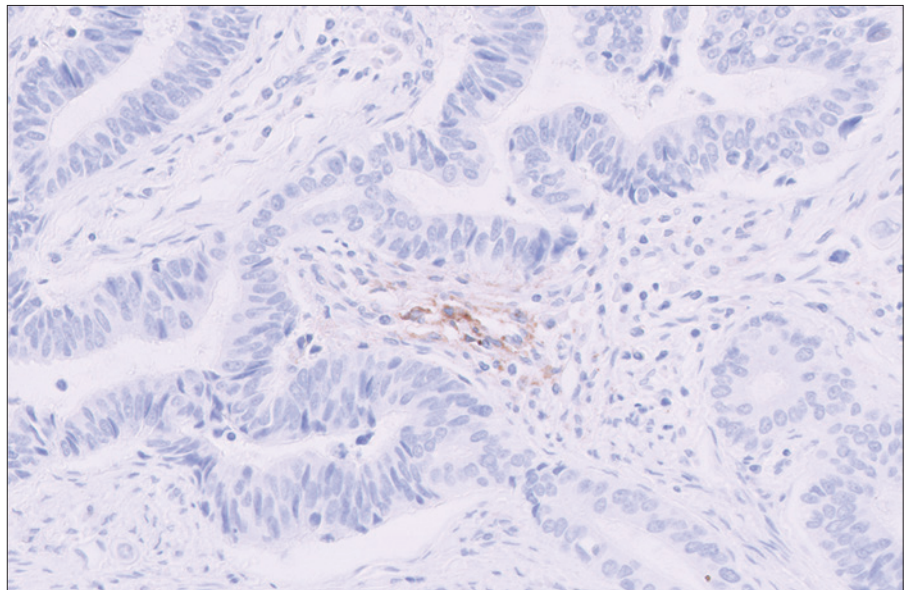


### Case 13: Near-Cutoff Positive (CPS Range 1–10)



**Figure 54:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 4, however any numerical CPS between 2–5 could be assigned to this image (20× magnification).

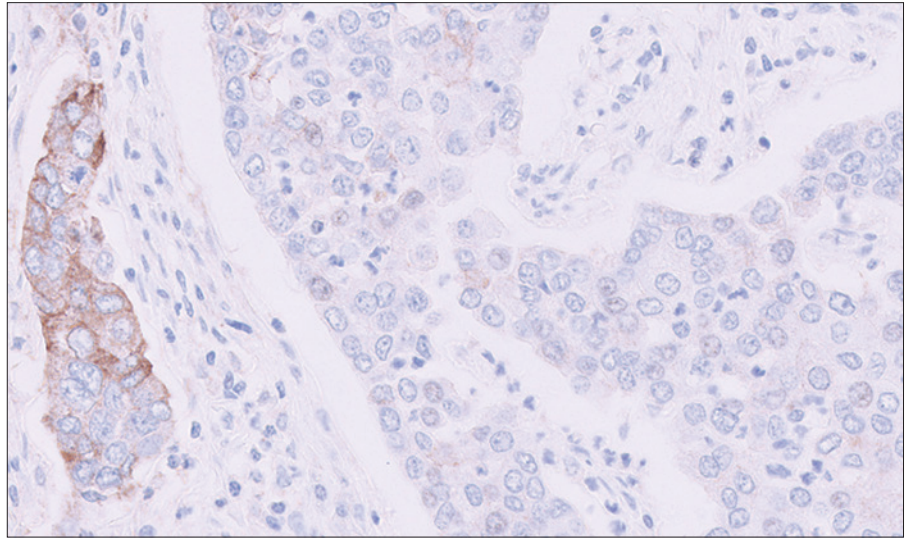
### Case 14: Near-Cutoff Positive (CPS Range 1–10)



**Figure 55:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 5, however any numerical CPS between 4–6 could be assigned to this image (20× magnification).

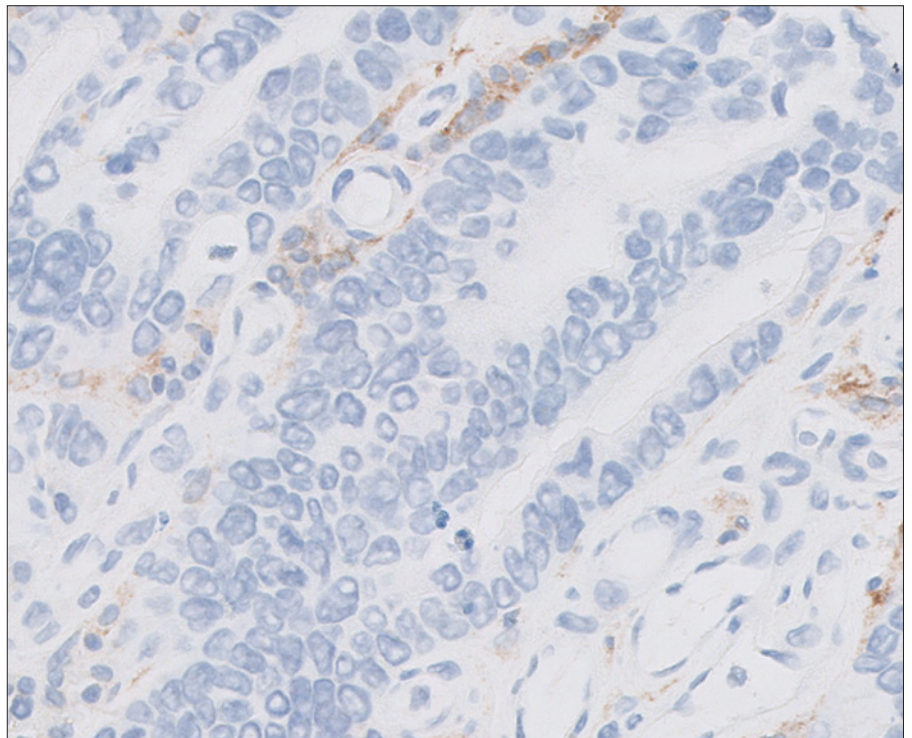


### Case 15: Near-Cutoff Positive (CPS Range 1–10)



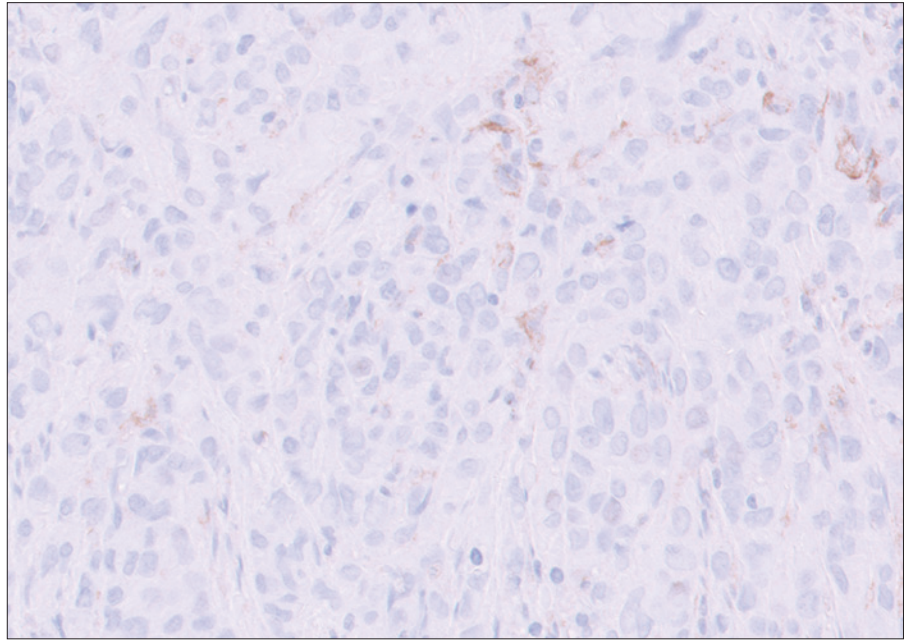
**Figure 56:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 5, however any numerical CPS between 4–6 could be assigned to this image (20× magnification).

### Case 16: Near-Cutoff Positive (CPS Range 1–10)



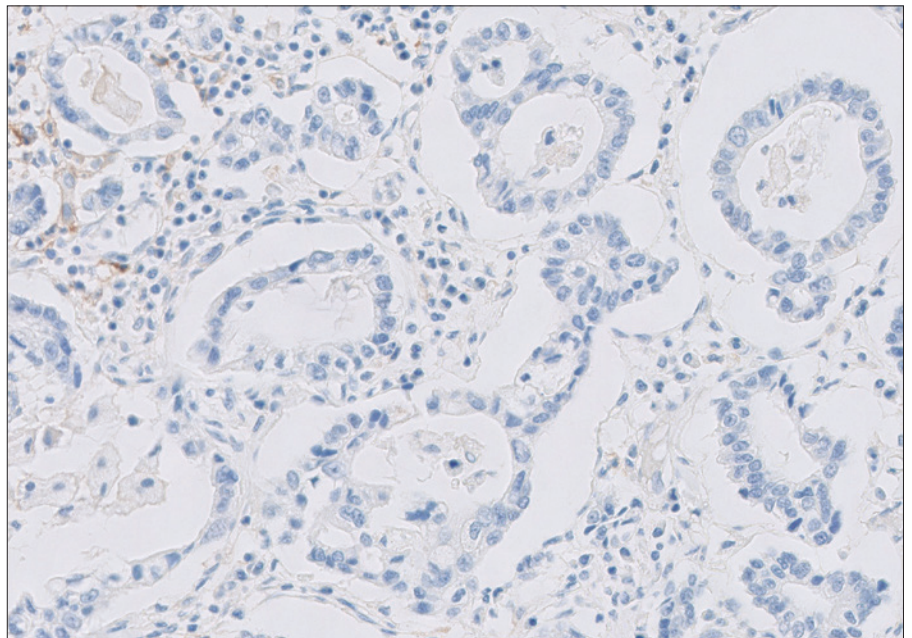
**Figure 57:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 6, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).

### Case 17: Near-Cutoff Positive (CPS Range 1–10)



**Figure 58:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 7, however any numerical CPS between 6–9 could be assigned to this image (20× magnification).

### Case 18: Near-Cutoff Positive (CPS Range 1–10)

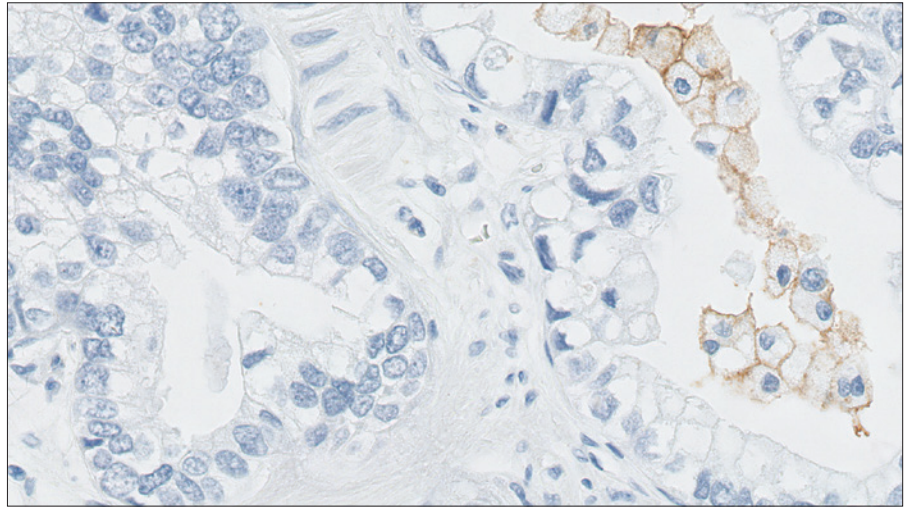


**Figure 59:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).



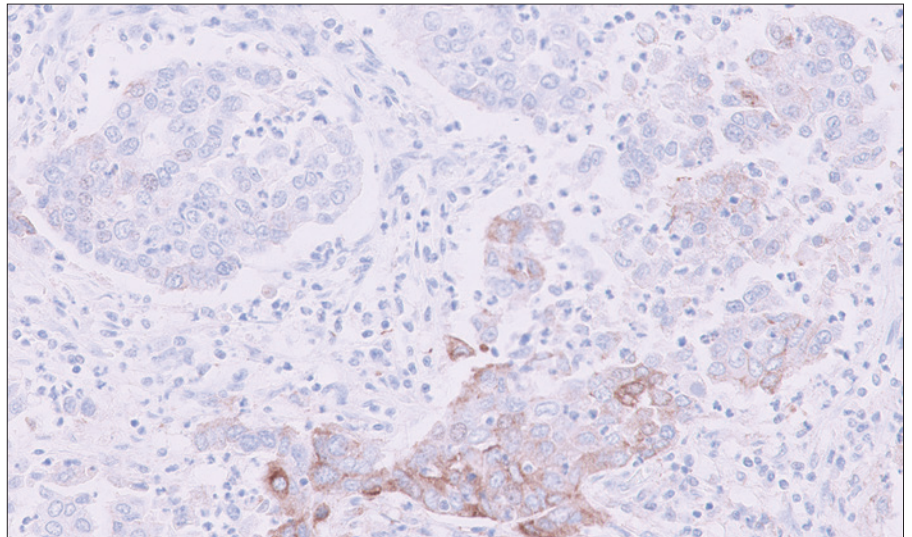
## CPS $\geq$ 1 Case Examples

### Case 19: CPS $\geq$ 1



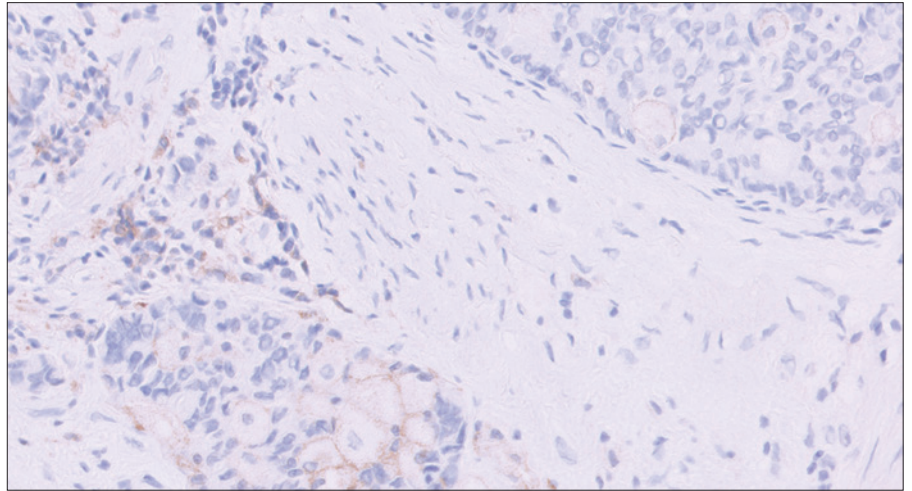
**Figure 60:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 12, however any numerical CPS between 10–14 could be assigned to this image (20 $\times$  magnification).

### Case 20: CPS $\geq$ 1



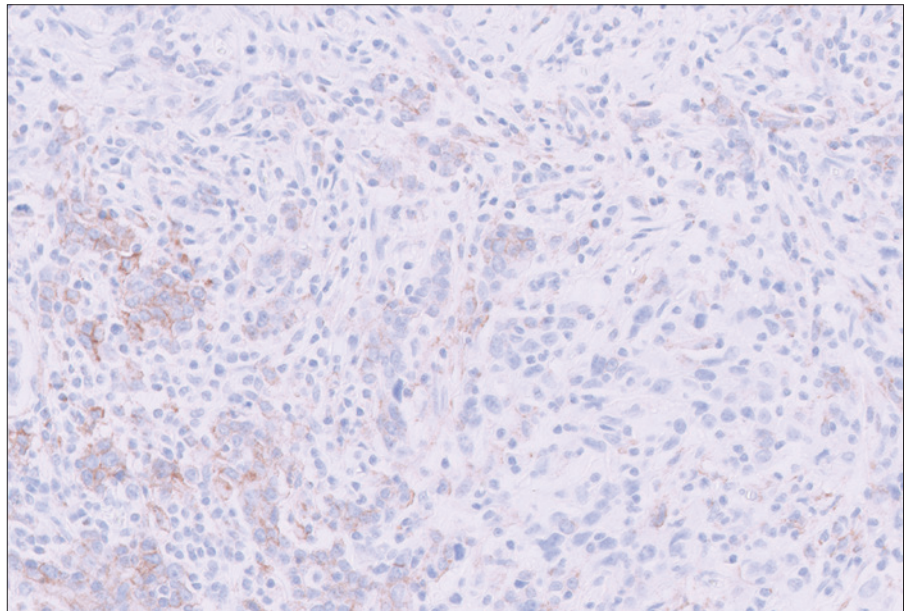
**Figure 61:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 12, however any numerical CPS between 10–15 could be assigned to this image (20 $\times$  magnification).

**Case 21: CPS  $\geq$  1**



**Figure 62:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 14, however any numerical CPS between 12–16 could be assigned to this image (20 $\times$  magnification).

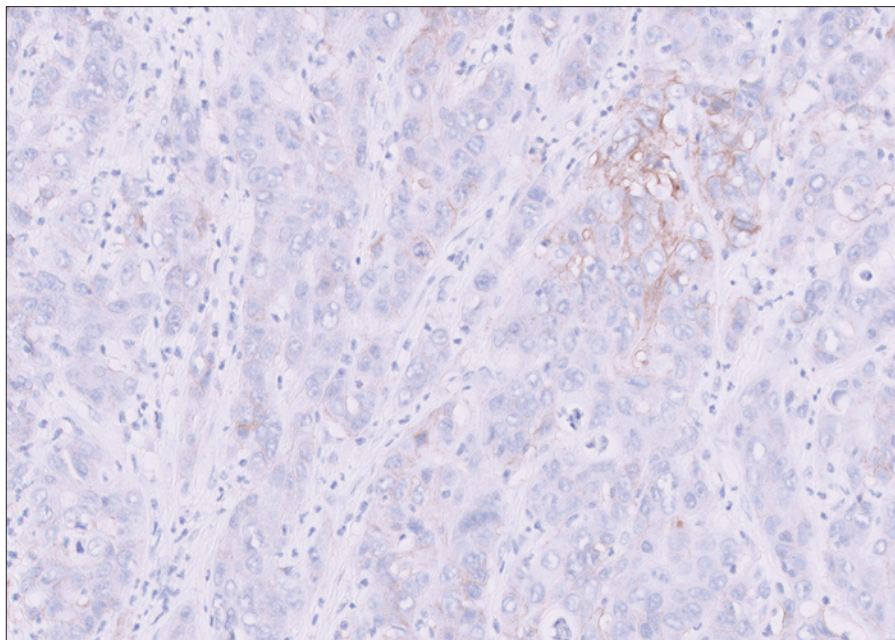
**Case 22: CPS  $\geq$  1**



**Figure 63:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 15, however any numerical CPS between 12–18 could be assigned to this image (20 $\times$  magnification).

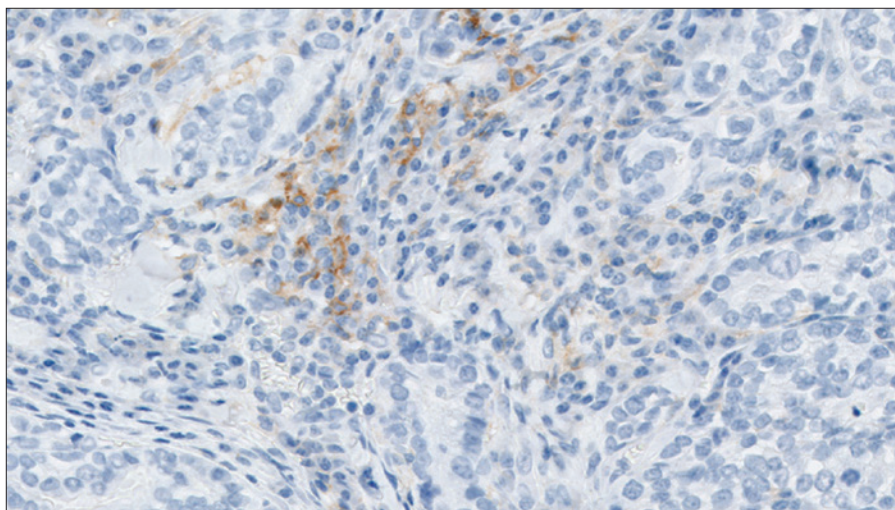


**Case 23: CPS  $\geq$  1**



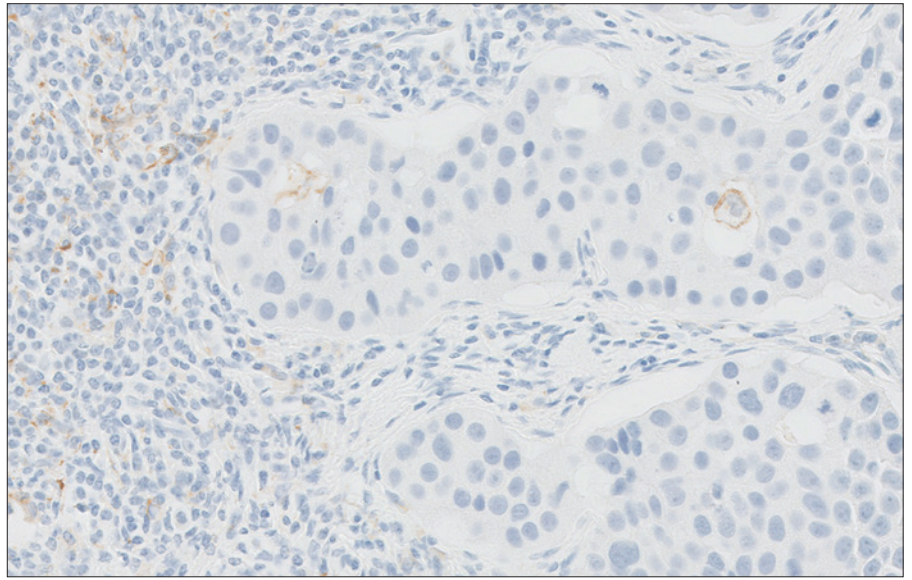
**Figure 64:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 15, however any numerical CPS between 11–18 could be assigned to this image (20 $\times$  magnification).

**Case 24: CPS  $\geq$  1**



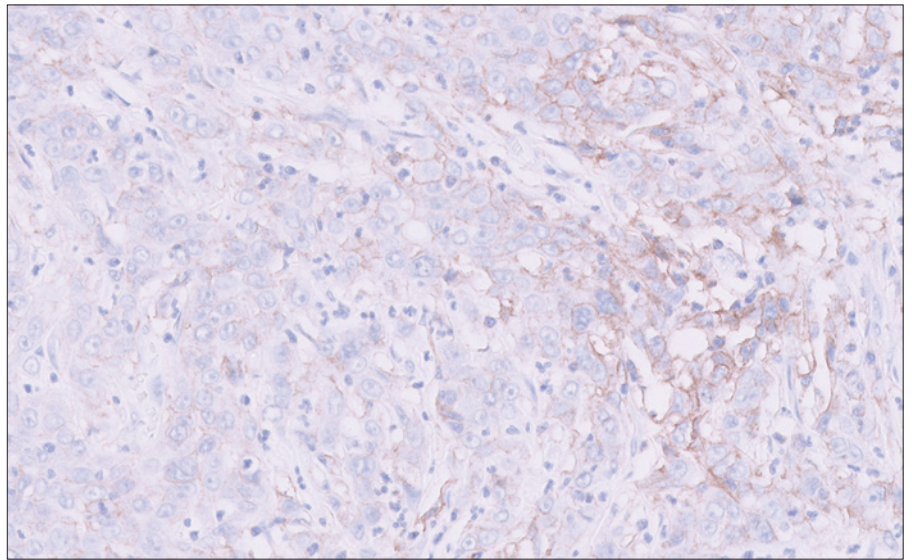
**Figure 65:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 17, however any numerical CPS between 15–19 could be assigned to this image (20 $\times$  magnification).

**Case 25: CPS  $\geq$  1**



**Figure 66:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 18, however any numerical CPS between 16–20 could be assigned to this image (20 $\times$  magnification).

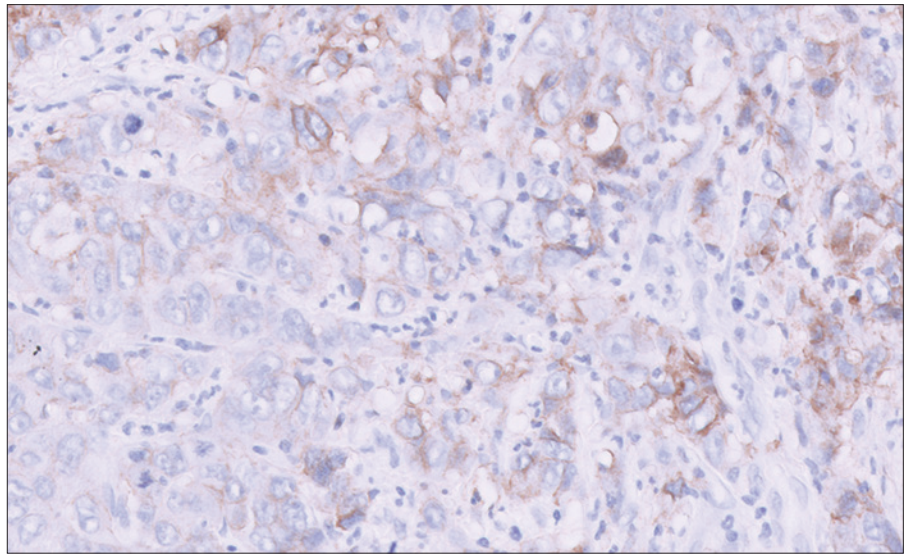
**Case 26: CPS  $\geq$  1**



**Figure 67:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 35, however any numerical CPS between 30–40 could be assigned to this image (20 $\times$  magnification).

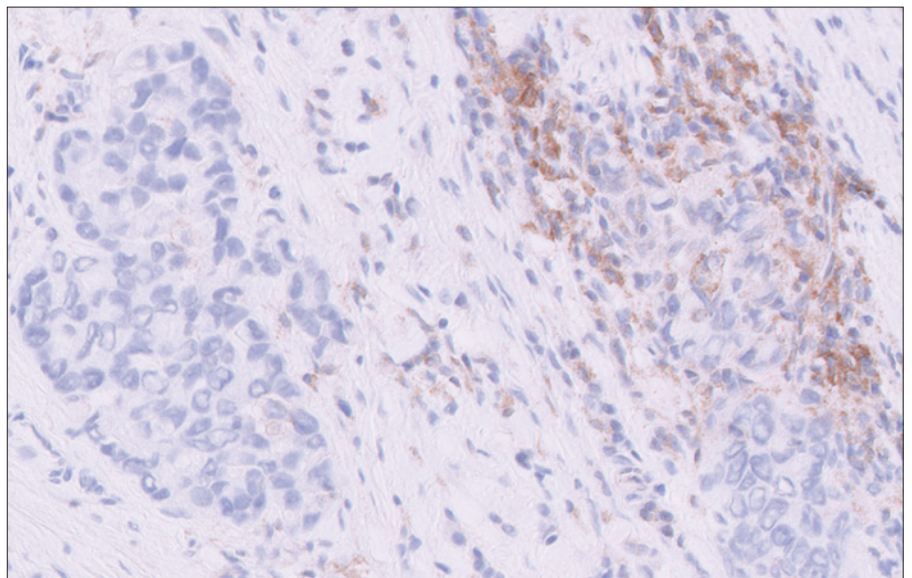


**Case 27: CPS  $\geq$  1**



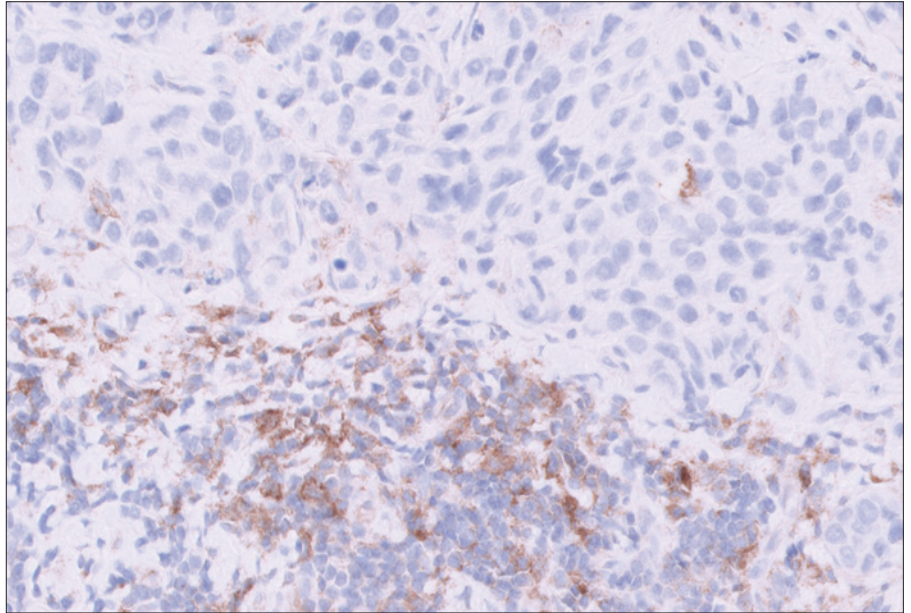
**Figure 68:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 40, however any numerical CPS between 30–50 could be assigned to this image (20 $\times$  magnification).

**Case 28: CPS  $\geq$  1**



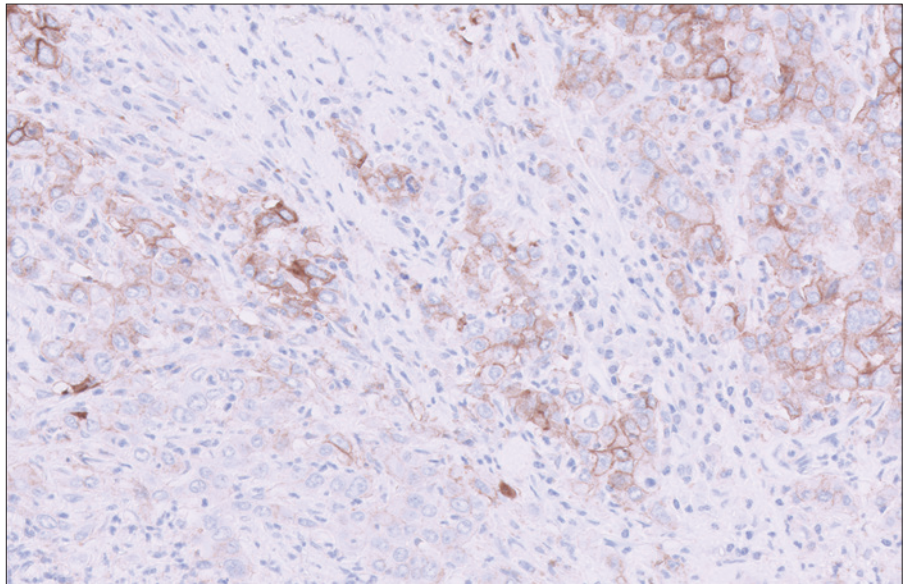
**Figure 69:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 50, however any numerical CPS between 40–60 could be assigned to this image (20 $\times$  magnification).

**Case 29: CPS  $\geq$  1**



**Figure 70:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 50, however any numerical CPS between 45–65 could be assigned to this image (20 $\times$  magnification).

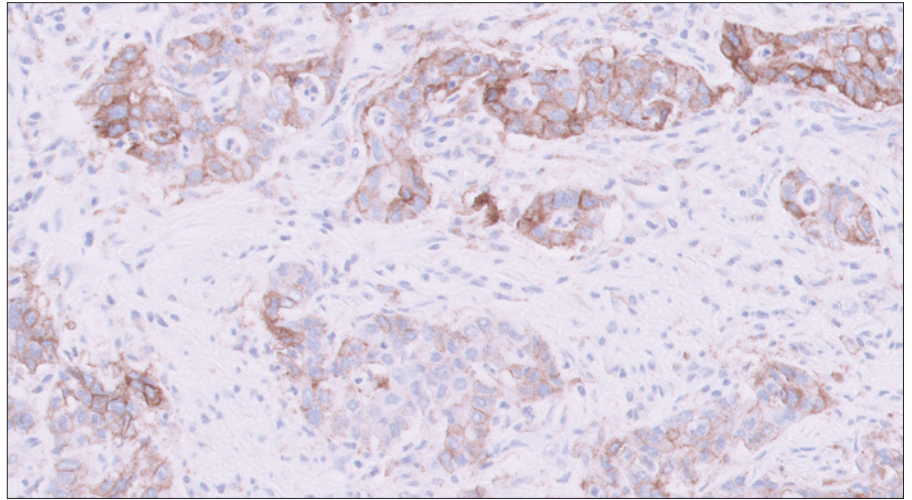
**Case 30: CPS  $\geq$  1**



**Figure 71:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 65, however any numerical CPS between 55–75 could be assigned to this image (20 $\times$  magnification).

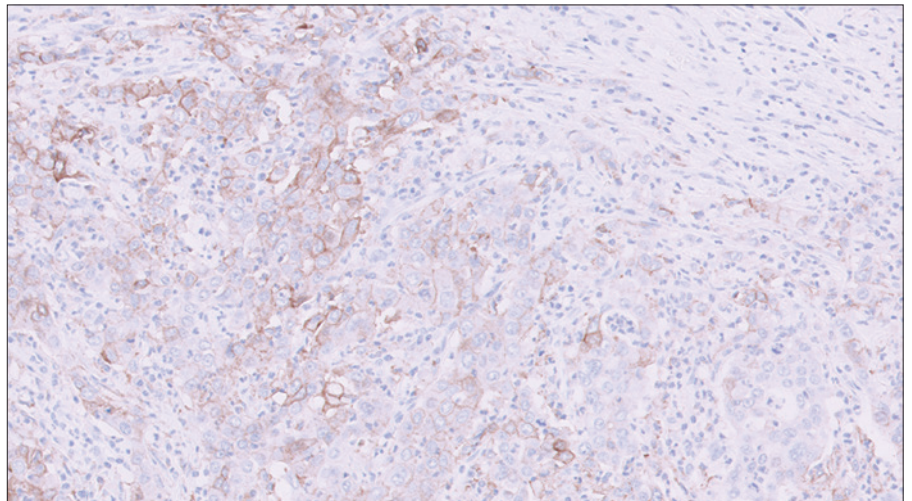


**Case 31: CPS  $\geq$  1**



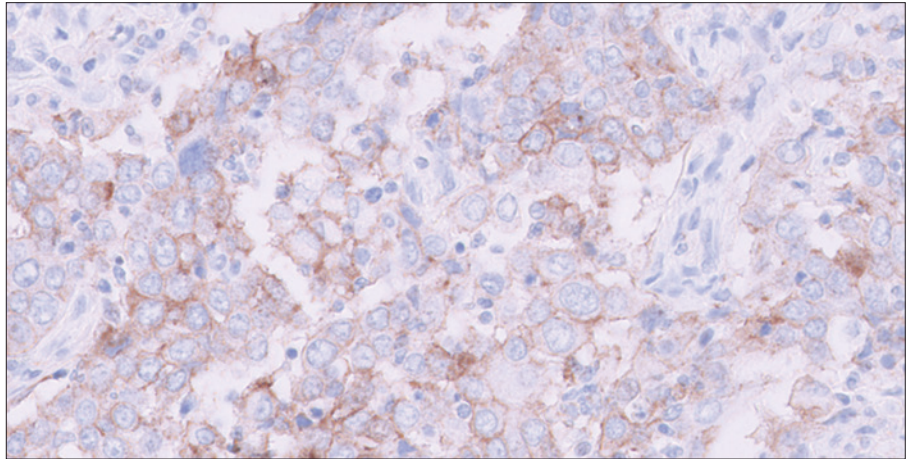
**Figure 72:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 70, however any numerical CPS between 60–75 could be assigned to this image (20 $\times$  magnification).

**Case 32: CPS  $\geq$  1**



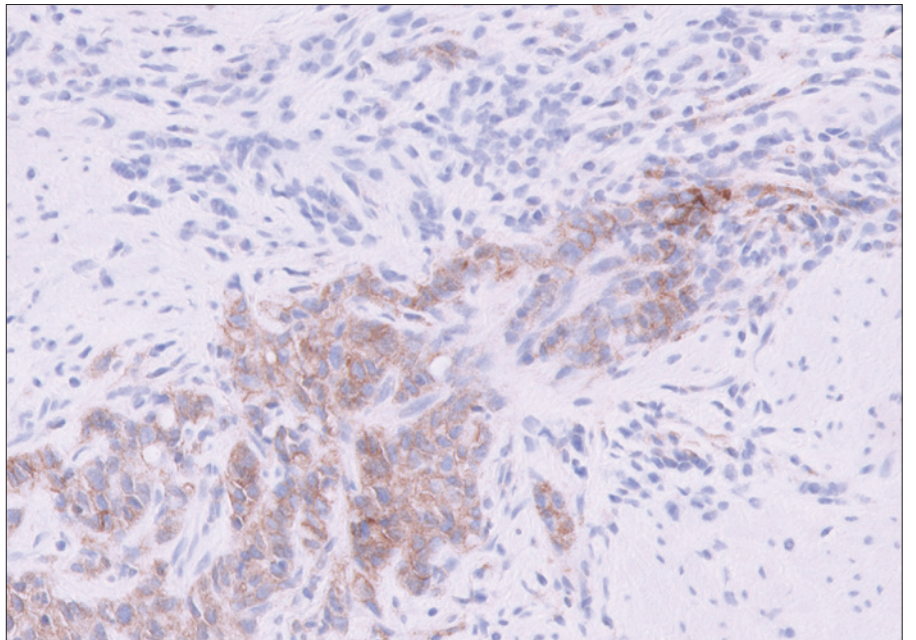
**Figure 73:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 75, however any numerical CPS between 65–80 could be assigned to this image (20 $\times$  magnification).

**Case 33: CPS  $\geq$  1**



**Figure 74:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 75, however any numerical CPS between 65–85 could be assigned to this image (20 $\times$  magnification).

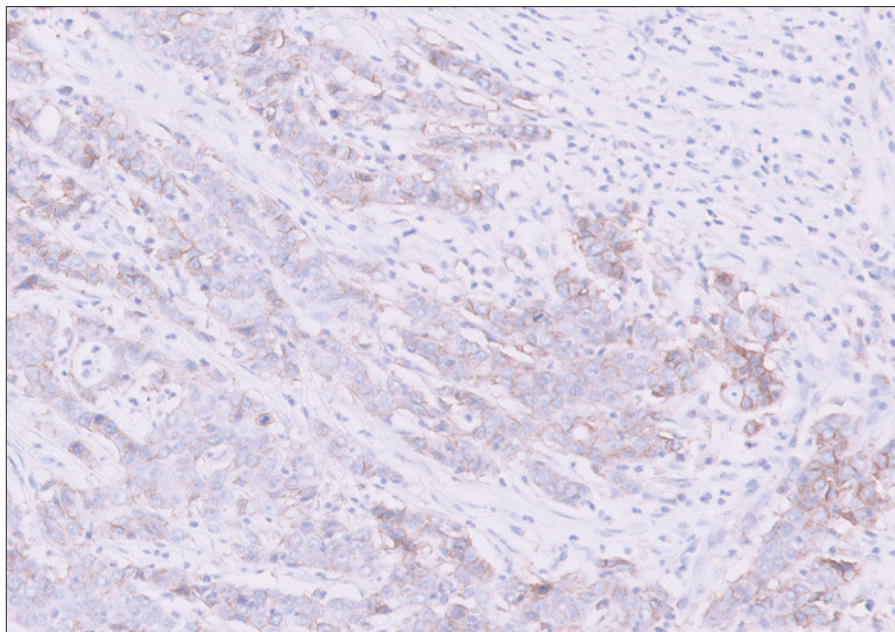
**Case 34: CPS  $\geq$  1**



**Figure 75:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 75, however any numerical CPS between 70–85 could be assigned to this image (20 $\times$  magnification).

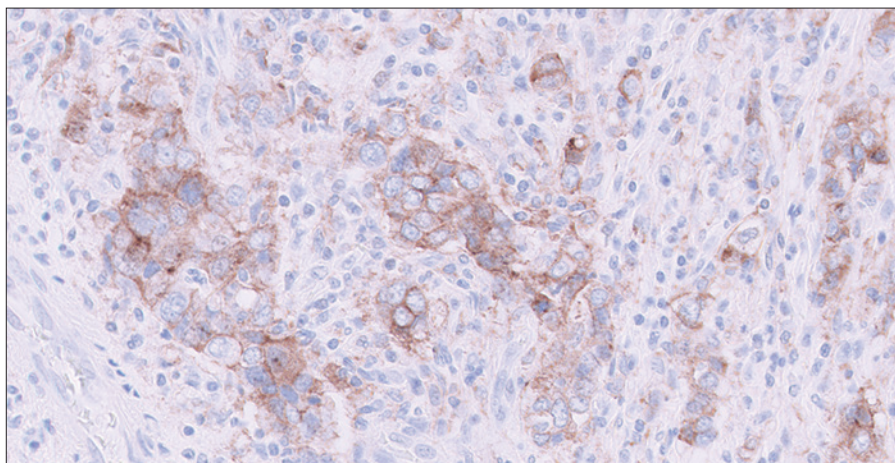


**Case 35: CPS  $\geq$  1**



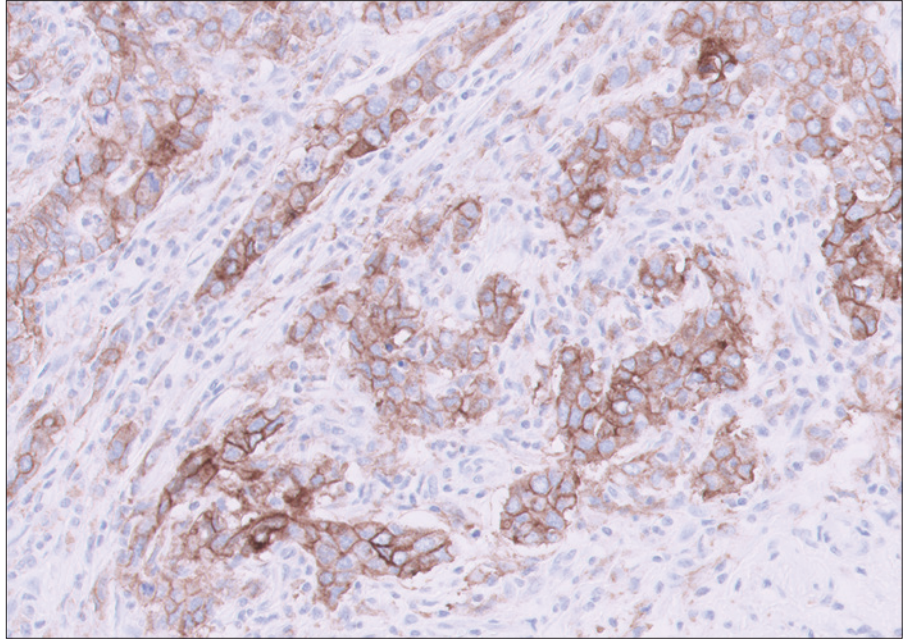
**Figure 76:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 75, however any numerical CPS between 65–85 could be assigned to this image (20 $\times$  magnification).

**Case 36: CPS  $\geq$  1**



**Figure 77:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 95, however any numerical CPS between 85–98 could be assigned to this image (20 $\times$  magnification).

**Case 37: CPS  $\geq 1$**



**Figure 78:** Gastric or GEJ adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting a CPS of 100 (20× magnification).



# Control Cell Line (CCL) Appendix

## Passing CCL

### Passing PD-L1 Negative CCL

- No cells with membrane staining\*
- Nonspecific staining < 1+ intensity\*

\* Note that staining of a few cells in the MCF-7 cell pellet may occasionally be observed. The following acceptance criteria are applicable: the presence of  $\leq 10$  total cells with distinct cell membrane staining, and/or nonspecific staining with  $\geq 1+$  intensity within the boundaries of the MCF-7 cell pellet are acceptable

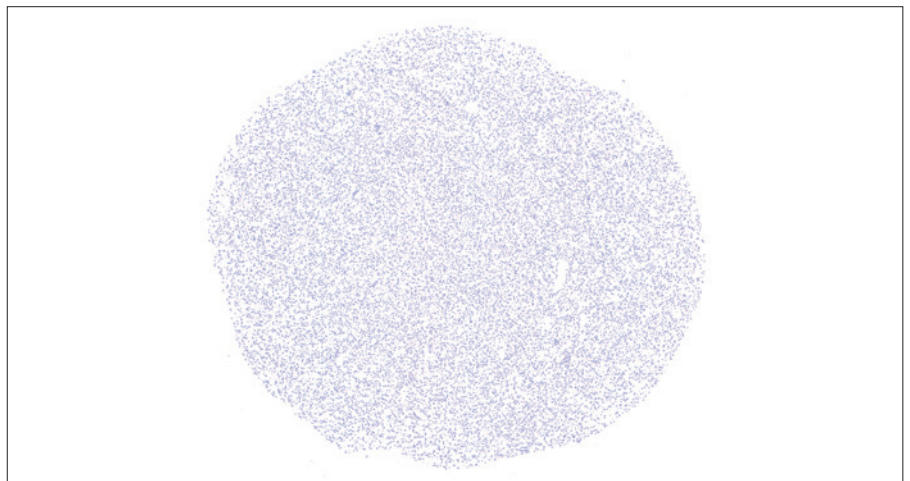


Figure 79: Ideal MCF-7 cell pellet (2 $\times$  magnification).

### Passing PD-L1 Positive CCL

- Cell membrane staining of  $\geq 70\%$  of cells
- $\geq 2+$  average staining intensity of cells with membrane staining
- Nonspecific staining < 1+ intensity



Figure 80: Ideal NCI-H226 cell pellet (2 $\times$  magnification).

## Borderline Passing CCL

## Borderline Passing vs. Passing PD-L1 Positive CCL

### Borderline Passing PD-L1 positive CCL



Figure 81: 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 staining cells in the pellet, the cell pellet can be split into quadrants and inspected at 20× magnification.

Quadrant 1

Quadrant 2

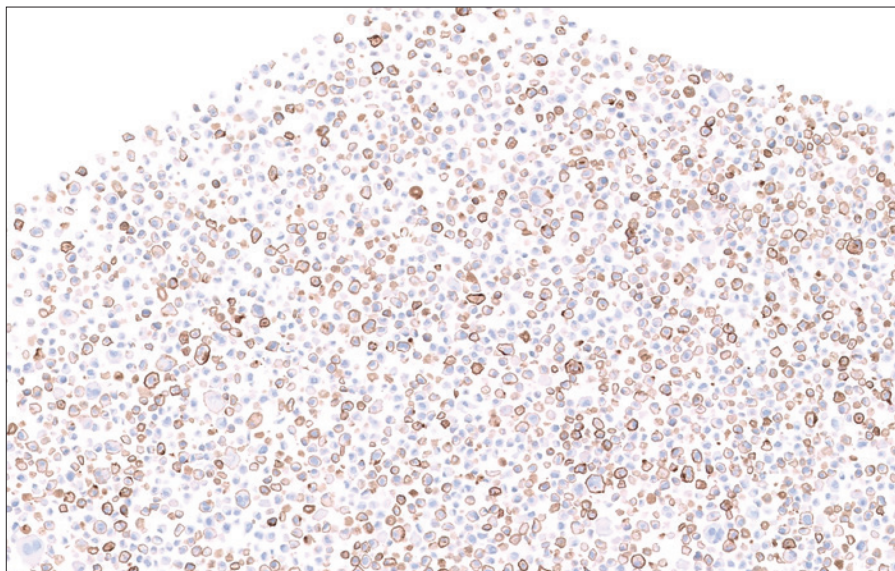


Quadrant 3

Quadrant 4

### Quadrant 1

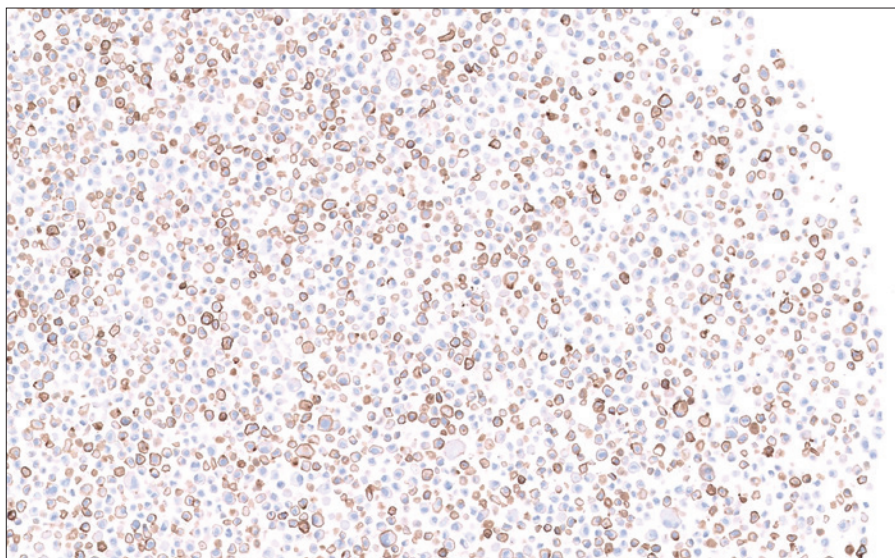
In Quadrant 1 approximately 70% of cells exhibit membrane staining, and the average staining intensity of all staining cells in this quadrant is  $\geq 2+$ .



**Figure 82:** 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 staining cells in this quadrant is  $\geq 2+$ .

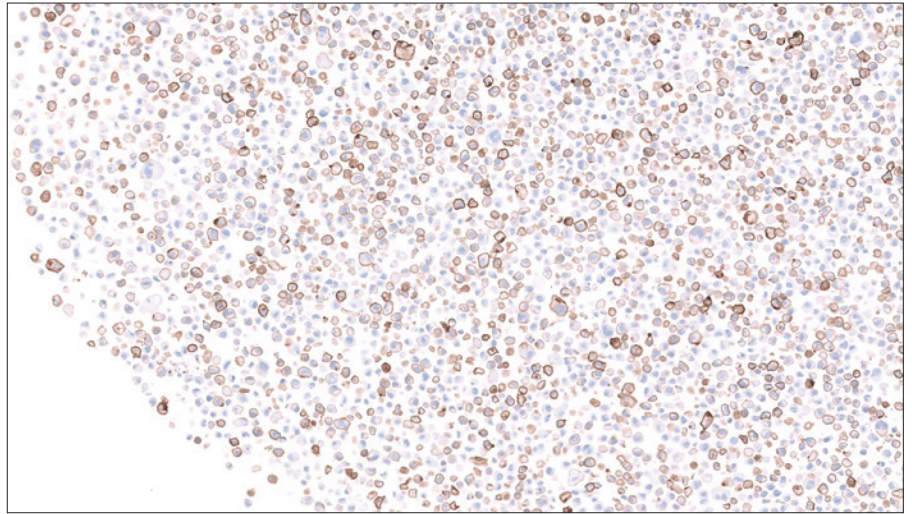


**Figure 83:** NCI-H226 cell pellet (5× magnification).



### Quadrant 3

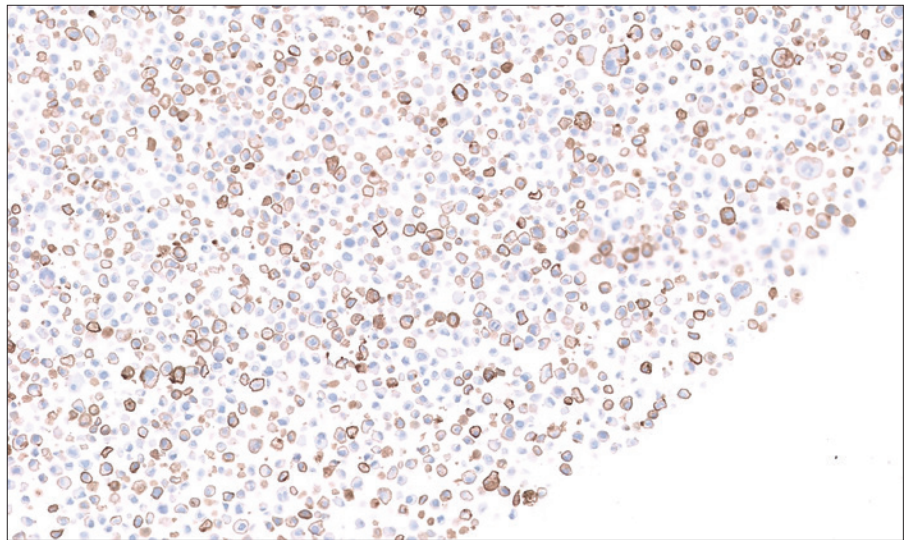
In Quadrant 3 approximately 70% of cells exhibit membrane staining, and the average staining intensity of all staining cells in this quadrant is  $\geq 2+$ .



**Figure 84:** NCI-H226 cell pellet (5 $\times$  magnification).

### Quadrant 4

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

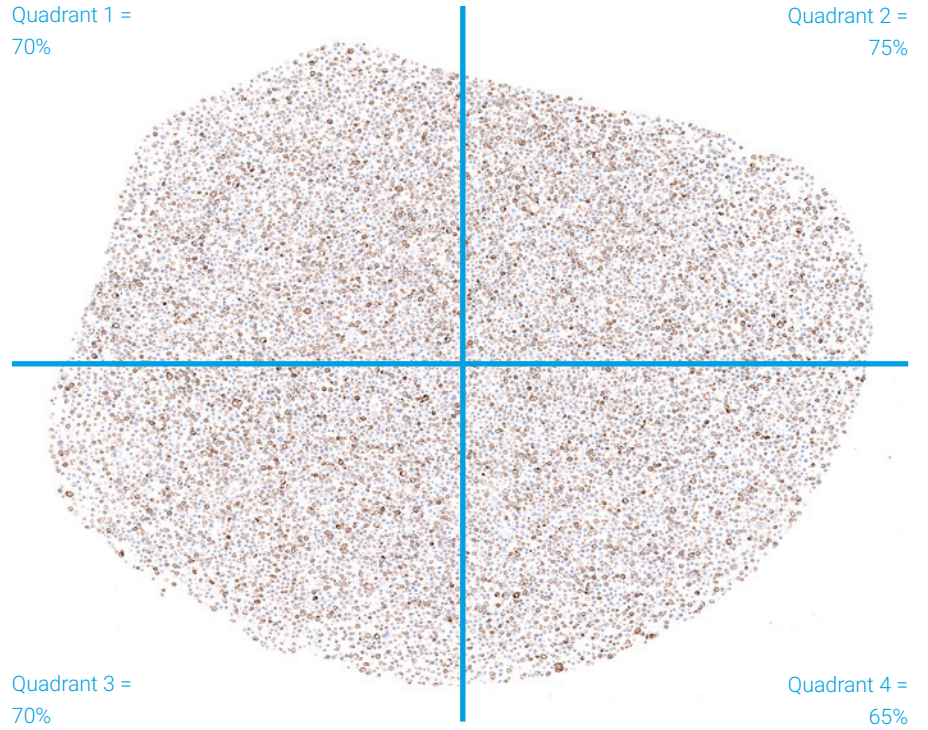


**Figure 85:** NCI-H226 cell pellet (5 $\times$  magnification).



### 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 with membrane staining in the pellet is  $\geq 2+$  intensity



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

- The overall percentage of cells with membrane staining = 70%
- The average staining intensity of all cells with membrane staining in the cell pellet is  $\geq 2+$

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 cells with membrane staining\*
- Nonspecific staining < 1+ intensity\*

\* Note that staining of a few cells in the MCF-7 cell pellet may occasionally be observed. The following acceptance criteria are applicable: the presence of  $\leq 10$  total cells with distinct cell membrane staining, and/or nonspecific staining with  $\geq 1+$  intensity within the boundaries of the MCF-7 cell pellet are acceptable

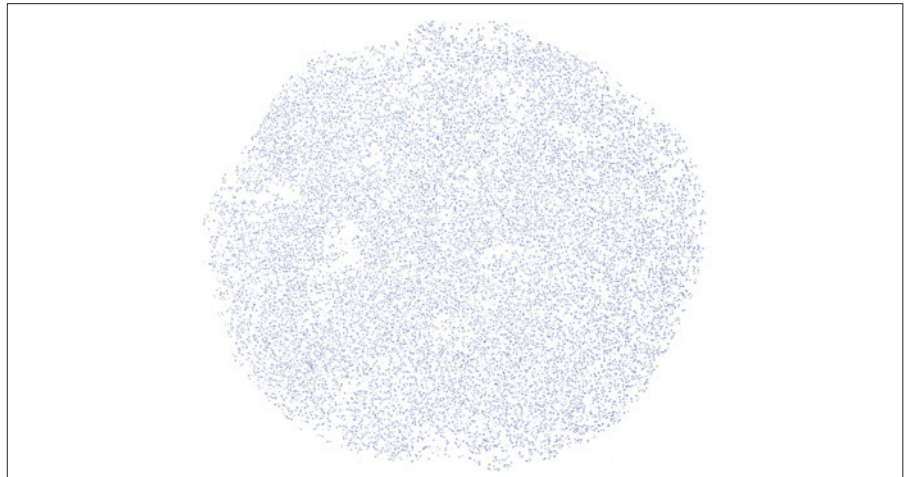


Figure 86: MCF-7 cell pellet (2× magnification).

#### Failed PD-L1 positive CCL

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

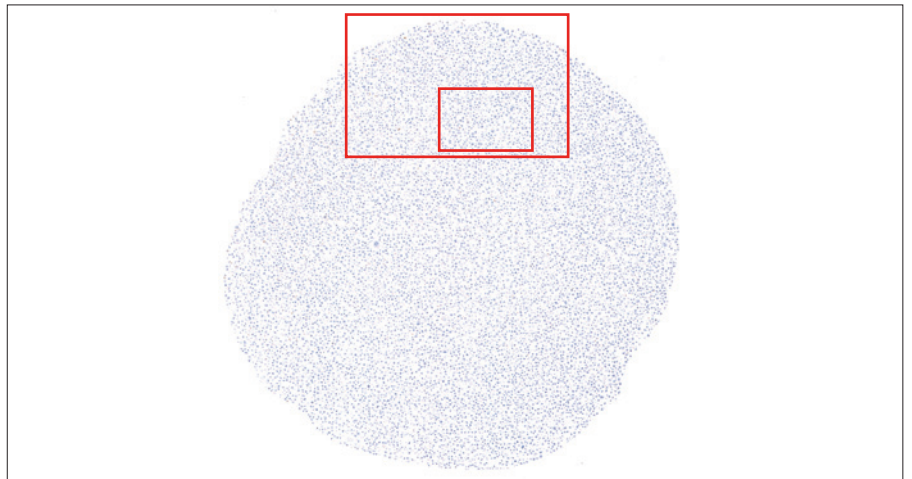


Figure 87: NCI-H226 cell pellet (2× magnification).

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

Failed PD-L1 positive CCL (10x)

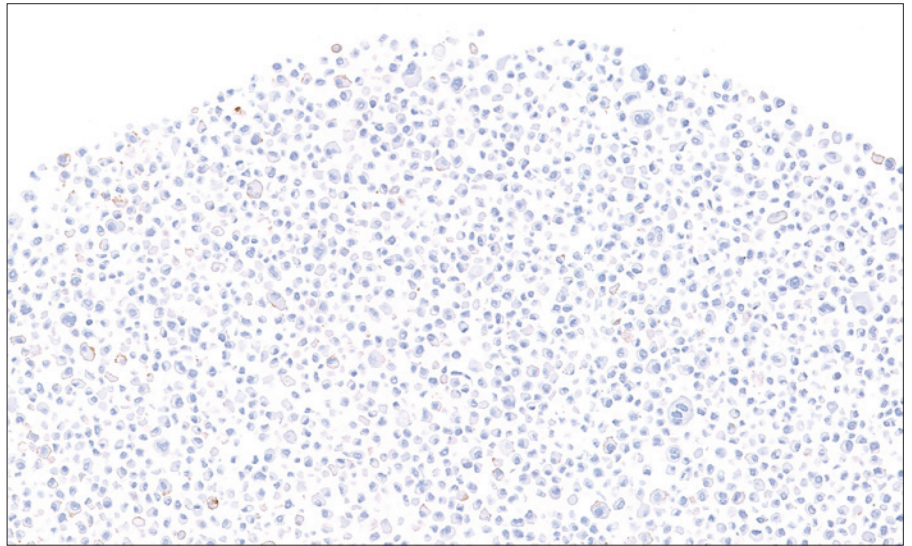


Figure 88: NCI-H226 cell pellet (10x magnification).

Failed PD-L1 positive CCL (20x)

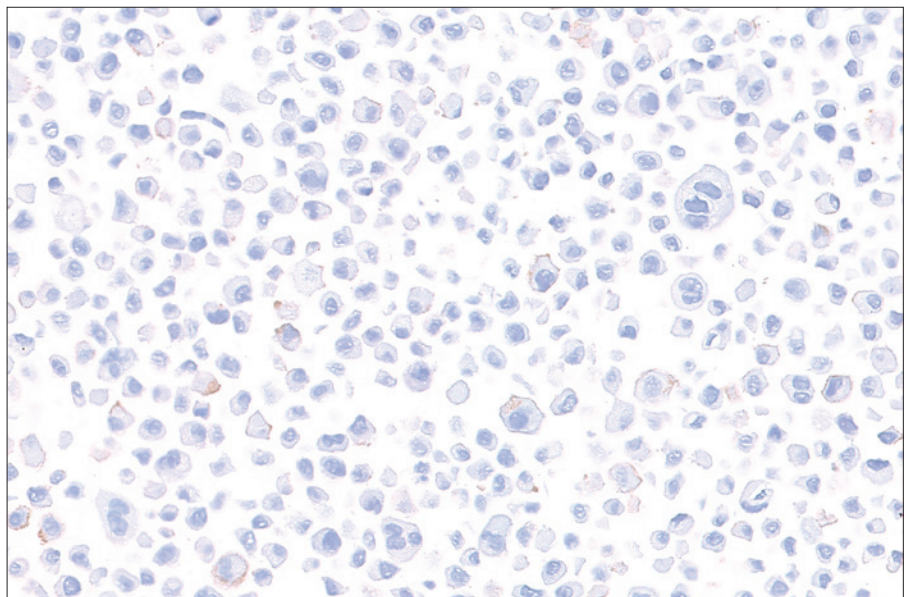


Figure 89: NCI-H226 cell pellet (20x magnification).

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

### Passing PD-L1 negative CCL

- No cells with membrane staining\*
- Nonspecific staining < 1+ intensity\*

\* Note that staining of a few cells in the MCF-7 cell pellet may occasionally be observed. The following acceptance criteria are applicable: the presence of  $\leq 10$  total cells with distinct cell membrane staining, and/or nonspecific staining with  $\geq 1+$  intensity within the boundaries of the MCF-7 cell pellet are acceptable

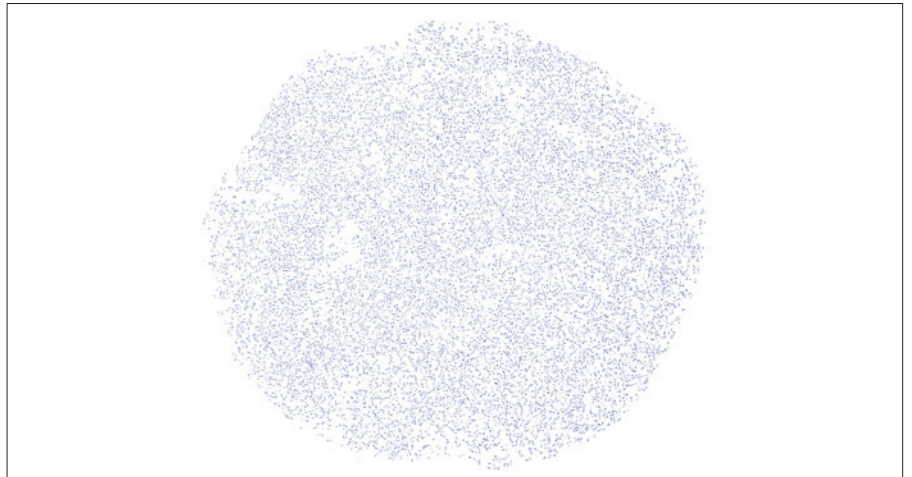


Figure 90: MCF-7 cell pellet (2x magnification).

### Failed PD-L1 positive CCL

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



Figure 91: NCI-H226 cell pellet (2x magnification).

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



Failed PD-L1 positive CCL (10x)

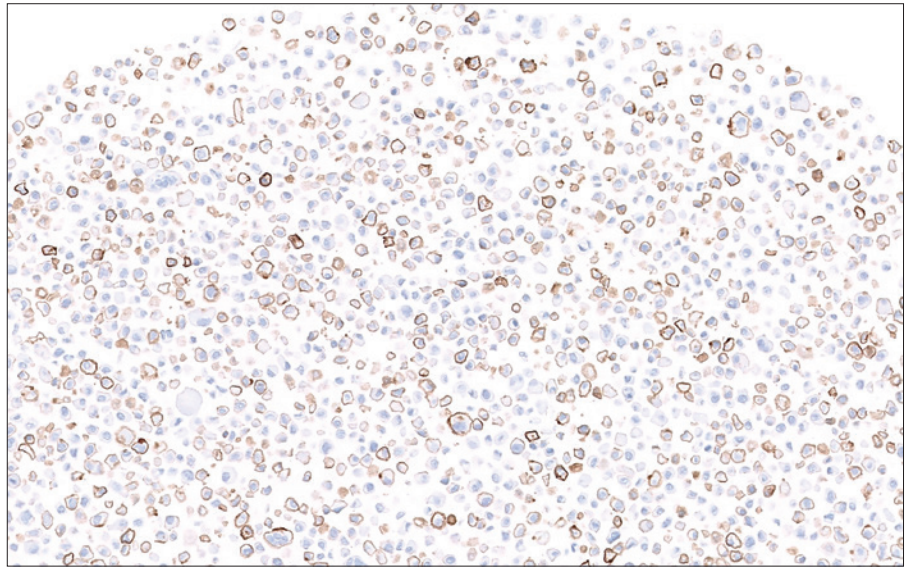


Figure 92: NCI-H226 cell pellet (10x magnification).

Failed PD-L1 positive CCL (20x)

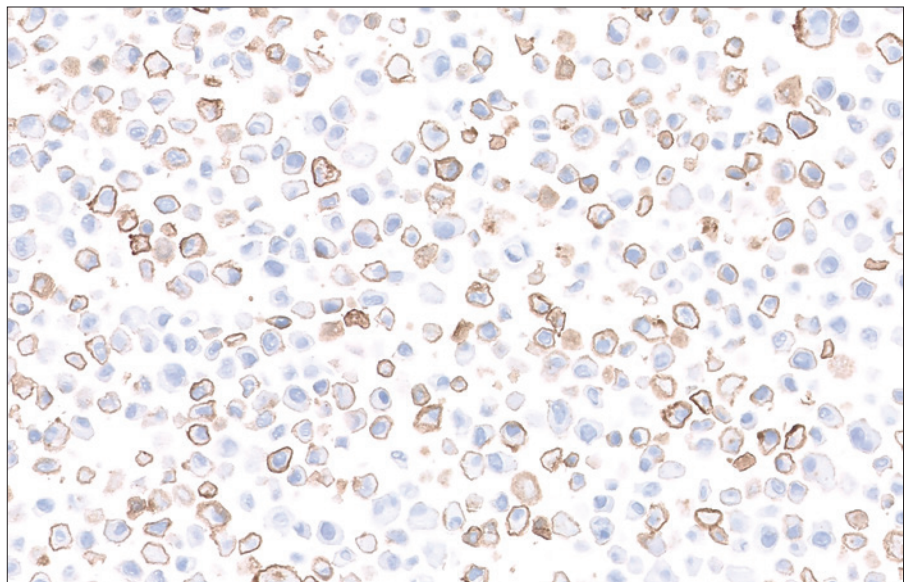


Figure 93: NCI-H226 cell pellet (20x magnification).

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

#### Passing PD-L1 positive CCL

- Cell membrane staining of  $\geq 70\%$  of cells
- $\geq 2+$  average staining intensity of cells with membrane staining
- Nonspecific staining  $< 1+$



Figure 94: NCI-H226 cell pellet (2x magnification).

#### Failed PD-L1 negative CCL

- Nonspecific (nuclear) staining is  $\geq 1+$  staining intensity
- There are  $> 10$  total cells with distinct cell membrane and/or nonspecific staining that is  $\geq 1+$  intensity

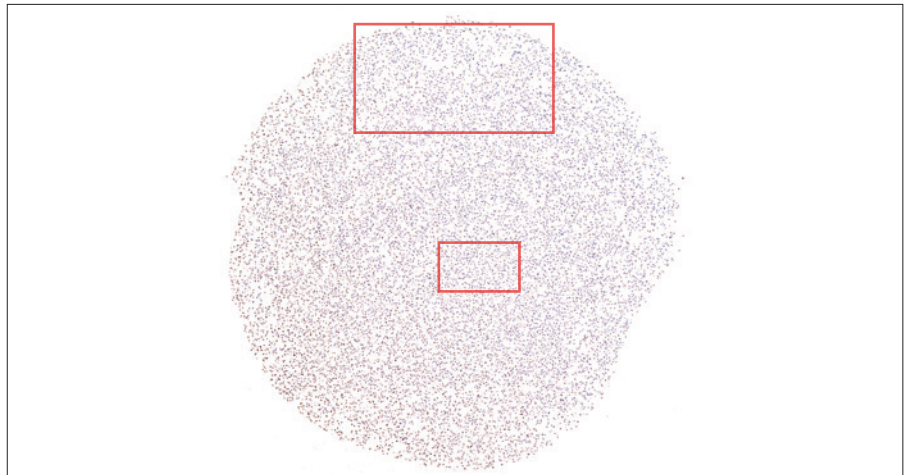


Figure 95: MCF-7 cell pellet (2x magnification).

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

Failed PD-L1 negative CCL (10x)

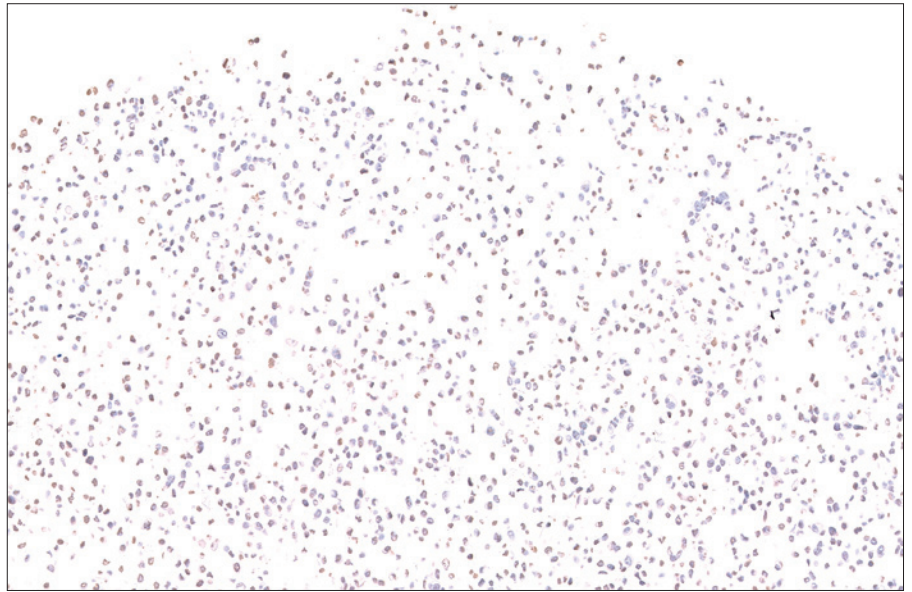


Figure 96: MCF-7 cell pellet (10x magnification).

Failed PD-L1 negative CCL (20x)

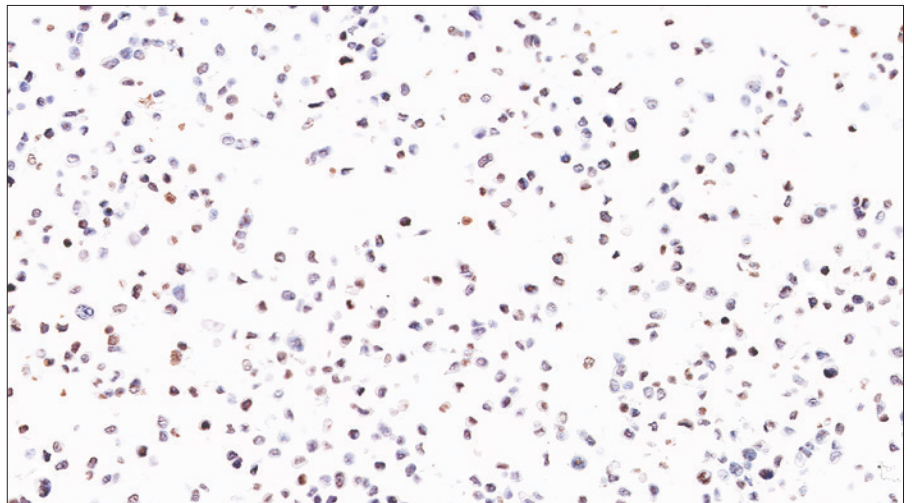


Figure 97: MCF-7 cell pellet (20x magnification).





# 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 protocol 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 kit-supplied Control Slide	Inadequate target retrieval	Verify that the 3-in-1 pretreatment procedure was correctly performed
	Inappropriate wash buffer used	Use only EnVision FLEX Wash Buffer (20×) (Code K8007)
Excessive nonspecific staining of slides	Paraffin incompletely removed	Verify that the 3-in-1 pretreatment procedure was correctly performed
	Slides dried after the 3-in-1 pretreatment procedure was performed	Ensure slides remain wet with 1× EnVision FLEX Wash Buffer after the 3-in-1 pretreatment procedure and after loading on the Autostainer Link 48. Ensure that the Autostainer Link 48 lid is properly closed to prevent reagent evaporation during the staining procedure
	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
	Warped Autostainer Link 48 slide racks used	Ensure that only level Autostainer Link 48 slide racks are used
Tissue detached from slides	Use of incorrect microscope slides	Use 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	Use only EnVision FLEX Wash Buffer (20×) (Code K8007)

Continued on next page

Problem	Probable Cause	Suggested Action
1× EnVision FLEX Target Retrieval Solution, Low pH is cloudy in appearance when heated	When heated the 1× EnVision FLEX Target Retrieval Solution, Low pH turns cloudy in appearance	This is normal and does not influence staining
1× EnVision FLEX Target Retrieval Solution, Low pH 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× EnVision FLEX Target Retrieval Solution, Low pH. Do not modify the pH of 1× EnVision FLEX Target Retrieval Solution, Low pH. If the pH is outside the acceptable range (6.1 ± 0.2), discard 1× EnVision FLEX Target Retrieval Solution, Low pH. Prepare new 1× EnVision FLEX Target Retrieval Solution, Low pH. Check the pH of the new 1× EnVision FLEX Target Retrieval Solution, Low pH
	Inferior quality water is used to dilute the EnVision FLEX Target Retrieval Solution concentrate	Ensure that distilled or deionized water is used to prepare 1× EnVision FLEX Target Retrieval Solution
	Incorrect target retrieval solution is used	Ensure that only EnVision FLEX Target Retrieval Solution, Low pH (50×) 1:50 (Code K8005) is used
Nonspecific > 1+ nuclear staining on PD-L1 and/or NCR slides	Specimen slides dried prior to initiating the Autostainer Link 48 staining procedure	Ensure slides remain wet with 1× EnVision FLEX Wash Buffer after deparaffinization, rehydration, and target retrieval (3-in-1) procedure and after loading on the Autostainer Link 48. Ensure that the Autostainer Link 48 lid is properly closed to prevent reagent evaporation during the staining procedure
	Improper manual slide rinsing with 1× EnVision FLEX Wash Buffer before loading on the Autostainer Link 48	Slide racks should be placed one rack at a time on the Autostainer Link 48 and then 1× EnVision FLEX Wash Buffer should be manually applied to the slides using a wash bottle. Ensure slides remain wet prior to initiating the Autostainer Link 48 procedure

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

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# Notes

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**a.** PD-L1 IHC 22C3 pharmDx [Instructions for Use]. Santa Clara, CA: Agilent Technologies, Inc.; 2023. **b.** Keytruda [package insert]. Merck Sharp & Dohme LLC, Rahway, NJ, USA; 2023. **c.** Data on file. Agilent Technologies, Inc.

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