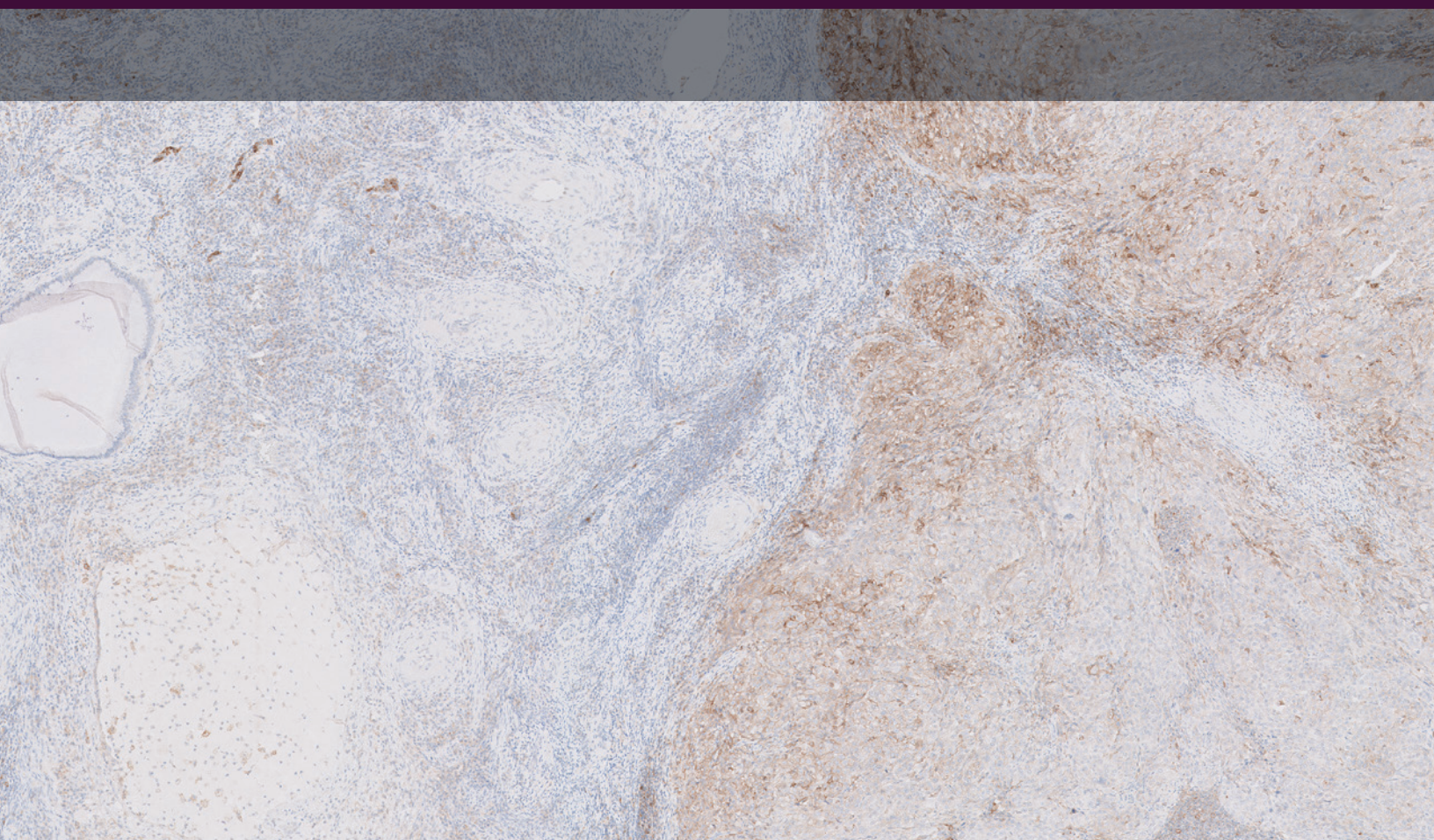


# PD-L1 IHC 22C3 pharmDx Interpretation Manual – Cervical Cancer

For in vitro diagnostic use





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## Intended Use

For in vitro diagnostic use.

PD-L1 IHC 22C3 pharmDx is a qualitative immunohistochemical assay using monoclonal mouse anti-PD-L1, Clone 22C3 intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) cervical cancer tissue using EnVision FLEX visualization system on Autostainer Link 48.

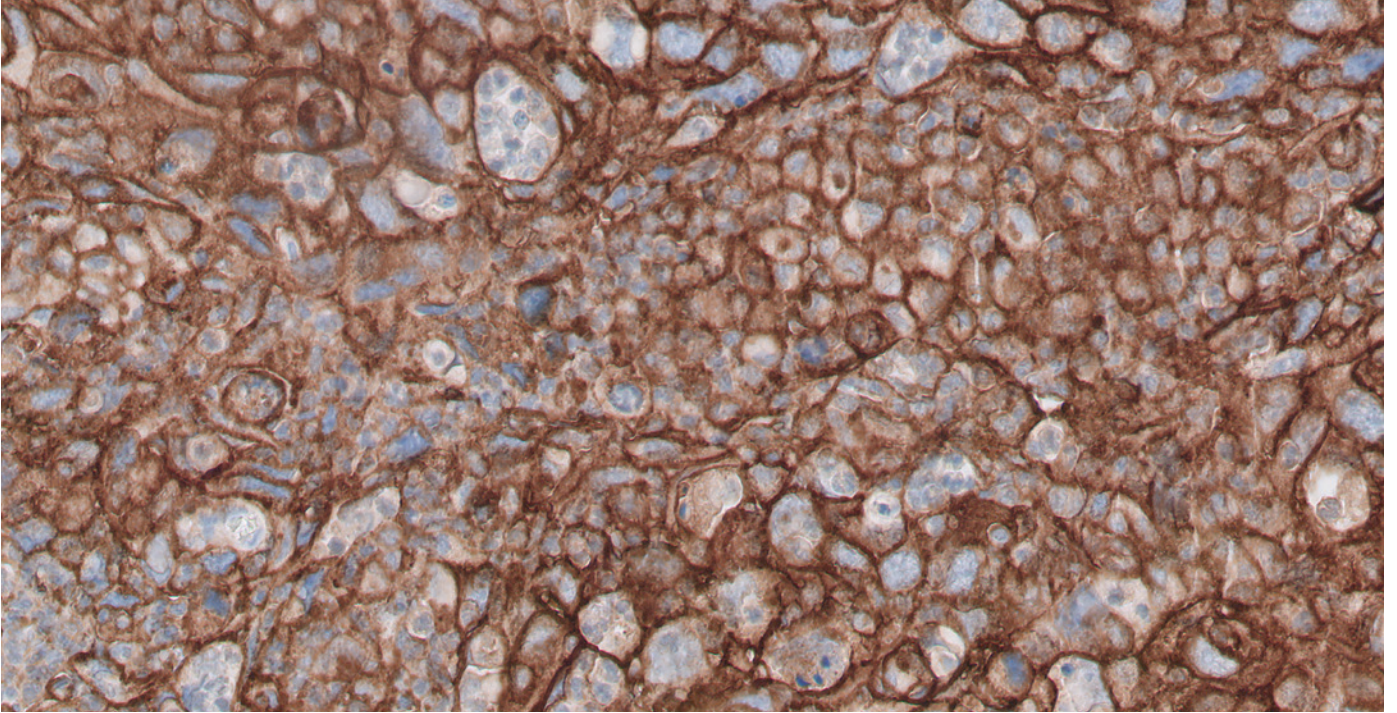
### **Cervical Cancer**

PD-L1 protein expression in cervical cancer is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. 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 cervical cancer patients for treatment with KEYTRUDA® (pembrolizumab). See the KEYTRUDA® product label for specific clinical circumstances guiding PD-L1 testing.

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

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



# Introduction

PD-L1 IHC 22C3 pharmDx is the only clinical trial-proven companion diagnostic indicated as an aid in identifying patients with cervical cancer for treatment with KEYTRUDA® (pembrolizumab). This Interpretation Manual is provided as a tool to help guide pathologists and laboratory personnel in achieving correct and reproducible results in assessing PD-L1 expression in formalin-fixed, paraffin-embedded cervical cancer specimens. PD-L1 expression evaluation may be used to identify patients for anti-PD-1 immunotherapy.

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 cervical cancer stains with PD-L1 IHC 22C3 pharmDx, example cases of various PD-L1 expression levels are provided as references. These example cases and in-depth recommendations for interpretation of cervical cancer specimens stained with PD-L1 IHC 22C3 pharmDx can help individual labs achieve reproducible and reliable results.

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

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

- CPS < 1
- CPS ≥ 1

For more details on staining and interpretation, please refer to the current version of the IFU provided with PD-L1 IHC 22C3 pharmDx, Code SK006 or visit [www.agilent.com](http://www.agilent.com).

## Assay Interpretation

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

## Reporting Results

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

## Photomicrographs

The included photomicrographs are of cervical cancer unless otherwise noted.

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

*Some tissue samples supplied by Asterand Bioscience.*

*Some data and biospecimens used in this project were provided by Sofia Bio LLC, Bulgaria and EastWest Biopharma, Ukraine with appropriate ethics approval and through Trans-Hit Biomarkers Inc.*

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

*Data and biospecimens used in this project were provided by US Biolab (Gaithersburg, MD, USA) and IOM Ricera (Viagrande, Italy) 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

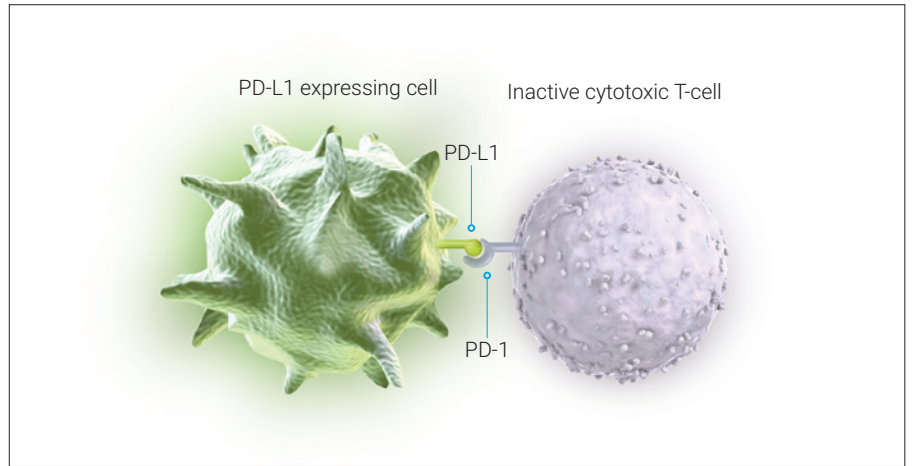
PD-1/PD-L1 interaction between tumor cells and activated T-cells (Figure 3) is a mechanistic pathway used by immunotherapeutic agents. When the tumor cell is unable to interact with the activated T-cell, the immune system remains active, helping to prevent immunosuppression.

### PD-L1 IHC 22C3 pharmDx Detects PD-L1 in Cervical Cancer Specimens

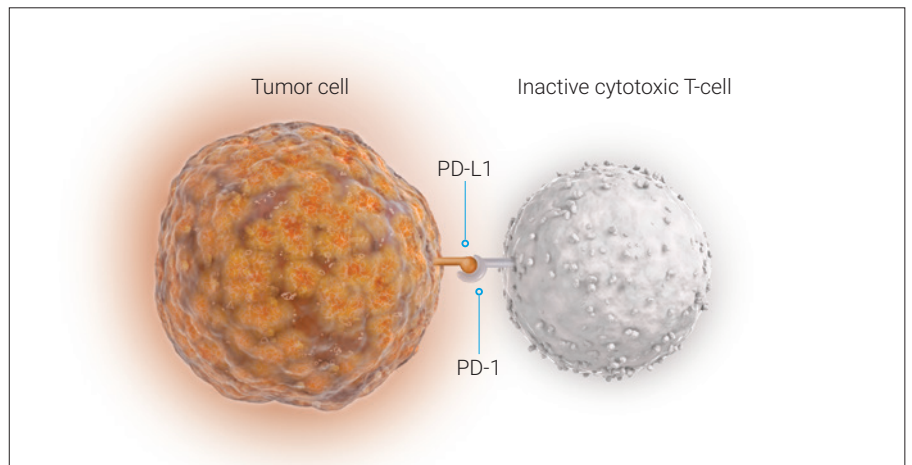
PD-L1 upregulation in cervical cancer is a biomarker for response to anti-PD-1 therapy. PD-L1 IHC 22C3 pharmDx is the only companion diagnostic used in the KEYTRUDA® (pembrolizumab) clinical trial (KEYNOTE-158) to evaluate the relationship between PD-L1 expression and clinical efficacy. KEYTRUDA is a humanized monoclonal PD-1-blocking antibody.



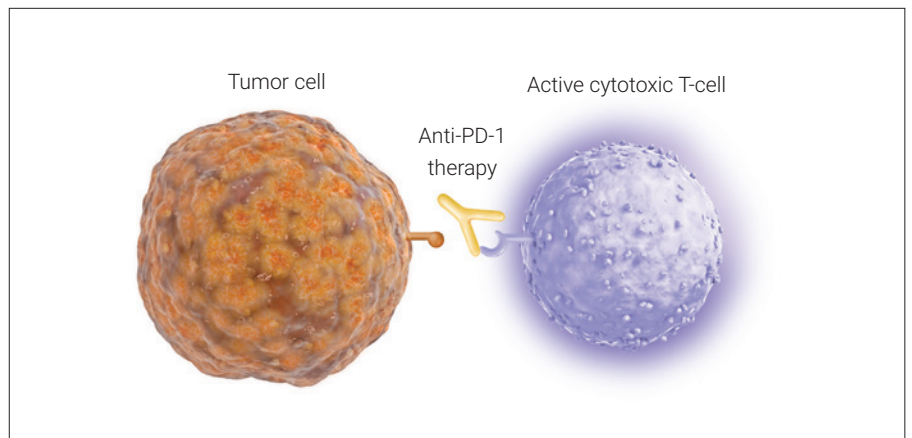
## The PD-1/PD-L1 Pathway



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



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



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

# PD-L1 IHC 22C3 pharmDx Overview

## What is PD-L1 IHC 22C3 pharmDx?

PD-L1 IHC 22C3 pharmDx is the only clinical trial-proven companion diagnostic indicated as an aid in identifying patients with cervical cancer for treatment with KEYTRUDA® (pembrolizumab). PD-L1 IHC 22C3 pharmDx is a qualitative immunohistochemical (IHC) assay intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) cervical cancer tissue samples using 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.

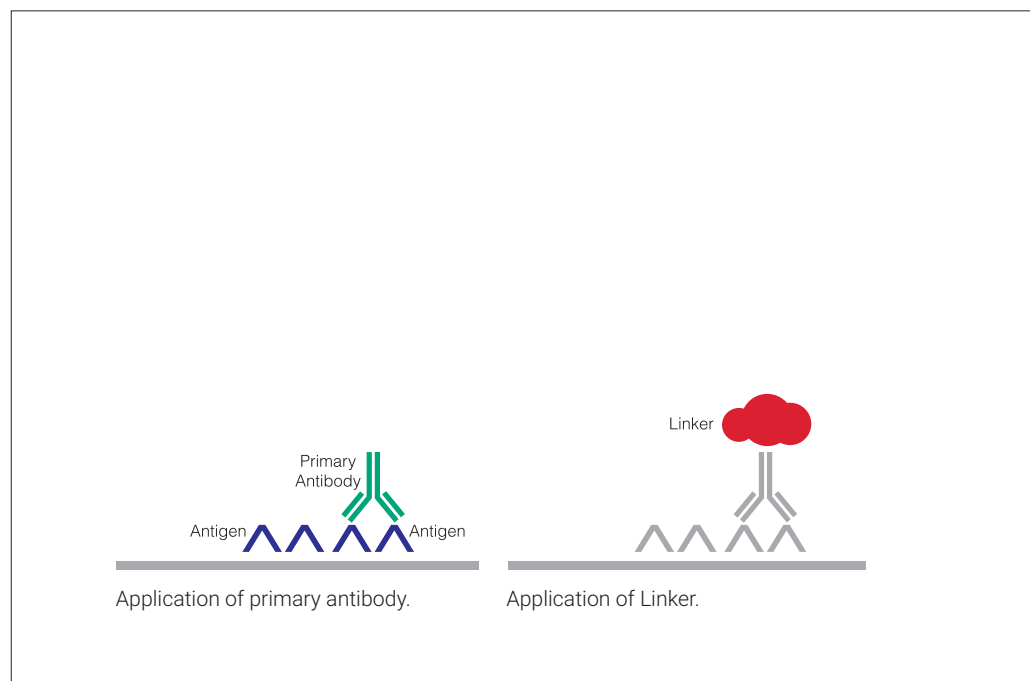


Figure 4: PD-L1 IHC 22C3 pharmDx staining procedure.

# Kit Configuration



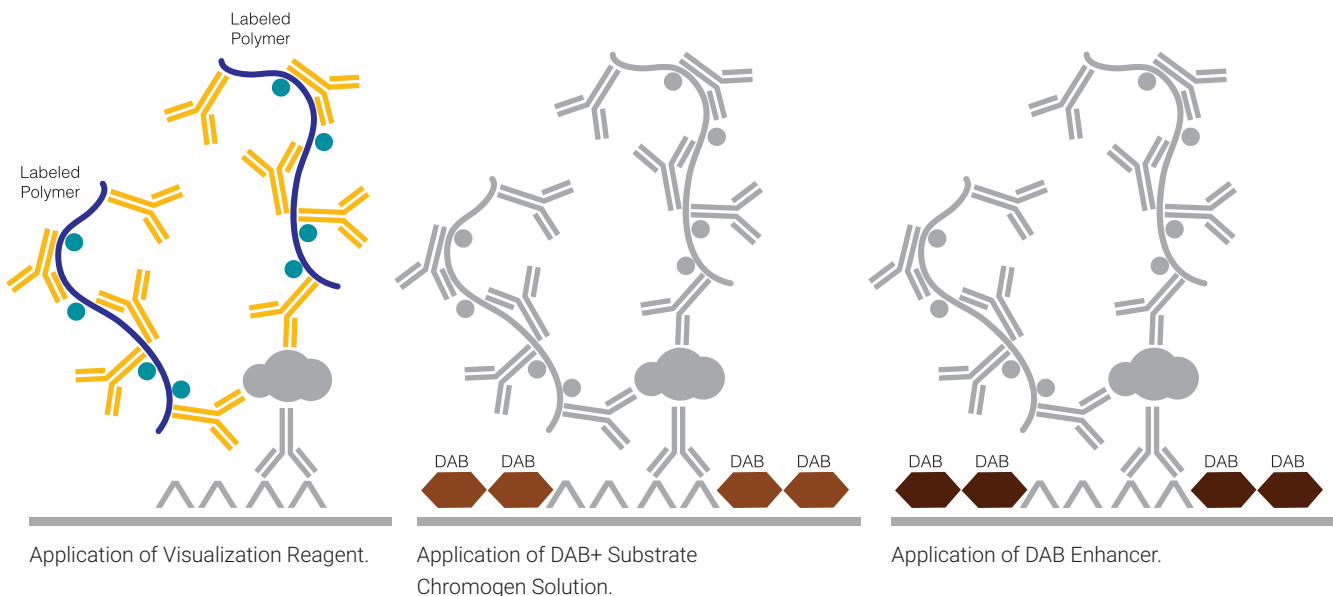
**Figure 5:** PD-L1 IHC 22C3 pharmDx components.

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 (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 factors: specimen collection and preparation prior to performing the test, and the actual performance of the test itself. Technical problems are generally related to procedural deviations and can be controlled and minimized through training and, where necessary, clarification of the product instructions.

## Specimen Preparation

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

## 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 tissue 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 tissue in the same manner. Control tissues processed differently from the patient specimen validate reagent performance only and do not verify tissue preparation.

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

### Optional Additional Lab-Supplied Control: Tonsil Tissue

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

### Tissue Processing

Formalin-fixed, paraffin-embedded 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 Fisherbrand Superfrost Plus charged slides, and then placed in a 58 ± 2 °C oven for 1 hour. Store tissue sections in the dark at 2–8 °C (preferred) or at room temperature in the dark up to 25 °C to preserve antigenicity, and stain within the time period given 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 pre-programmed in the DakoLink software.

## Reagent Storage

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

## Reagent Preparation

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

## EnVision FLEX Target Retrieval Solution, Low pH

Dilute EnVision FLEX Target Retrieval Solution, Low pH, (50×) 1:50 using distilled or deionized water (reagent-quality water). One 30 mL bottle of concentrate provides 1.5 L of working solution, which is sufficient to fill one PT Link tank. Discard 1× EnVision FLEX Target Retrieval Solution, Low pH after 3 uses or 5 days after dilution.

## EnVision FLEX Wash Buffer

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

## DAB+ Substrate-Chromogen Solution

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

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

### **Controls to Assess Staining Quality**

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

- One PD-L1 IHC 22C3 pharmDx Control Cell Line Slide stained with the primary antibody
- Positive and negative lab-supplied control tissues stained with the primary antibody and Negative Control Reagent
- Serial section of each patient specimen stained with the Negative Control Reagent

### **Deparaffinization, Rehydration, and Target Retrieval**

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

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

## Staining and Counterstaining

- Place the Autostainer racks with slides one rack at a time on the Autostainer Link 48
- Prior to initiating the staining procedure on the Autostainer Link 48, 1x EnVision FLEX Wash Buffer should be manually applied to the slides using a wash bottle for prevention of tissue drying. 1x EnVision FLEX Wash Buffer should not be applied directly on the tissue section but applied sufficiently to the slide so that the tissue section is amply covered. Slides should remain wet prior to the initiation of the staining procedure. 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  $\geq 175$  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, Code K8008

## Mounting

Use non-aqueous 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"/>
Specimen tissue sections 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"/>
EnVision FLEX Target Retrieval Solution, Low pH is prepared properly? pH of 1× Target Retrieval Solution must be 6.1 ± 0.2.	<input type="checkbox"/>	<input type="checkbox"/>
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?	<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 30. Before examining the patient specimen for PD-L1 staining, it is important to examine the controls to assess staining quality.

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

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) stained section is recommended for the evaluation of specimen adequacy. PD-L1 IHC 22C3 pharmDx and the H&E staining should be performed on serial sections from the same paraffin block of the specimen.

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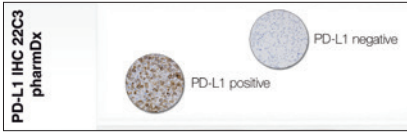
A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide for the specimen to be considered adequate for PD-L1 evaluation.

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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 and the staining intensity. If any staining of the Control Cell Line Slide is not satisfactory, all results with the patient specimens should be considered invalid.

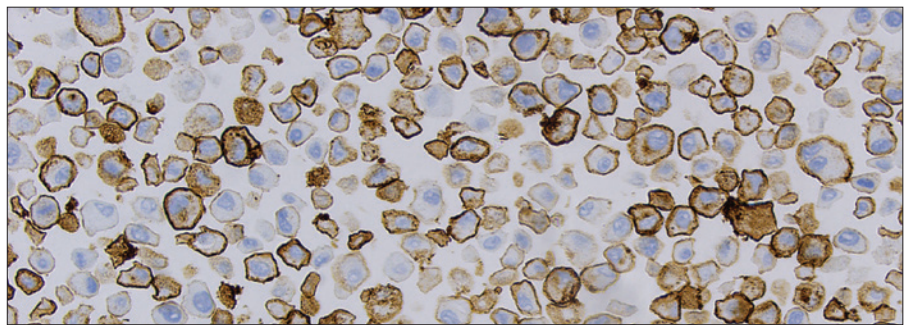
Evaluate the overall staining intensity using the following guide:

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

### Positive Control Cell Pellet

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

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



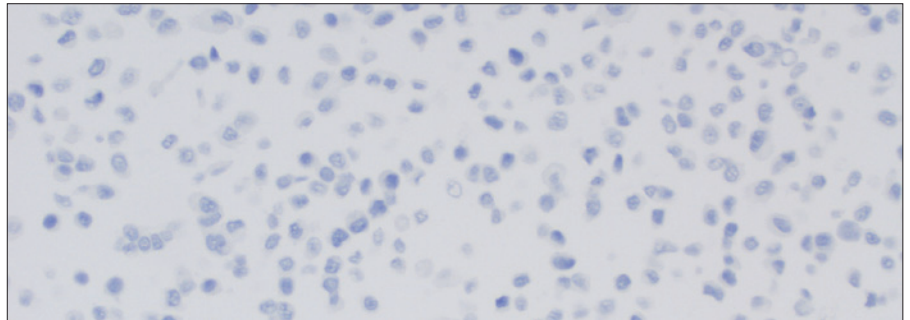
**Figure 7:** Positive cell pellet with acceptable staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20x magnification).

### 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.



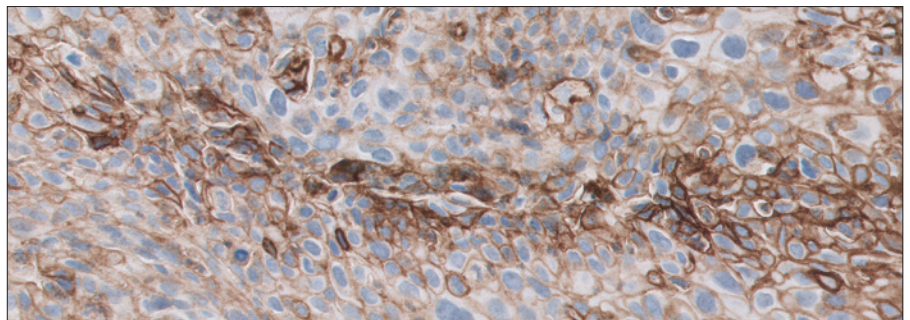
**Figure 8:** Negative cell pellet with no staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20x magnification).

See the Control Cell Line (CCL) Appendix on page 60 for images of passing, borderline and failing control cell line staining.

### Positive and Negative Lab-supplied Control Tissue

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

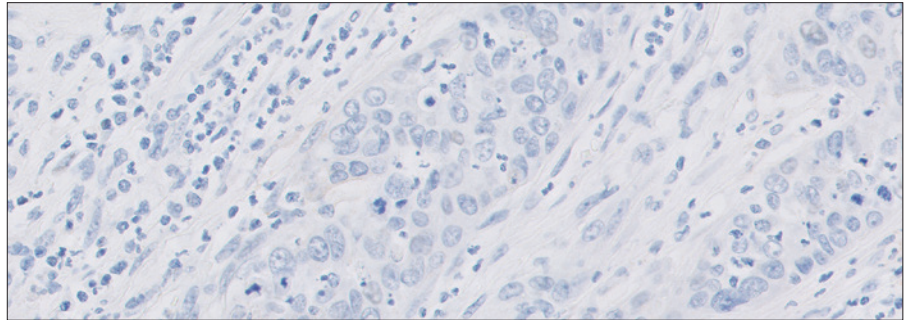
- Requirements for slide stained with PD-L1: Presence of brown cell membrane staining should be observed. Nonspecific staining, including nuclear staining, should be  $\leq 1+$
- Requirements for slide stained with Negative Control Reagent: No membrane staining. Nonspecific staining, including nuclear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



**Figure 9:** Ideal positive lab-supplied control tissue (20x magnification).

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

- 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+

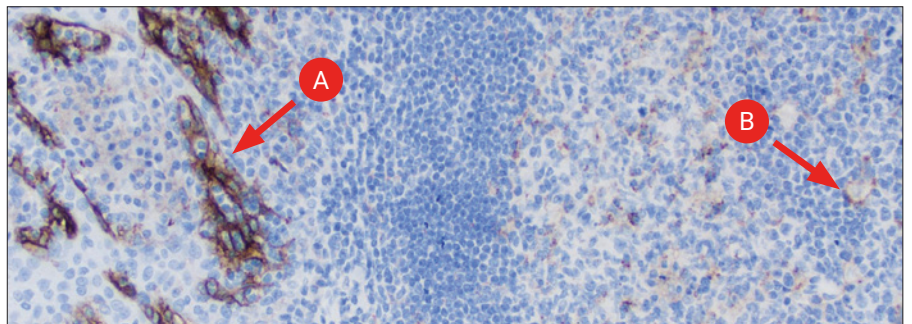


**Figure 10:** Ideal negative lab-supplied control tissue demonstrating lack of staining (20x magnification).

### Optional 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.



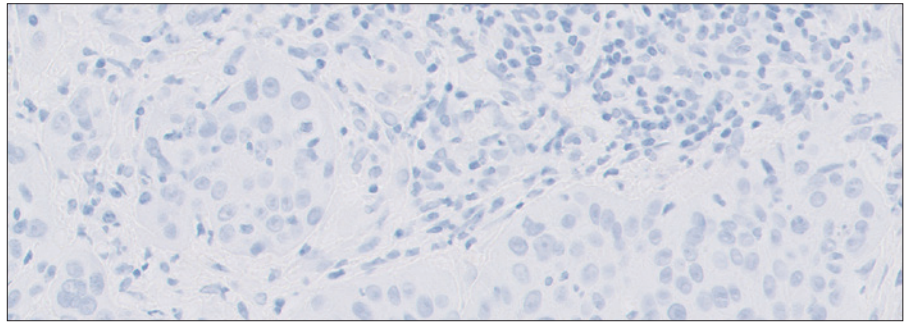
**Figure 11:** Tonsil stained with PD-L1 primary antibody exhibiting strong membrane staining in portions of the crypt epithelium (A) and weak-to-moderate membrane staining of follicular macrophages in the germinal centers (B) (10x magnification).

### 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 the absence of staining (Figure 12).

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

Nonspecific staining, including nuclear staining, should be  $\leq 1+$ .



**Figure 12:** Cervical cancer tissue stained with Negative Control Reagent (20x magnification).

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Negative Control Reagent stained slides indicate nonspecific staining and allow for better interpretation of patient specimens stained with the primary antibody.

---

# Slide Evaluation Flowchart

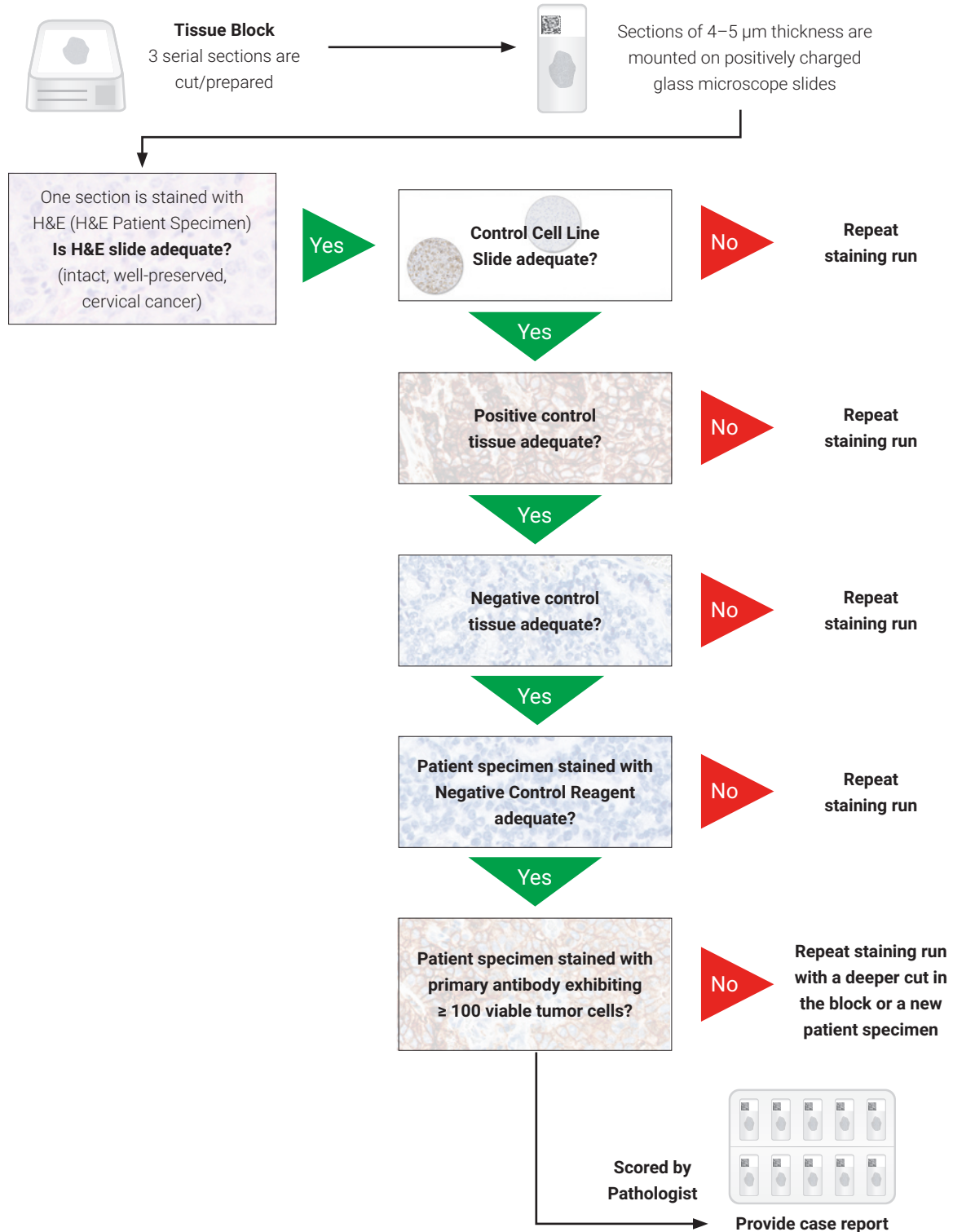


Figure 13: Recommended order of slide evaluation.

# Combined Positive Score

## Definition of Combined Positive Score (CPS)

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

CPS is defined accordingly:

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

*\* Macrophages and histiocytes are considered the same cells*

## CPS Numerator Inclusion and Exclusion Criteria

Any 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 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 scoring. Only MICs directly associated with the response against the tumor are scored.

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



## Determining Combined Positive Score

- At lower magnifications (4x, 10x), 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 and resection) for the specimen to be considered adequate for evaluation
- For specimens with less than 100 viable tumor cells, tissue from a deeper level of the block or potentially another block could have a sufficient number of tumor cells for evaluation of PD-L1 expression
- At higher magnification (20x), 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 26 for additional CPS inclusion/exclusion criteria)
  - Calculate CPS
- Evaluation of membrane staining should be performed at no higher than 20x magnification. Slide reviewer should not perform the CPS calculation at 40x magnification

**Table 1: CPS Numerator Inclusion/Exclusion Criteria**

Tissue Elements	Included in the Numerator	Excluded from the Numerator
Tumor Cells	Convincing partial or complete linear membrane staining (at any intensity) of viable invasive cervical tumor cells	<ul style="list-style-type: none"> <li>– Nonstaining tumor cells</li> <li>– Tumor cells with only cytoplasmic staining</li> <li>– Cervical intraepithelial neoplasia (CIN I-III)</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 cervical intraepithelial neoplasia (CIN I-III)</li> <li>– MICs associated with benign cells including squamous or glandular mucosa, cervical polyps, and microglandular hyperplasia</li> <li>– MICs (including lymphoid aggregates) associated with ulcers and other processes not associated with the tumor, such as cervicitis</li> <li>– Neutrophils, eosinophils, and plasma cells</li> </ul>
Other Cells	– Not included	<ul style="list-style-type: none"> <li>– Benign cells including squamous or glandular mucosa, cervical polyps, and microglandular hyperplasia</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

† Adjacent MICs are defined as being within the same 20x field as the tumor. However, MICs that are NOT directly associated with the response to the tumor should be excluded

‡ Macrophages and histiocytes are considered the same cells

**Table 2: CPS Denominator Inclusion/Exclusion Criteria**

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	All viable tumor cells	<ul style="list-style-type: none"> <li>– Any necrotic or nonviable tumor cells</li> <li>– CIN I-III</li> </ul>
Immune Cells	Not included	All immune cells of any type
Other Cells	Not included	<ul style="list-style-type: none"> <li>– Benign cells including squamous or glandular mucosa, cervical polyps, and microglandular hyperplasia</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.**

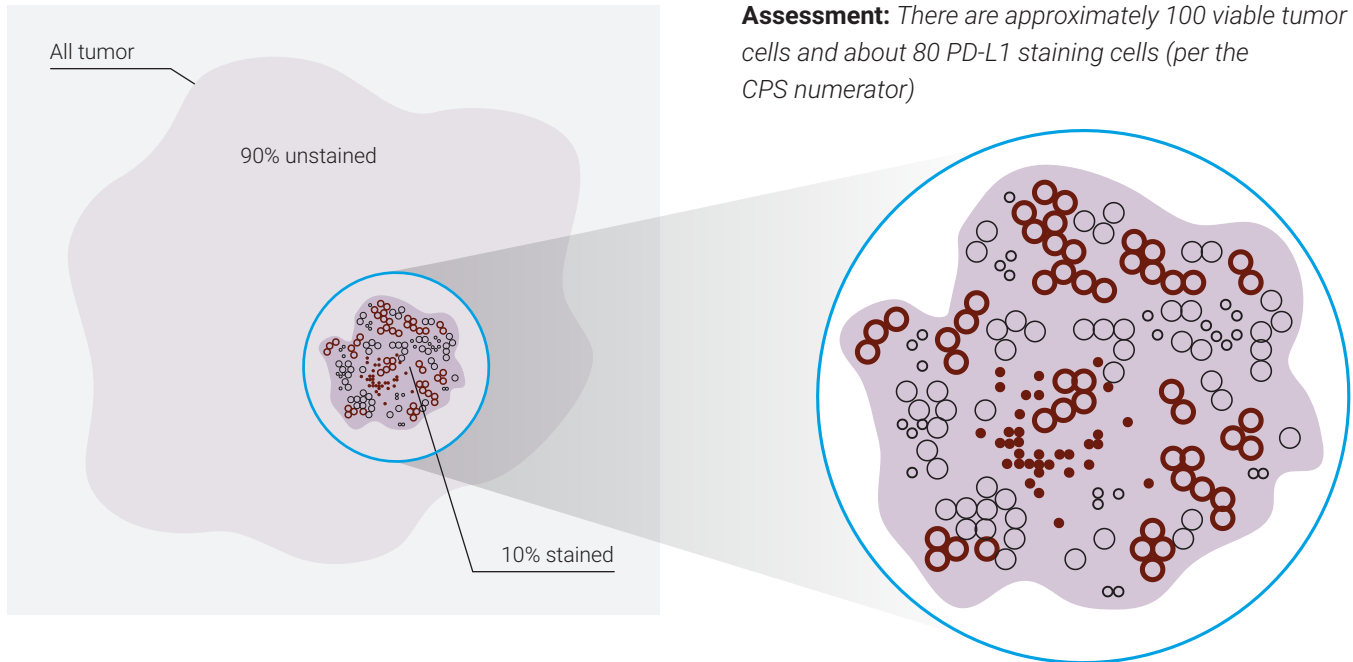
### Example 1: Calculation of Combined Positive Score in a Small Tumor Area with Staining

At lower magnifications (4x, 10x): Evaluate the tumor area for convincing staining as described in “Determining Combined Positive Score” on page 25.

**Assessment:** 10% of area with staining, 90% of area without staining

At higher magnification (20x): Confirm there is no staining in areas that appeared void of staining at lower magnifications. Evaluate the area of staining to estimate the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages). Also estimate the total number of viable tumor cells (PD-L1 staining and nonstaining tumor cells).

**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:

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

CPS of entire tumor area: 10% x 80 = ~CPS 8

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

### Clinical Interpretation: CPS ≥1

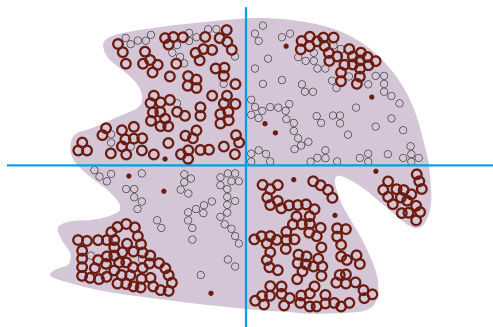
\* Including tumor cells, lymphocytes, macrophages

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

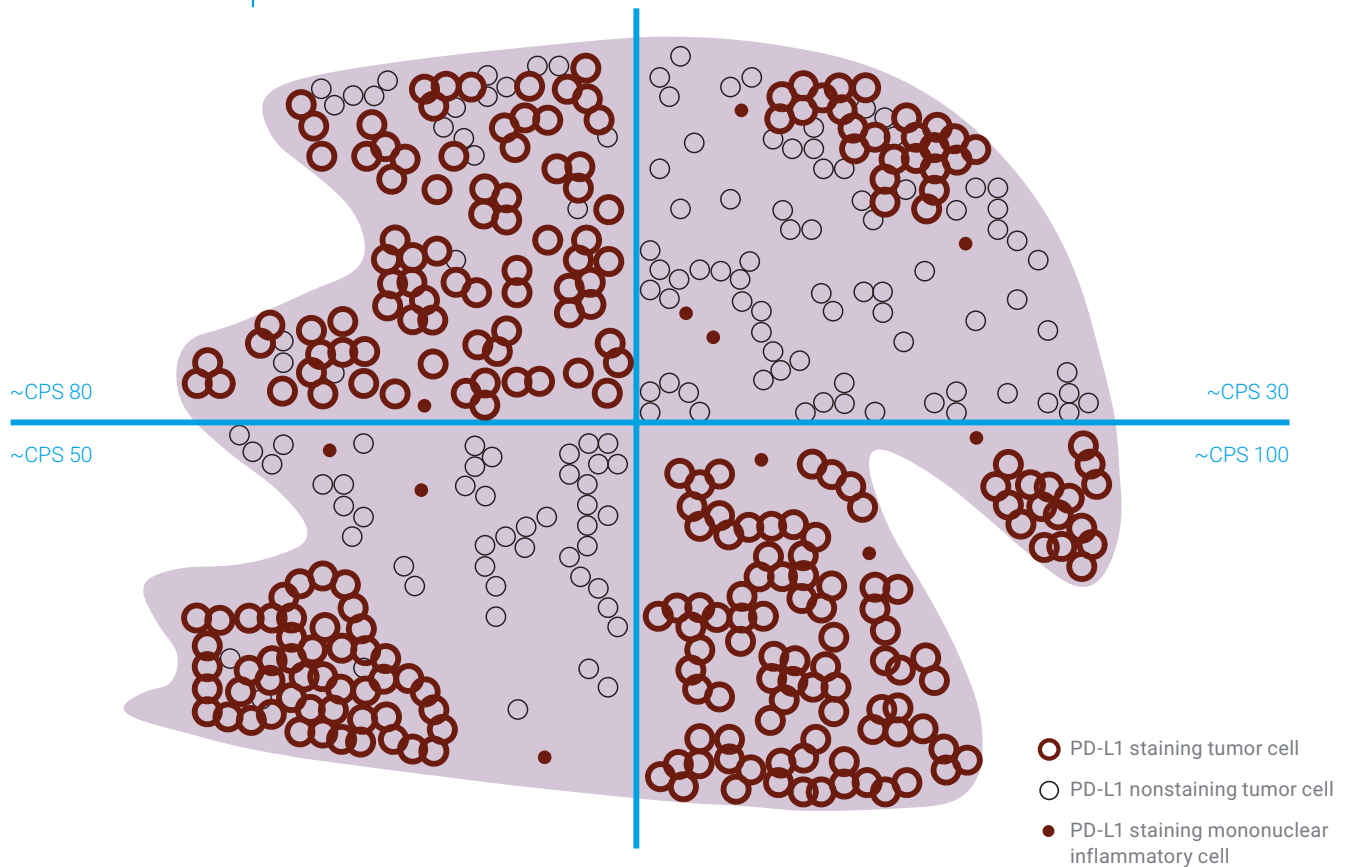
### Example 2: Calculation of Combined Positive Score in a Heterogeneous Tumor Area

At lower magnifications (4x, 10x): Visually divide the tumor area into regions with equal numbers of tumor cells.

At higher magnification (20x): 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:  
 $(80+30+50+100 / 4) \times 100 \approx \text{CPS } 65$

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

Clinical Interpretation: CPS  $\geq$  1

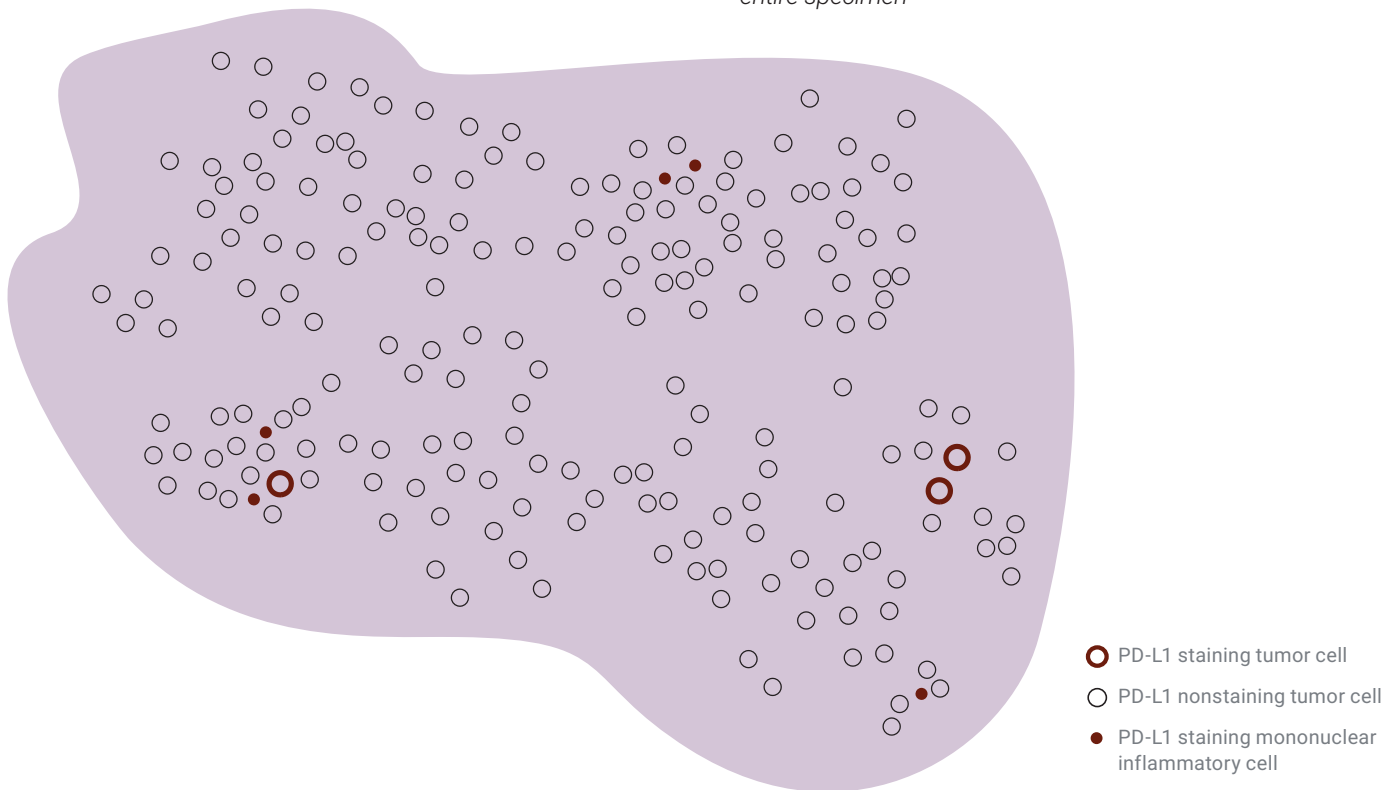
Figure 15: Example with heterogeneous tumor area.

### Example 3: Calculation of Combined Positive Score for a Near Cut-off Specimen (CPS 0–10)

At lower magnifications (4x, 10x): Evaluate the specimen for convincing staining as described in “Determining Combined Positive Score” on page 25.

At higher magnification (20x): 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:

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

Clinical Interpretation: CPS ≥1

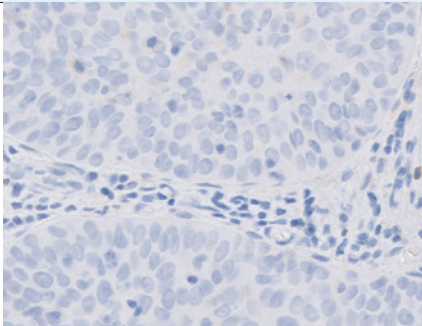
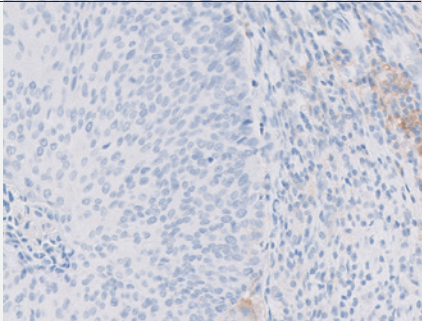
\* Including tumor cells, lymphocytes, macrophages

Figure 16: Example of near cut-off specimen (CPS 0–10).

# Interpretation of CPS

The Combined Positive Score describes the PD-L1 expression level of the specimen. See the table below for scoring guideline examples.

**Table 3:** CPS and corresponding PD-L1 Expression Levels

CPS	Expression Level	Image (20x magnification)
<p>&lt; 1</p>	<p>CPS is less than 1</p>	
<p>≥ 1</p>	<p>CPS is greater than or equal to 1</p>	

## Identifying Patients With Cervical Cancer for Treatment

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

### Clinical Validation of PD-L1 IHC 22C3 pharmDx in Previously Treated Patients With Cervical Cancer\*

The clinical validity of PD-L1 IHC 22C3 pharmDx in evaluating PD-L1 expression (CPS  $\geq$  1) in previously treated patients with cervical cancer\* is based on the KEYTRUDA KEYNOTE-158 study sponsored by Merck Sharp & Dohme LLC. Specimens from previously treated patients with cervical cancer were tested for PD-L1 expression using PD-L1 IHC 22C3 pharmDx. Eighty-five percent of enrolled cervical cancer patients had tumors that expressed PD-L1 with a Combined Positive Score (CPS) of greater than or equal to 1 (CPS  $\geq$  1) (Table 4). Clinical efficacy of KEYTRUDA treatment in patients is presented in the Clinical Performance Evaluation section on page 74.

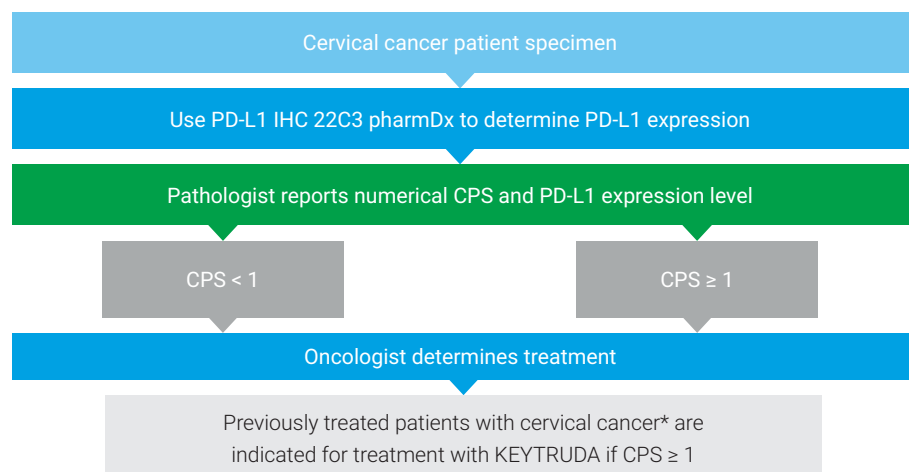
Table 4: PD-L1 Prevalence in Patients with Cervical Cancer Enrolled in KEYNOTE-158

PD-L1 Expression	CPS < 1	CPS $\geq$ 1
Prevalence, % (n)	15% (15)	85% (82)

Note: Prevalence calculation based on patients with known PD-L1 expression; 1 patient (1% of the cohort) had unknown PD-L1 expression level.

## PD-L1 IHC 22C3 pharmDx Testing Scheme

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



\* Patients with disease progression on or after chemotherapy

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

# Reporting Results

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

## PD-L1 IHC 22C3 pharmDx Summary of Sample Tested

Date of Run: \_\_\_\_\_

PD-L1 IHC 22C3 pharmDx Lot: \_\_\_\_\_

Staining Run Log ID: \_\_\_\_\_

Specimen ID: \_\_\_\_\_

Patient Identifiers: \_\_\_\_\_

Type of Service: IHC Stain with Manual Interpretation

Other: \_\_\_\_\_

PD-L1 Included in Cervical Cancer Comprehensive Panel: Yes:  No:

Type of Tissue: Cervical Cancer:  Other:

## PD-L1 Testing Results

Control Cell Line Slide Results: Pass:  Fail:

Adequate Tumor Cells Present ( $\geq 100$  cells):

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

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

CPS  $\geq 1$ :  CPS  $< 1$ :

Comments to Treating Physician:

- \* The PD-L1 IHC 22C3 pharmDx assay was validated only at CPS  $\geq 1$  cutoff for the Cervical Cancer indication. The CPS raw score has not been validated for clinical use.
- KEYTRUDA® (pembrolizumab) is indicated for the treatment of patients with advanced (recurrent or metastatic) cervical cancer whose tumors have PD-L1 expression [Combined Positive Score (CPS) greater than or equal to 1] as determined by an approved test. See KEYTRUDA prescribing information for details.



# Combined Positive Score Summary and Examples

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

By definition, PD-L1 staining cells in cervical cancer are:

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

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

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

This section will define and illustrate all scoring inclusions and exclusions for accurate determination of Combined Positive Score. All images are cervical cancer unless otherwise noted in the figure caption.

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

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

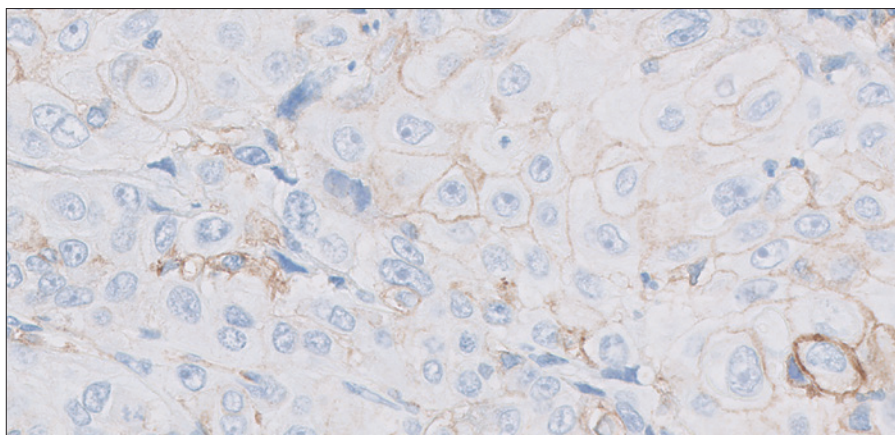
Tumor cells, lymphocytes, and macrophages exhibiting appropriate PD-L1 expression are defined as PD-L1 staining cells. All PD-L1 staining cells are included in the CPS numerator for determination of the Combined Positive Score (see Tables 1 and 2 on page 26 for additional CPS inclusion/exclusion criteria).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

### Tumor Cells

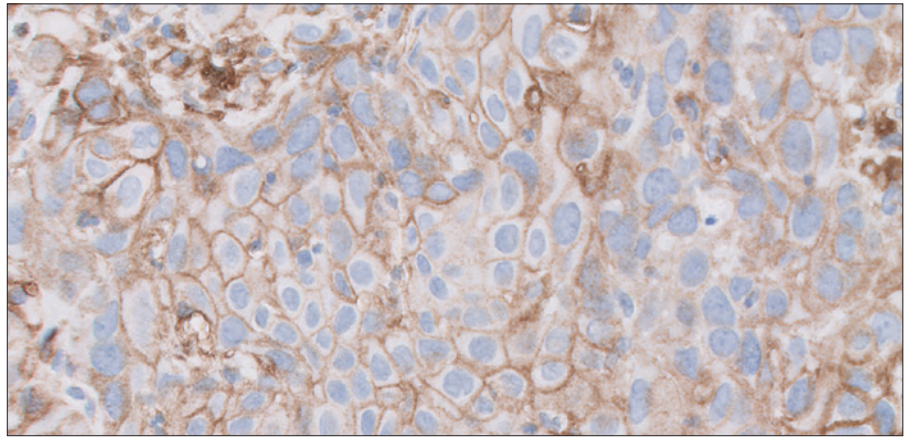
#### Linear Membrane Staining

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 20x magnification.

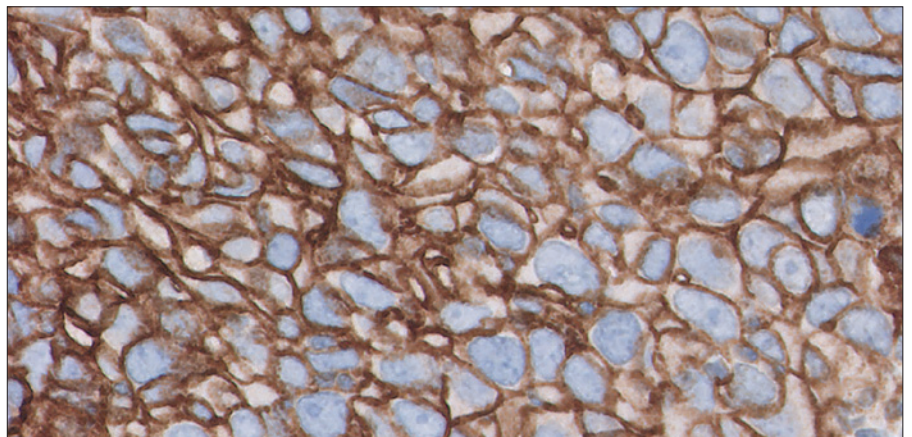
Convincing staining of tumor cells (linear membrane staining) is often heterogeneous, with various staining intensities present.



**Figure 18a:** 1+ linear membrane PD-L1 staining of tumor cells (20x magnification).



**Figure 18b:** 2+ linear membrane PD-L1 staining of tumor cells (20x magnification).



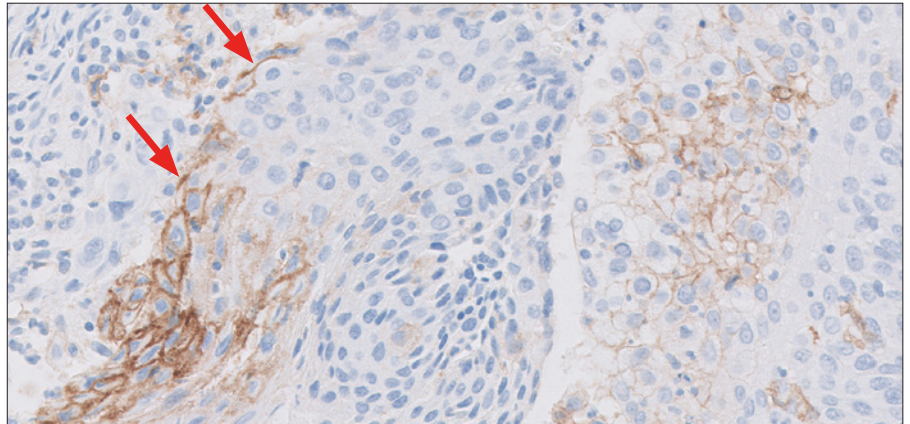
**Figure 18c:** 3+ linear membrane PD-L1 staining of tumor cells (20x magnification).

**Key point**

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

### Partial Linear Membrane Staining

Tumor cells can exhibit partial linear membrane staining. Any partial linear membrane staining observed at any intensity and observed at a 20x magnification must be included in the CPS numerator.



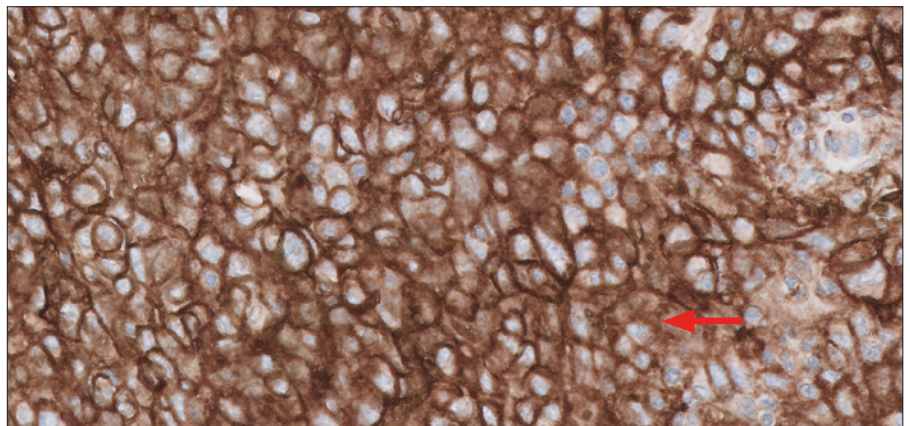
**Figure 19:** Partial linear membrane PD-L1 staining of tumor cells (arrows) (20x magnification).

#### Key point

**Convincing partial 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 20x magnification should be included in the CPS numerator. Tumor cells exhibiting only cytoplasmic staining are excluded from the CPS numerator. However, linear PD-L1 staining of tumor cells can be smooth or granular. If linear membrane staining is distinct from cytoplasmic staining, then the cell should be included in the score.



**Figure 20:** PD-L1 staining of linear membrane distinct from cytoplasmic staining (arrow) (20x magnification).

#### Key point

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

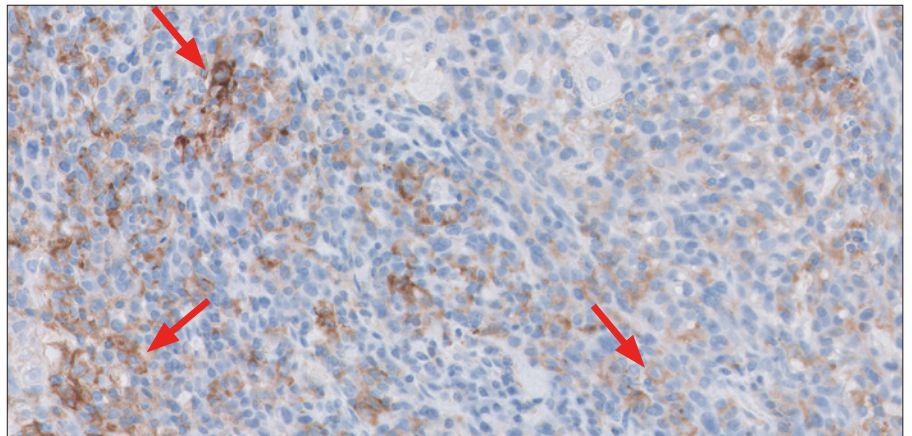
## Immune Cells

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

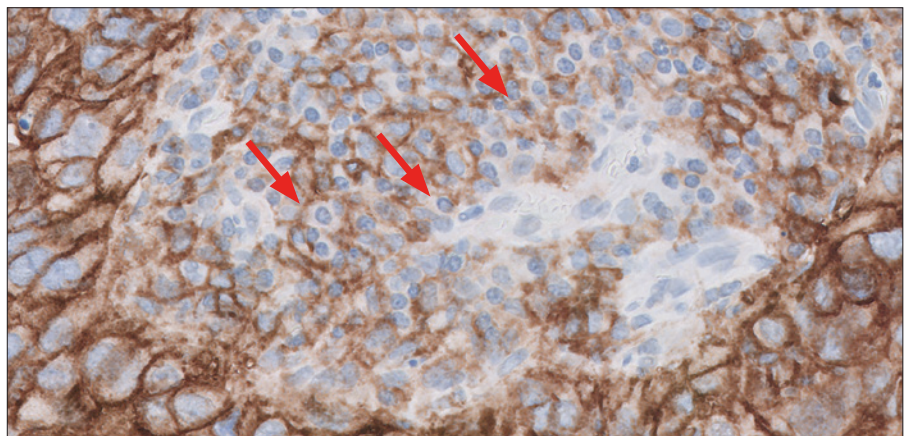
Tumor-associated lymphocytes and macrophages (mononuclear inflammatory cells, MICs) exhibiting membrane and/or cytoplasmic staining at a 20x magnification ( $\geq 1+$  intensity) are considered PD-L1 staining cells and should be included in the CPS numerator. Tumor-associated MICs are present within 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 21a:** PD-L1 staining of tumor-associated lymphocytes (arrows) (20x magnification).



**Figure 21b:** PD-L1 staining of tumor-associated macrophages (arrows) (20x magnification).

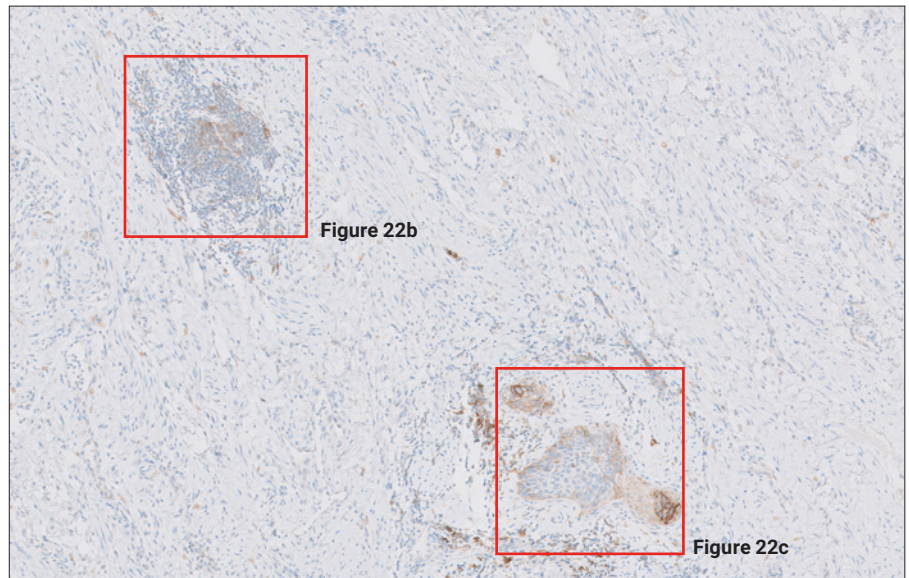
### Key point

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

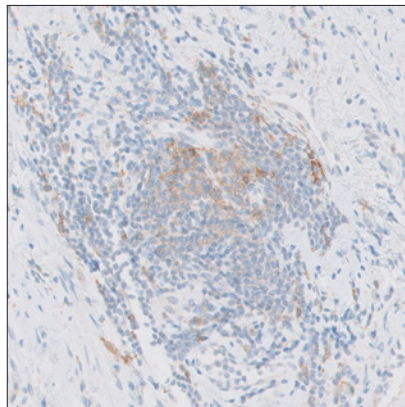
### Immune Cell Inclusion/Exclusion: 20x Rule

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 20x magnification field of view. In cases where it is difficult to tell if MICs are tumor-associated, the following is suggested as a guideline:

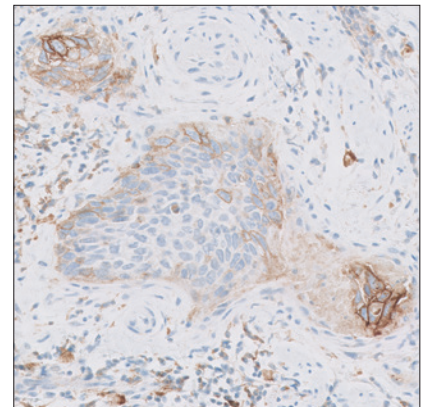
Move the slide so that the tumor is in the approximate center of a 20x field. Immune cells surrounding the tumor in this field should be included in scoring. Immune cells outside of this field should be excluded from scoring as long as they do not surround neighboring tumor cells. See Figures 22a–22c for an example of determining which MICs are included in the CPS numerator.



**Figure 22a:** At 5x magnification, two areas of PD-L1 staining mononuclear inflammatory cells are visible. Following the instructions above, zoom in to 20x magnification on each field to determine which immune cells to include in the numerator (5x magnification).



**Figure 22b:** Tumor cells are absent from this 20x field containing PD-L1 staining mononuclear inflammatory cells, thus none of these cells should be included in the numerator (20x magnification).



**Figure 22c:** When positioning the tumor cells in the approximate center of a 20x field, PD-L1 staining mononuclear inflammatory cells that are present within the same field should be included in the numerator (20x magnification).

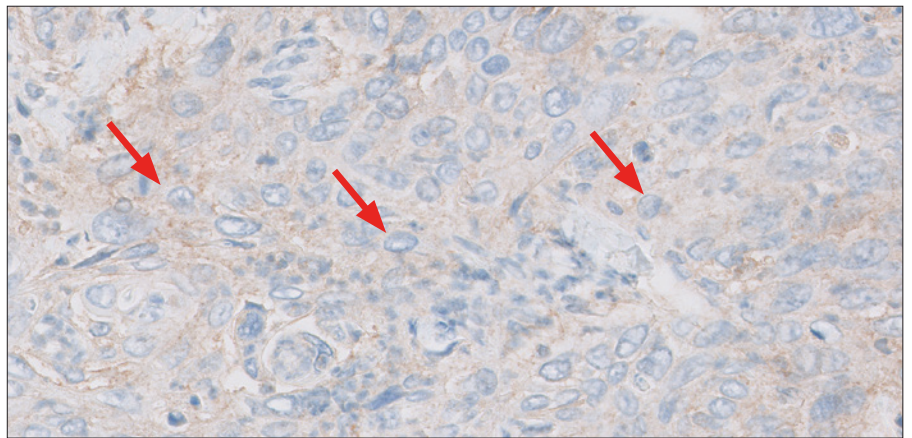
## Cells Excluded from CPS

Only tumor cells exhibiting PD-L1 membrane staining and MICs exhibiting PD-L1 membrane and/or cytoplasmic staining should be included in the CPS numerator. Below are 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 26 to view all exclusion criteria.

### Tumor Cells with Only Cytoplasmic Staining

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

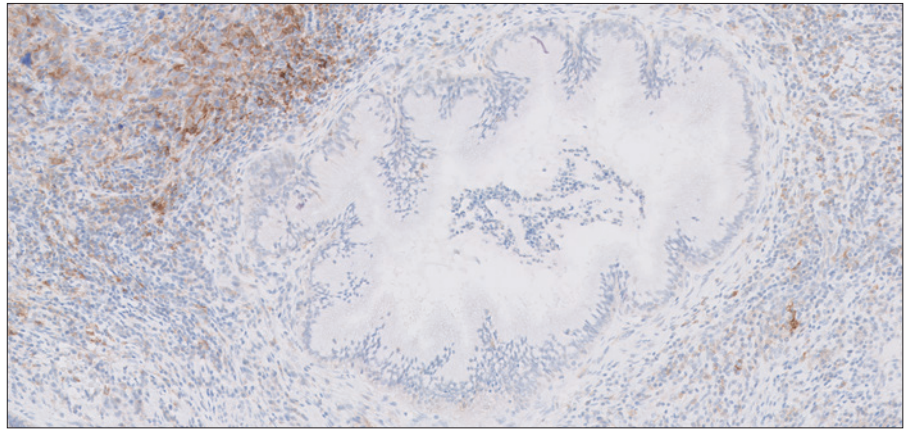


**Figure 23:** Tumor cells exhibiting only cytoplasmic PD-L1 staining (arrows) (20x magnification).

### Key point

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

## Benign Glands

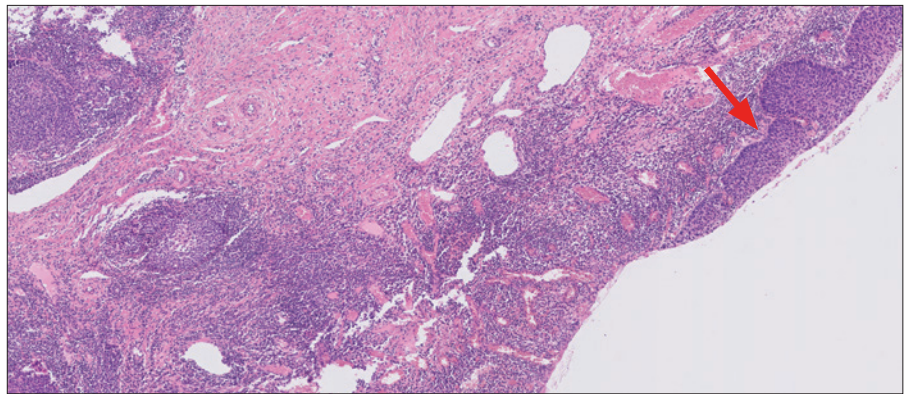


**Figure 24:** Benign endocervical gland excluded from the score (10x magnification).

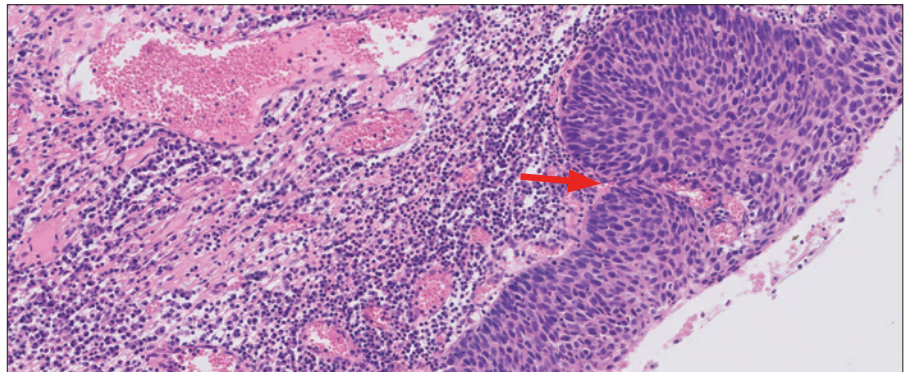
### Key point

Benign endocervical glands may stain for PD-L1. Any immune cells associated with benign endocervical glands should be excluded from the score

## Cervical Intraepithelial Neoplasia (CIN)

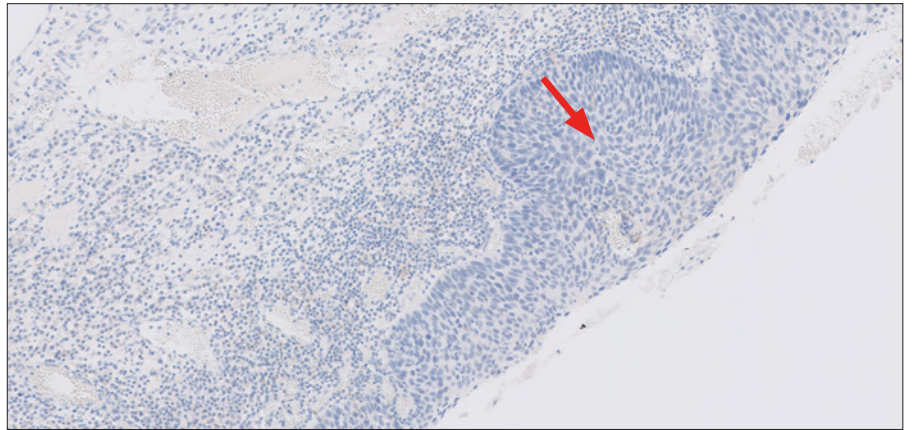


**Figure 25a:** Hematoxylin and eosin (H&E) section demonstrating cervical carcinoma in situ (CIN III) (arrow) (2x magnification).



**Figure 25b:** Hematoxylin and eosin (H&E) section demonstrating cervical carcinoma in situ (CIN III) (arrow) (10x magnification).



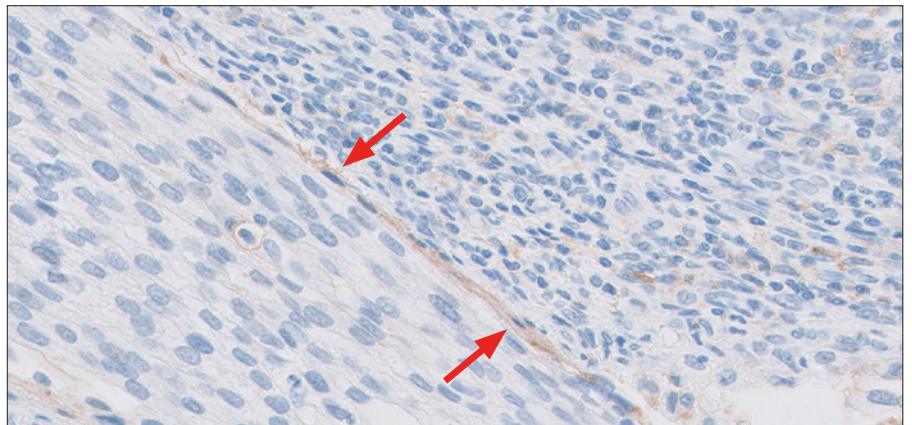


**Figure 25c:** Any PD-L1 staining in CIN should be excluded from the score (arrow). Nonstaining CIN III is excluded from the denominator (10x magnification).

**Key point**

PD-L1 staining may be present in CIN and should be excluded from the score

Stromal Cells



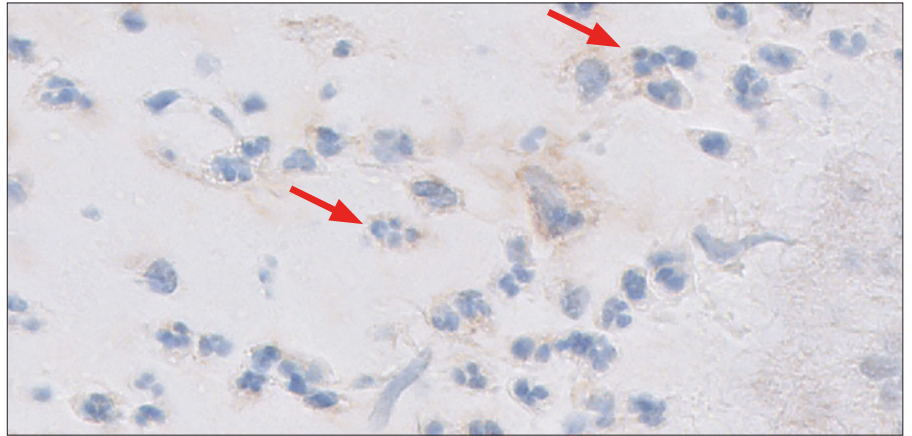
**Figure 26:** PD-L1 staining on stromal cells (arrows) (20x magnification).

**Key point**

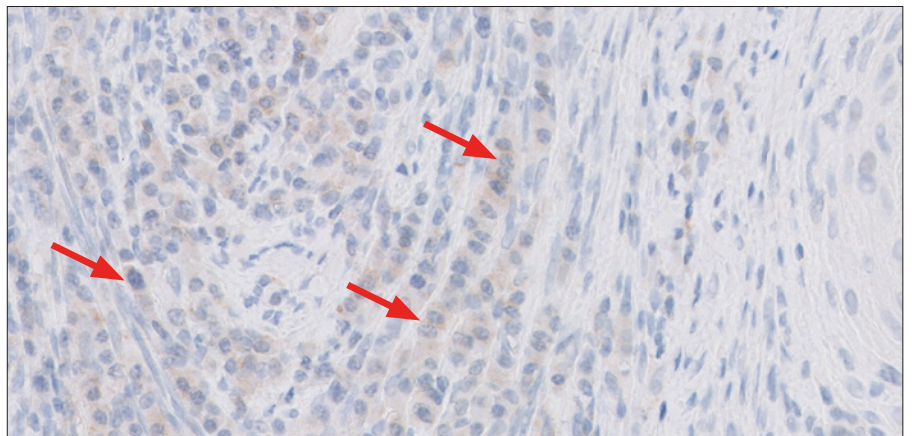
PD-L1 staining stromal cells should be excluded from the score

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



**Figure 27a:** PD-L1 staining on neutrophils (arrows) (20x magnification).



**Figure 27b:** PD-L1 staining on plasma cells (arrows) (20x magnification).

#### Key point

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

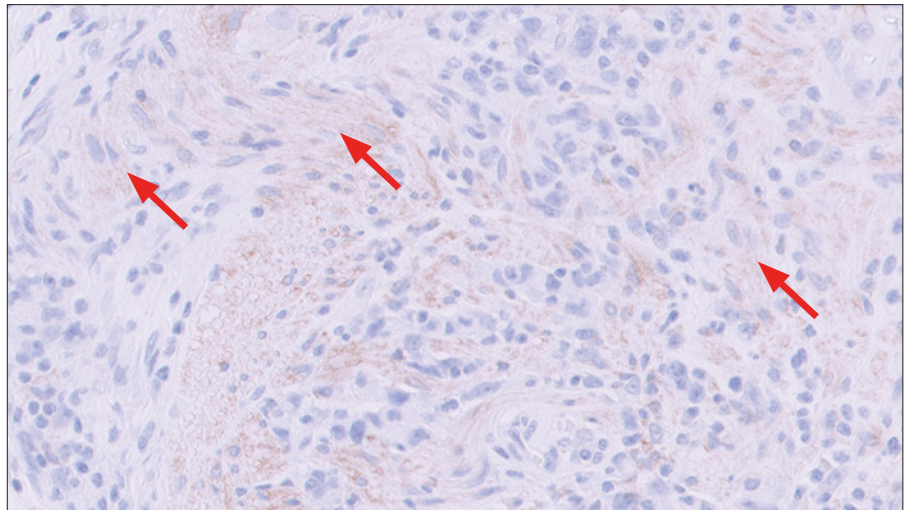
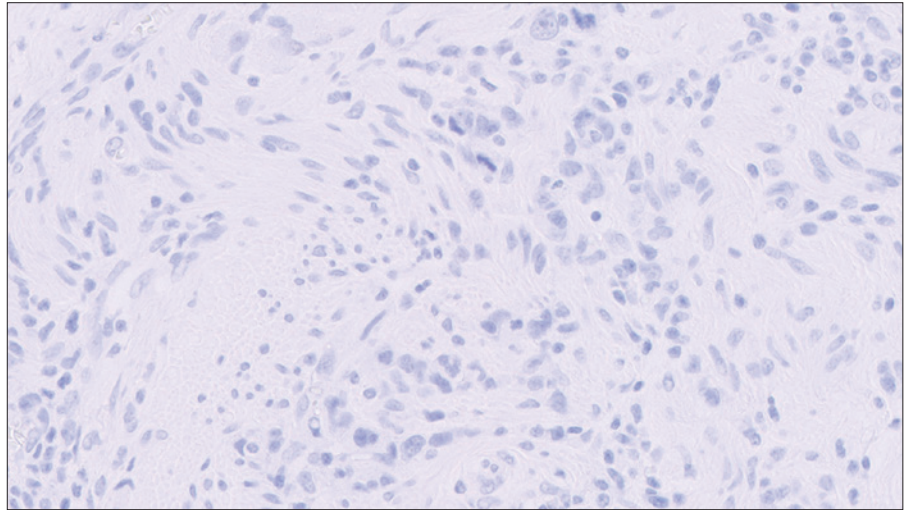


# Artifacts

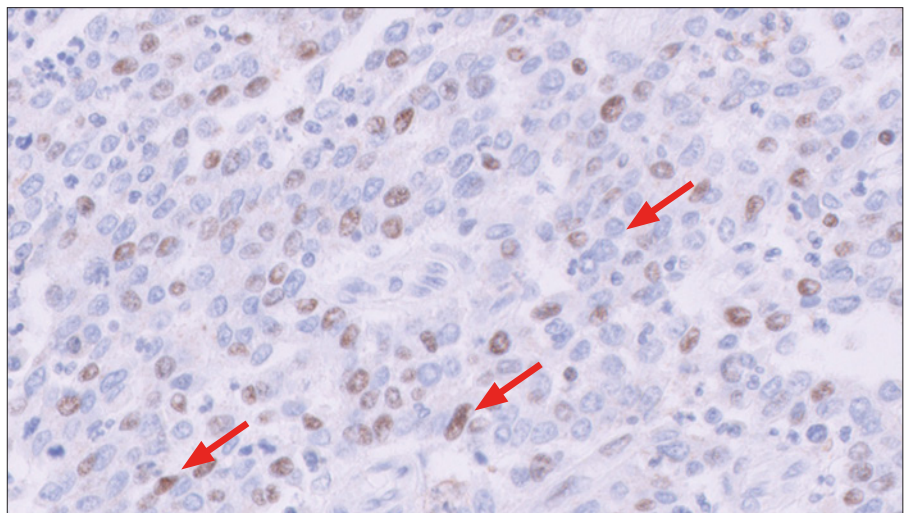
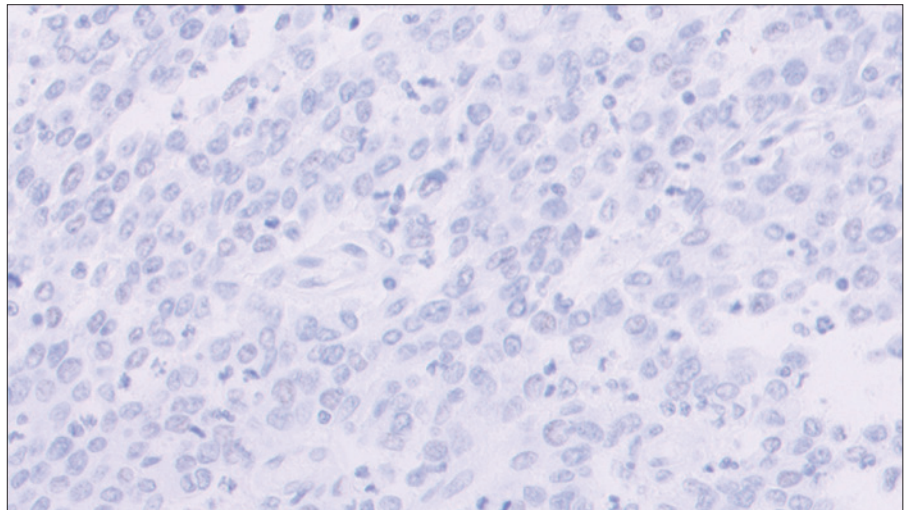
The following pages provide examples of artifacts you may see when staining with PD-L1 IHC 22C3 pharmDx.

## 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 34a:** 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 (20x magnification).



**Figure 34b:** 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 (arrows)(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 slide should be considered evaluable (20x magnification).

## Nonspecific Staining

Nonspecific 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:

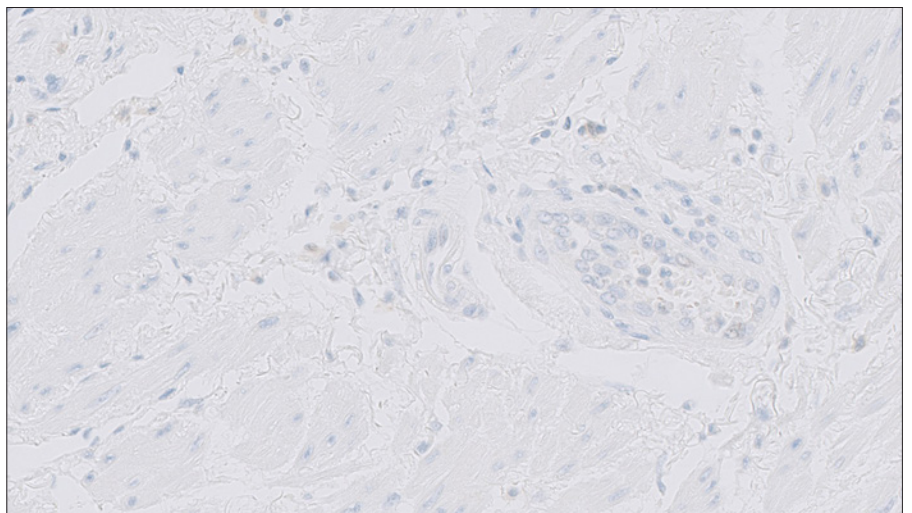
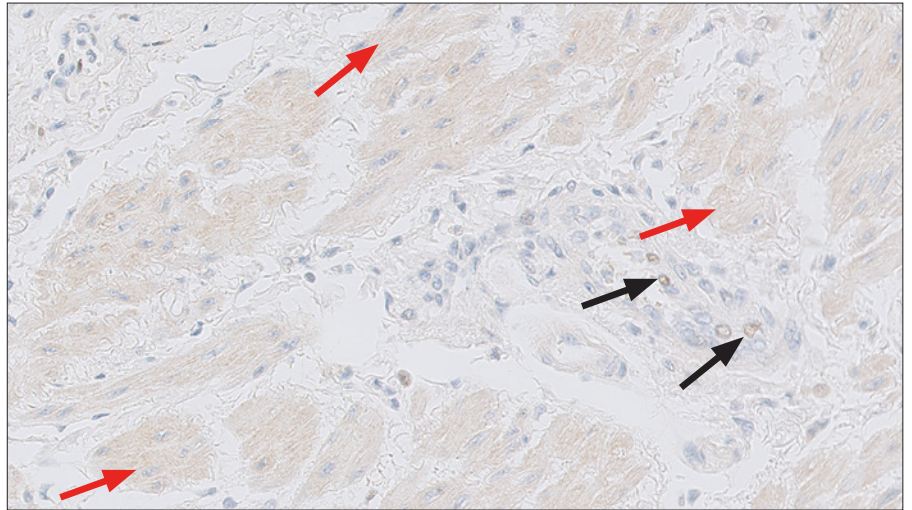
- Pre-analytic fixation and processing of the specimen, including use of fixatives other than neutral buffered formalin (not recommended)
- Incomplete removal of paraffin from the section
- Incomplete rinsing of reagents from slides during staining
- Drying of slides; ensure slides remain wet with buffer while loading onto Autostainer Link 48, prior to initiating run and 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.

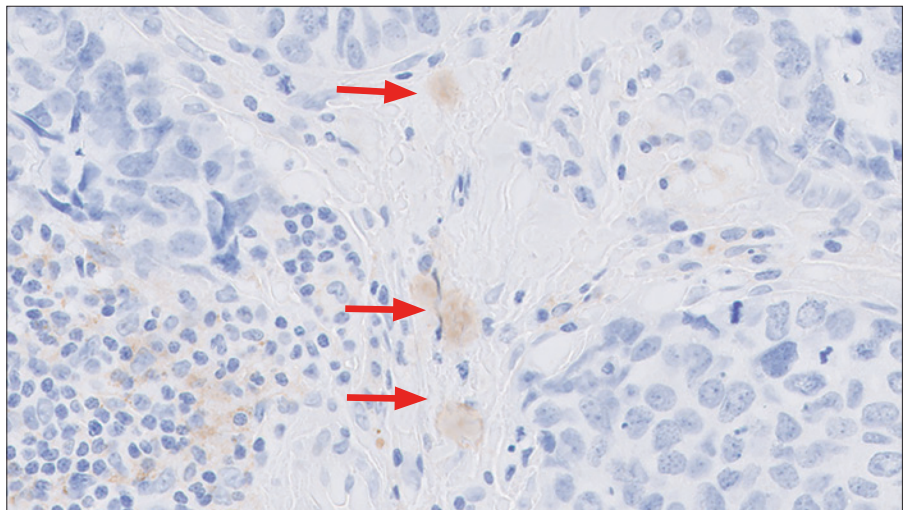
All specimens must have  $\leq 1+$  nonspecific staining to be considered acceptable for PD-L1 expression evaluation.

When staining of cell nuclei is present in both the specimen slide stained with the PD-L1 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.

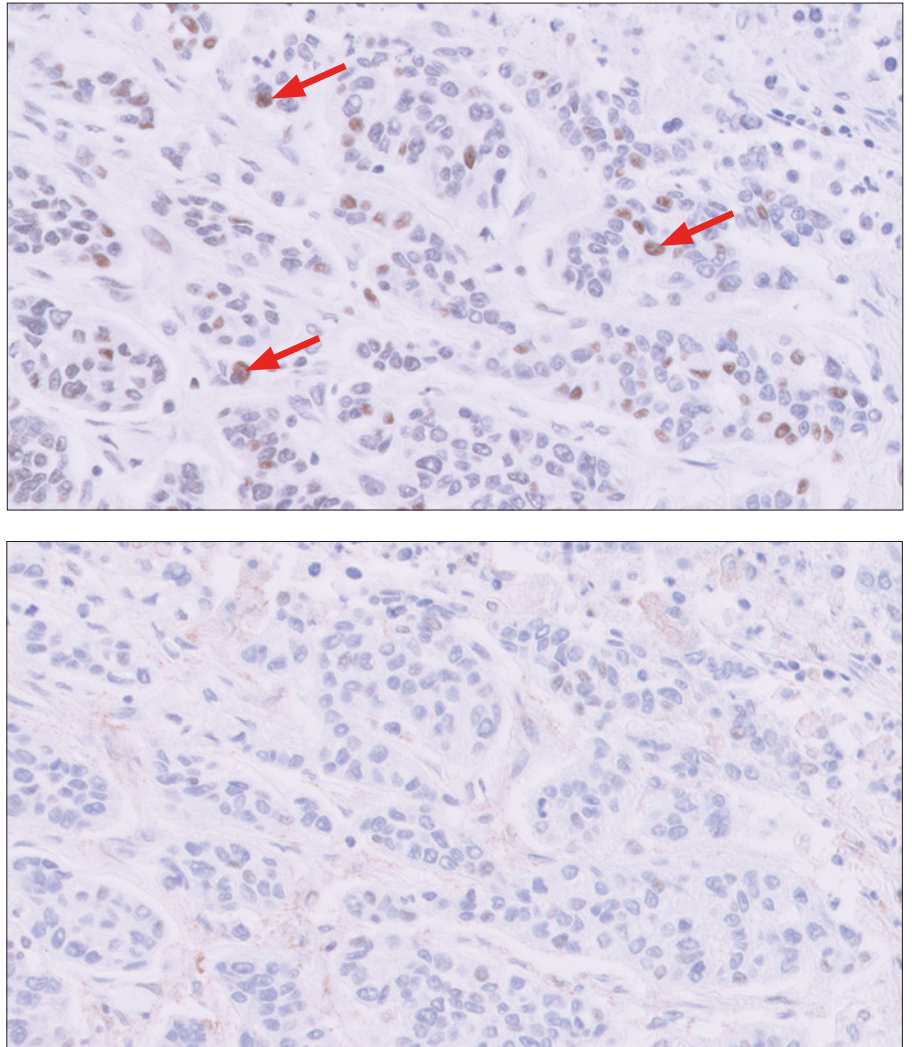


**Figure 35a:** Esophageal adenocarcinoma specimen stained with NCR (top) exhibiting acceptable ( $\leq 1+$  intensity) nonspecific staining on smooth muscle cells (red arrows) and occasional endothelial cell nuclei (black arrows). Note that in this case, the nonspecific staining is not also present in the PD-L1 slide (bottom) (20x magnification).



**Figure 35b** Negative Control Reagent (NCR) exhibiting nonspecific staining in TNBC (arrows) (20x magnification).





**Figure 35c:** Gastric or GEJ adenocarcinoma specimen exhibiting nonspecific nuclear staining (arrows) that is present in both the NCR-(top) and PD-L1 (bottom) stained 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 (20x magnification).

#### Key Point

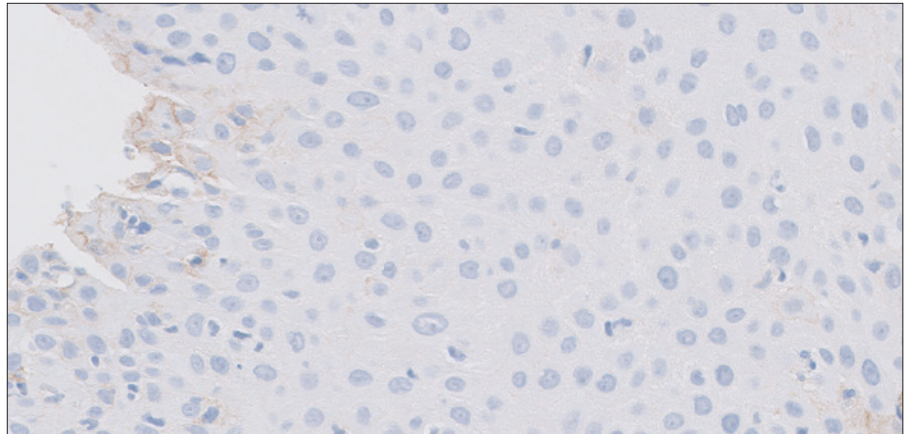
All specimens must have  $\leq 1+$  nonspecific staining, including nuclear staining

## Edge Artifact

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

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

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

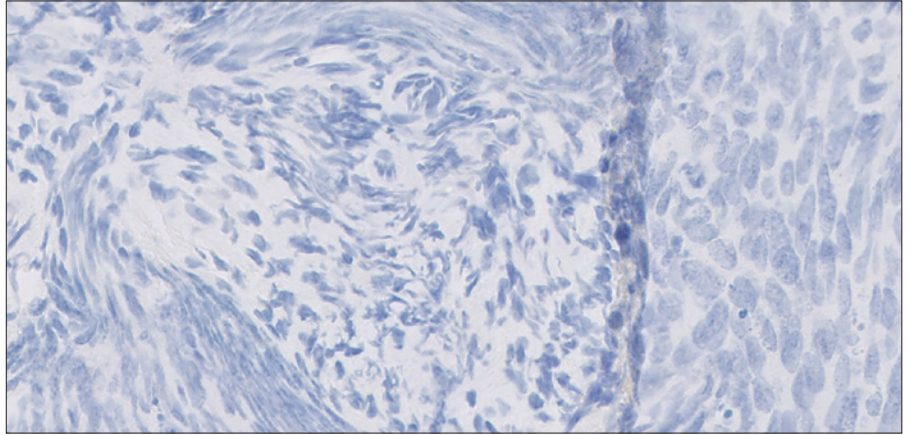


**Figure 29:** Edge artifact staining should be excluded from the score (20x magnification).

### Key point

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

## Crush Artifact



**Figure 30:** Crush artifact (20x magnification).

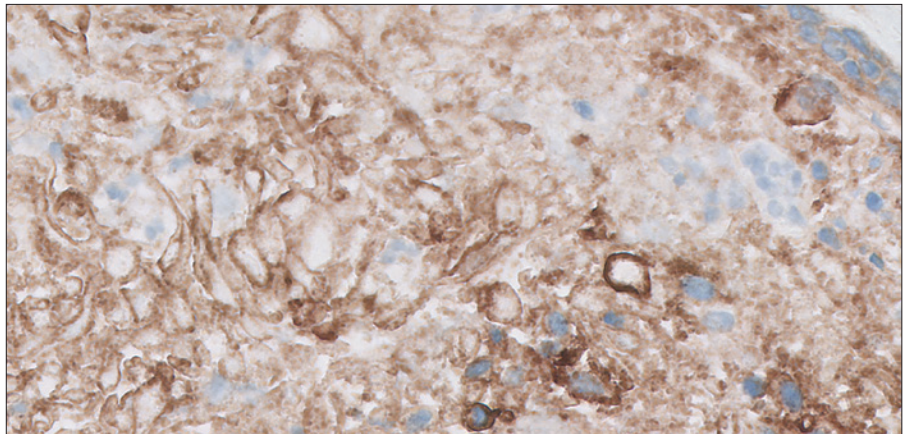
Areas of the examined section exhibiting cytologically and morphologically distorted secondary crush artifact may show exaggerated staining and should be excluded from the score.

### Key point

Scoring of crush artifact should be avoided

## Necrosis

Cytological and morphological changes consistent with necrosis often demonstrate marked PD-L1 staining and should be excluded from the score.



**Figure 31:** Staining of necrosis should be excluded from the score (20x magnification).

### Key point

PD-L1 staining of necrotic areas should be excluded from the CPS calculation

# CPS Case Examples

## CPS < 1 Case Examples

### Case 1: CPS < 1

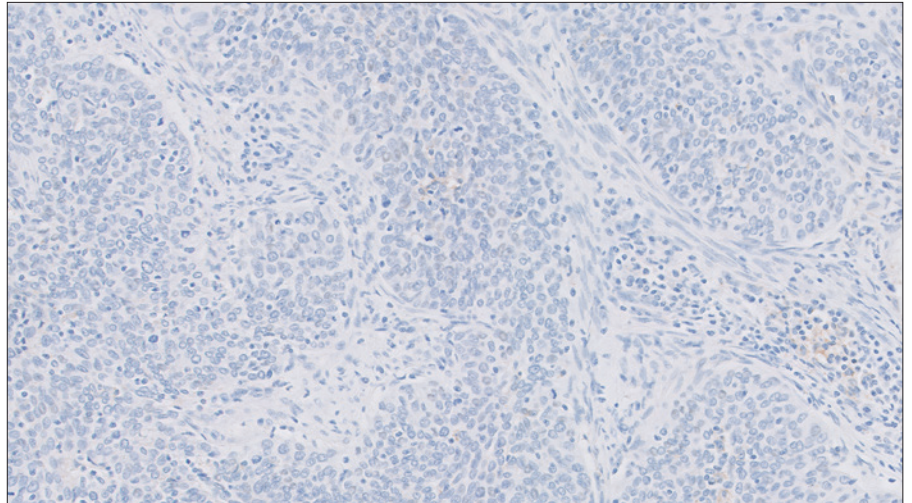


Figure 32a: 10x magnification

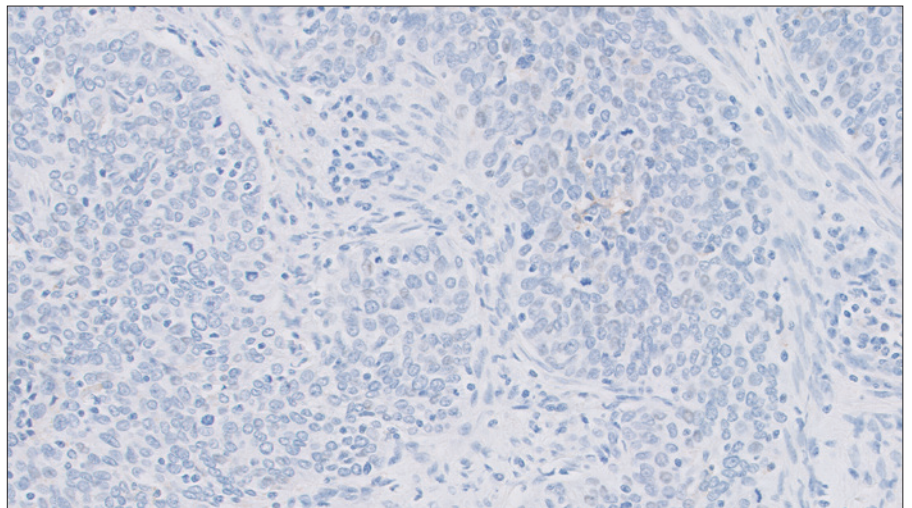
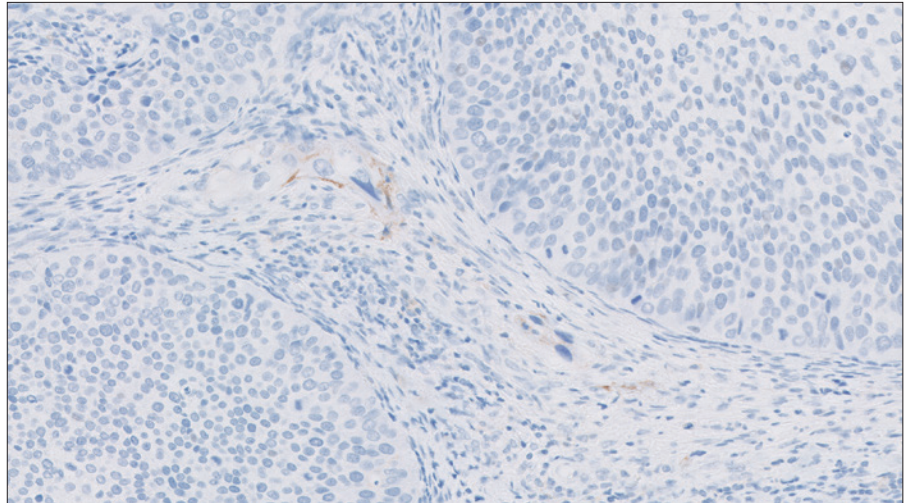


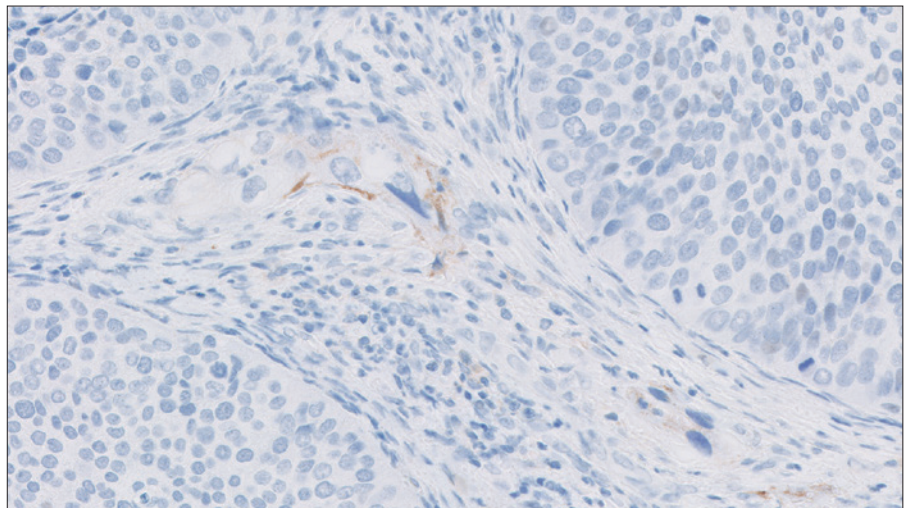
Figure 32b: 20x magnification

PD-L1 antibody exhibiting CPS < 1 at 20x magnification.

**Case 2: CPS < 1**



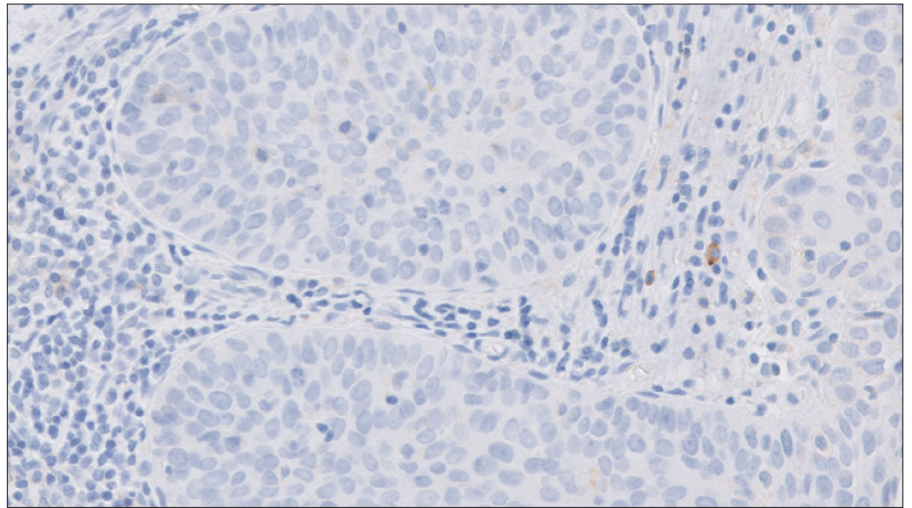
**Figure 33a:** 10x magnification



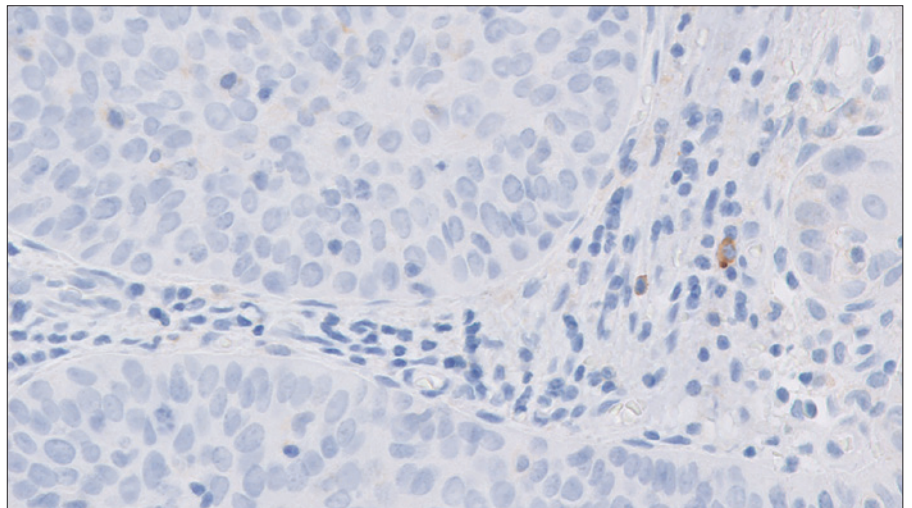
**Figure 33b:** 20x magnification

PD-L1 antibody exhibiting CPS < 1 at 20x magnification.

**Case 3: CPS < 1**



**Figure 34a:** 10x magnification



**Figure 34b:** 20x magnification

PD-L1 antibody exhibiting CPS < 1 at 20x magnification.

## CPS $\geq$ 1 Case Examples

### Case 4: CPS $\geq$ 1

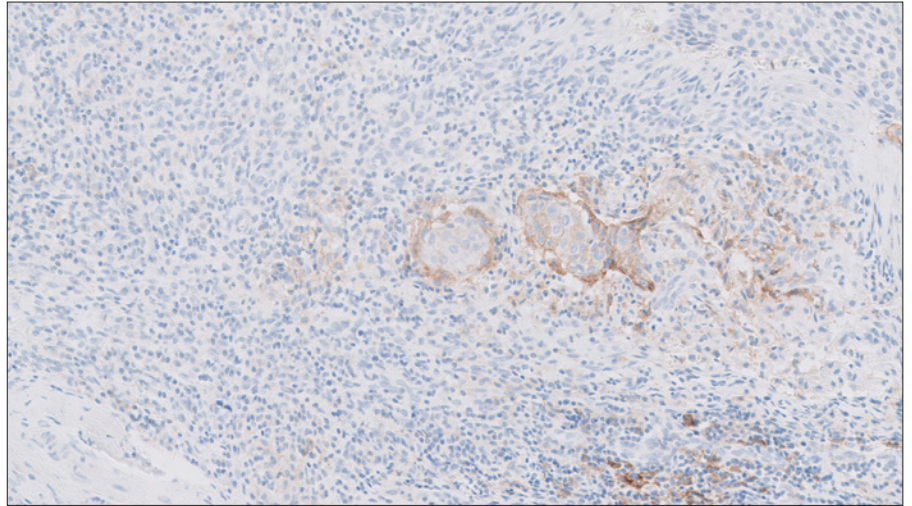


Figure 35a: 10x magnification

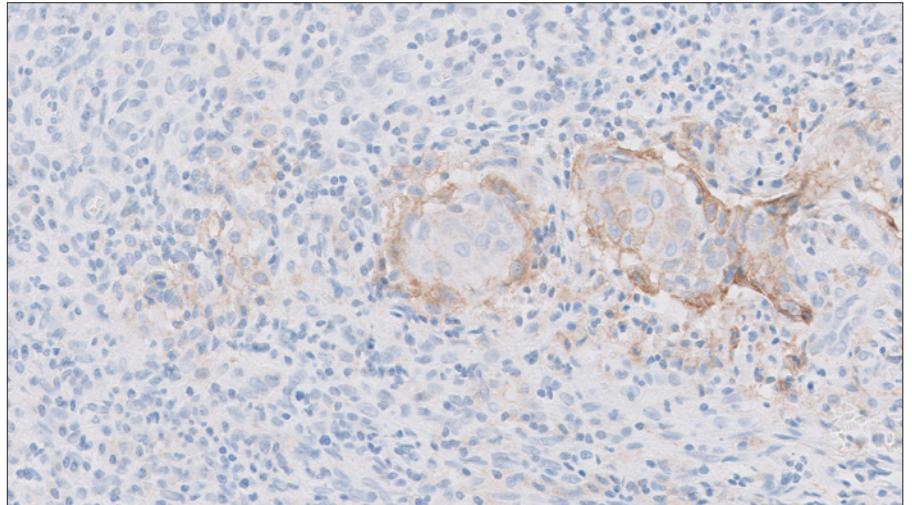
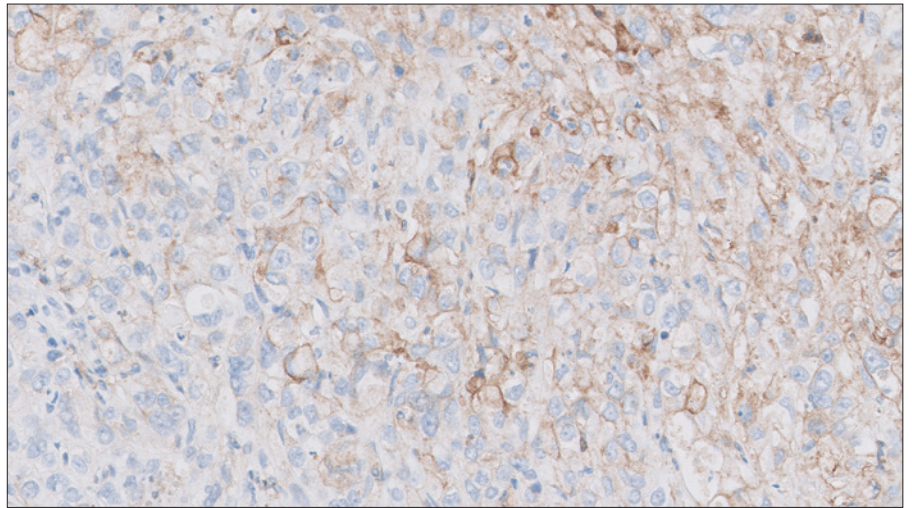


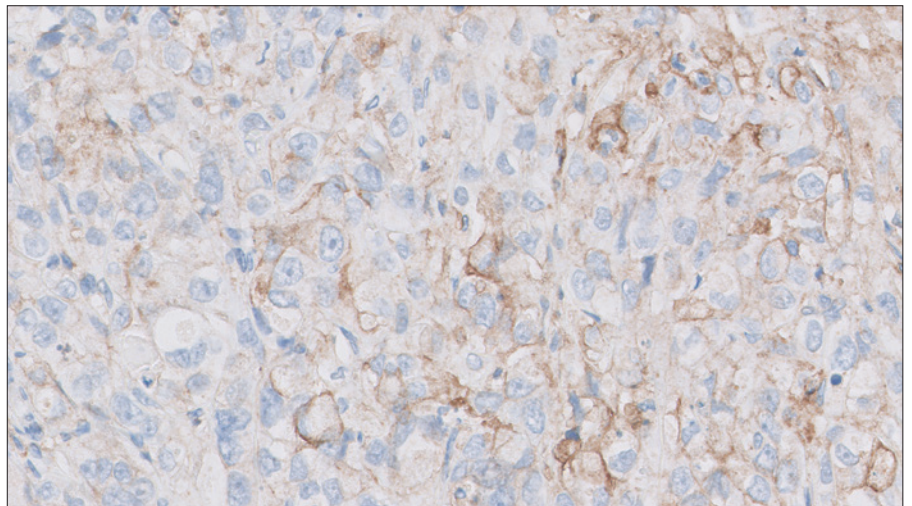
Figure 35b: 20x magnification

PD-L1 antibody exhibiting CPS  $\geq$  1 at 20x magnification.

**Case 5: CPS  $\geq 1$**



**Figure 36a:** 10x magnification

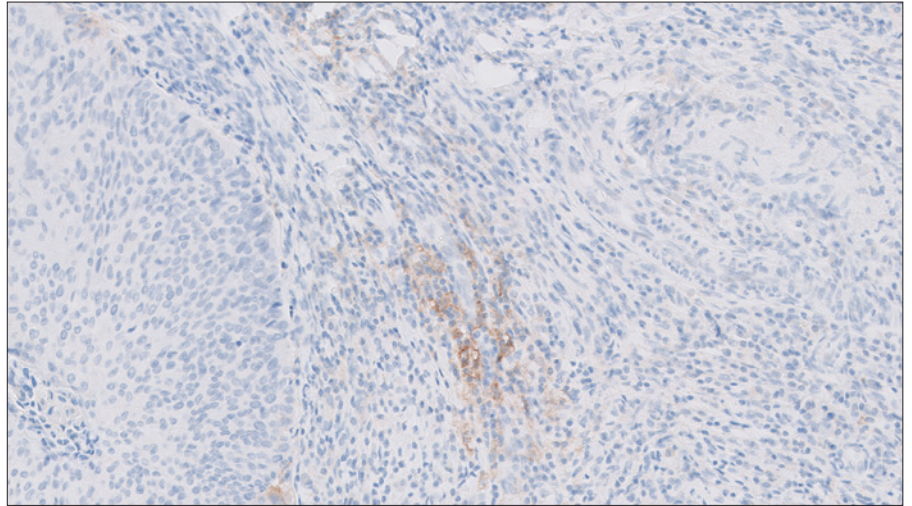


**Figure 36b:** 20x magnification

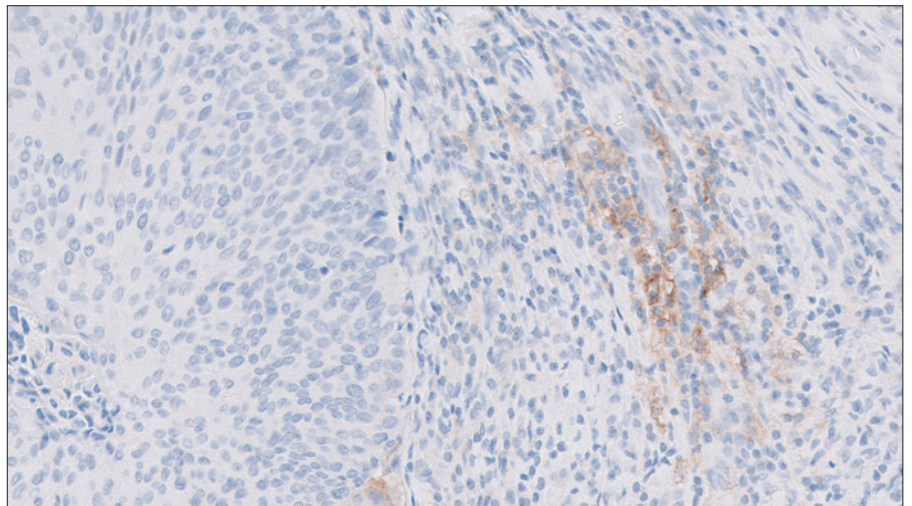
PD-L1 antibody exhibiting CPS  $\geq 1$  at 20x magnification.



**Case 6: CPS  $\geq$  1**



**Figure 37a:** 10x magnification



**Figure 37b:** 20x magnification

PD-L1 antibody exhibiting CPS  $\geq$  1 at 20x magnification.

## Near Cut-off Case Examples (CPS 0–10)

### Challenging Case 1: Near Cut-off (CPS 0–10)

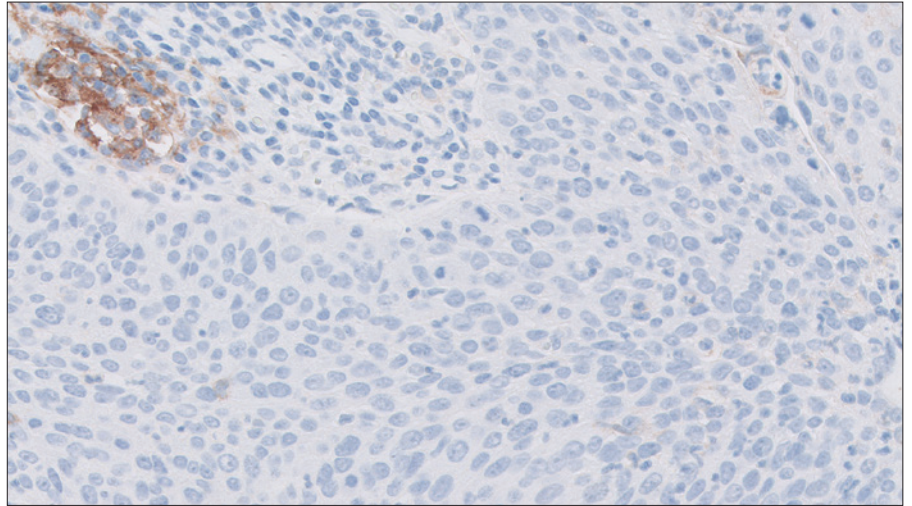


Figure 38: CPS 4 at 20x magnification.

### Challenging Case 2: Near Cut-off (CPS 0–10)

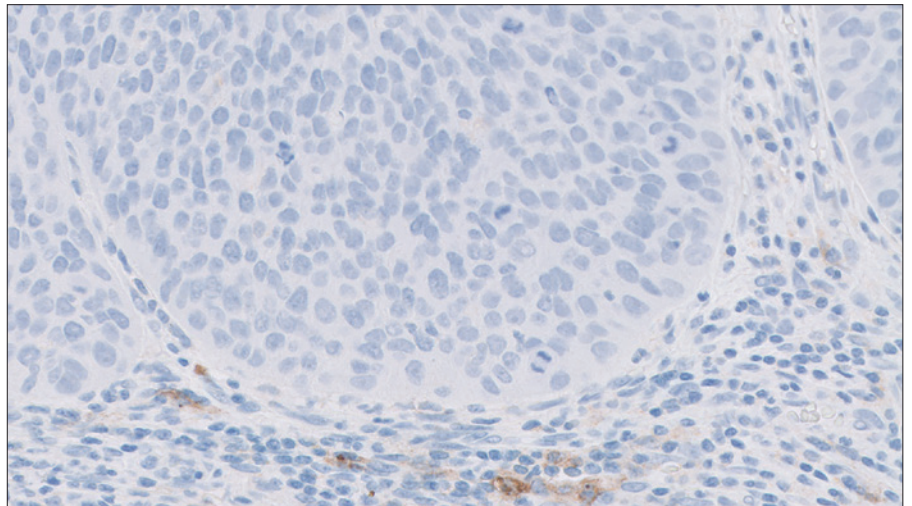


Figure 39: CPS 1 at 20x magnification.

### Challenging Case 3: Near Cut-off (CPS 0–10)

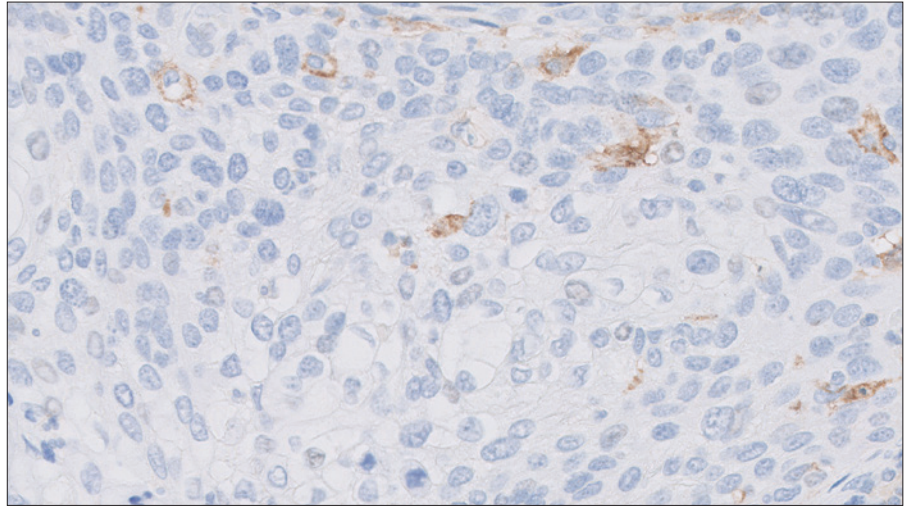


Figure 40: CPS 3 at 20x magnification.

# 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.

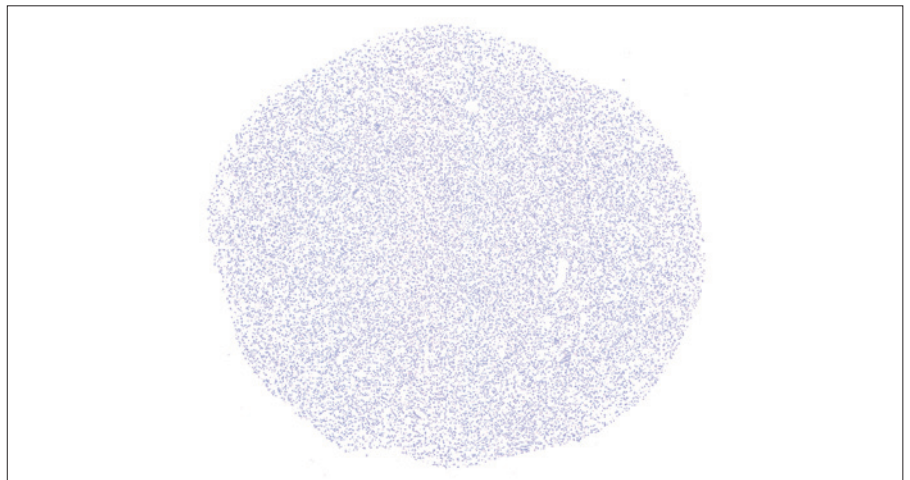


Figure 59: Ideal MCF-7 cell pellet (2x magnification).

### 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 is < 1+ intensity



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

## Borderline Passing CCL

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

#### Borderline Passing PD-L1 positive CCL



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

### Evaluation Strategy for Borderline Passing PD-L1 Positive CCL

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

Quadrant 1

Quadrant 2

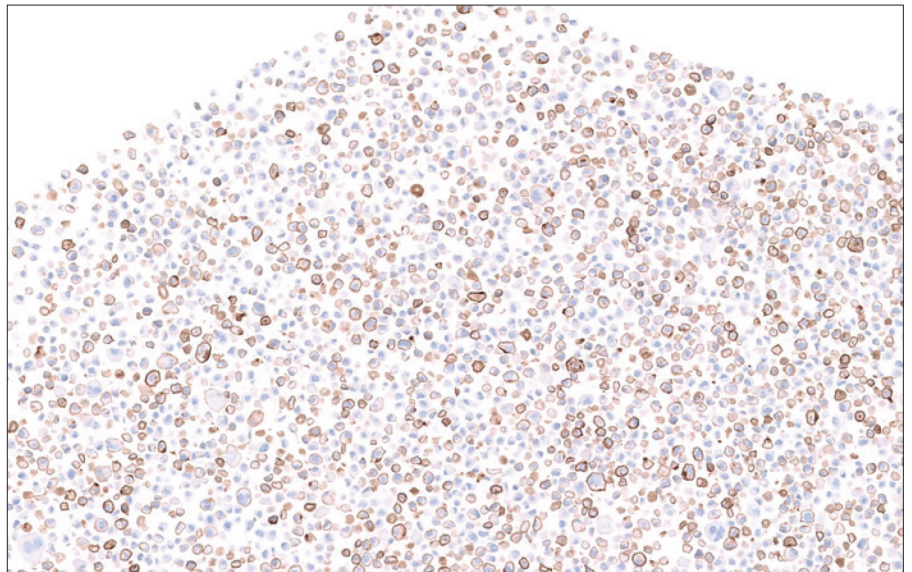


Quadrant 3

Quadrant 4

### Quadrant 1

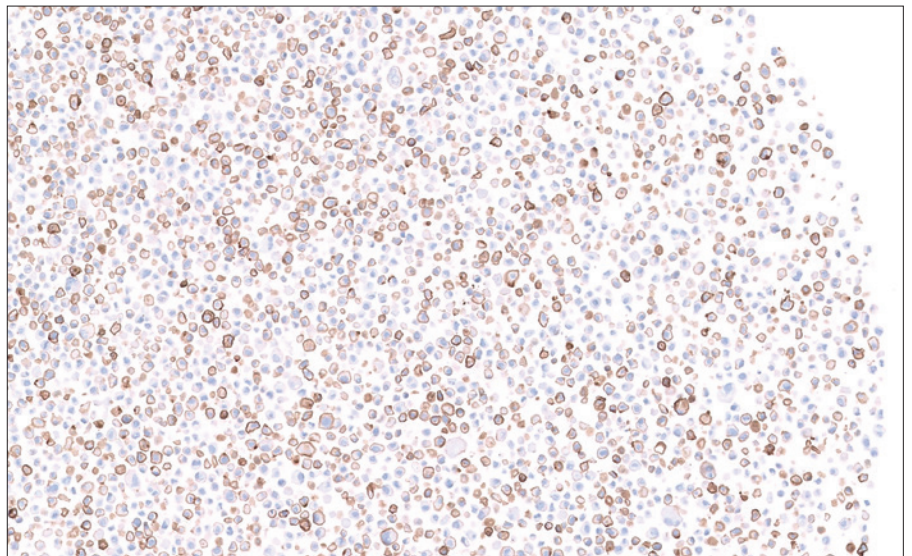
In Quadrant 1 approximately 70% of cells exhibit membrane staining, and the average staining intensity of all staining cells in this quadrant is  $\geq 2+$ .



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

### Quadrant 2

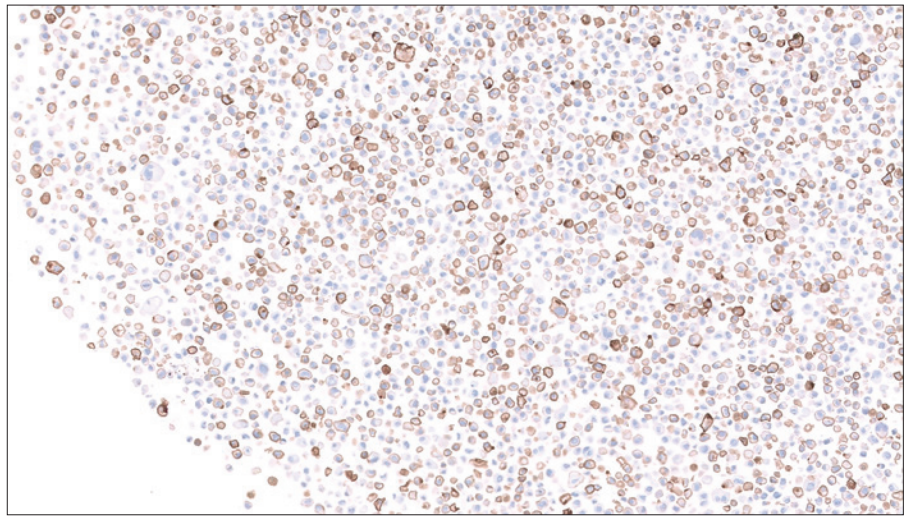
In Quadrant 2 approximately 75% of cells exhibit membrane staining, and the average staining intensity of all staining cells in this quadrant is  $\geq 2+$ .



**Figure 63:** NCI-H226 cell pellet (5× magnification).

### Quadrant 3

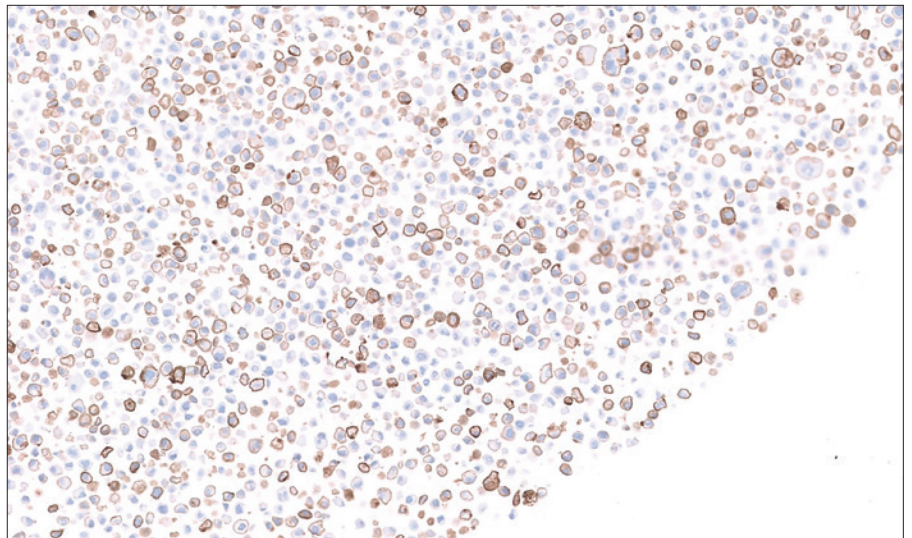
In Quadrant 3 approximately 70% of cells exhibit membrane staining, and the average staining intensity of all staining cells in this quadrant is  $\geq 2+$ .



**Figure 64:** NCI-H226 cell pellet (5× magnification).

### Quadrant 4

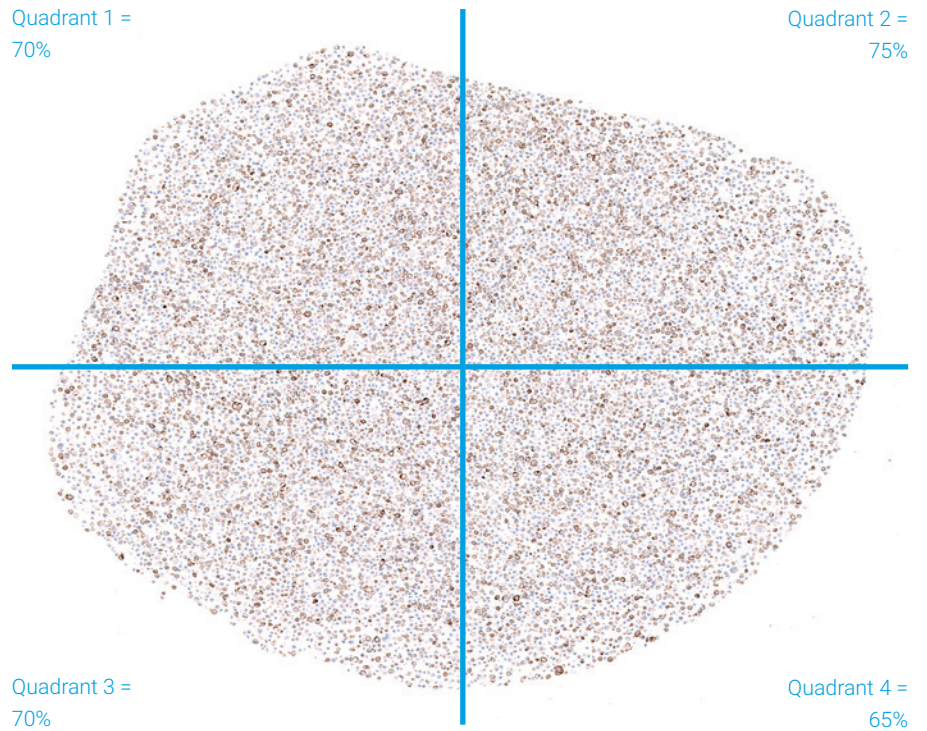
In Quadrant 4 approximately 65% of cells exhibit membrane staining, and the average staining intensity of all staining cells in this quadrant is  $\geq 2+$ .



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

### Calculation

1. Calculate the average percentage of cells exhibiting membrane staining across all 4 quadrants to estimate the total percentage of cells exhibiting membrane staining across the entire PD-L1 positive CCL pellet
2. Determine whether the average staining intensity across all cells 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 226 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.

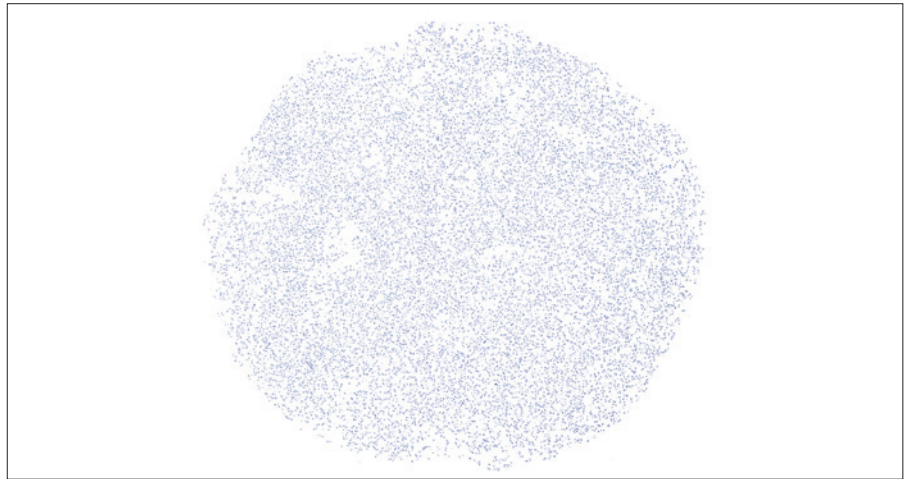


Figure 66: MCF-7 cell pellet (2x magnification).

#### 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+

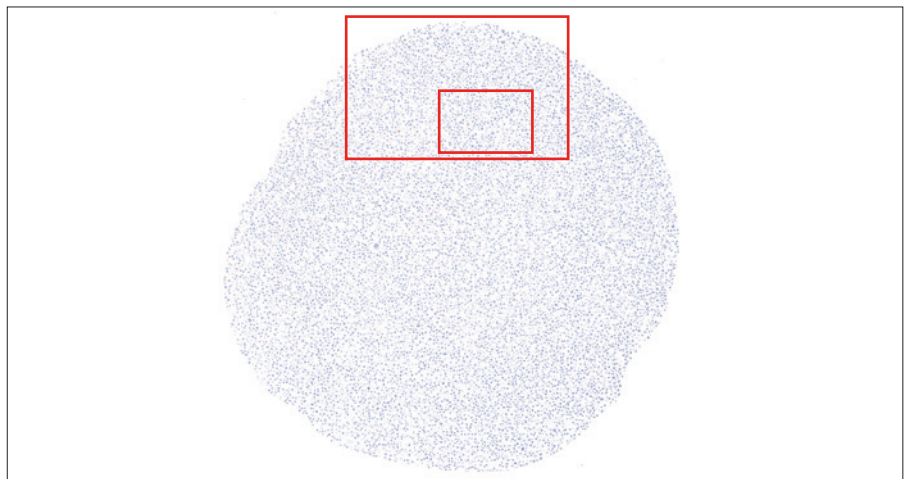


Figure 67: NCI-H226 cell pellet (2x magnification).

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

Failed PD-L1 positive CCL (10x)

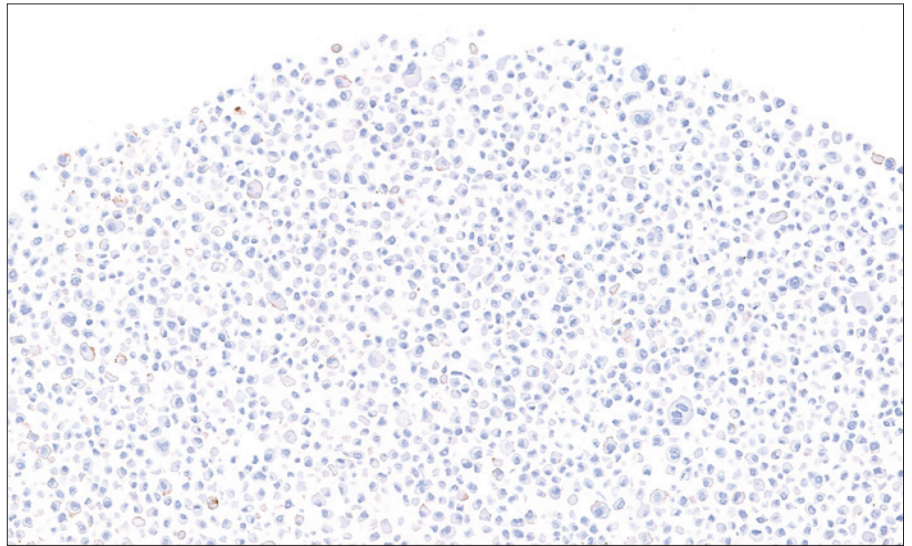


Figure 68: NCI-H226 cell pellet (10x magnification).

Failed PD-L1 positive CCL (20x)

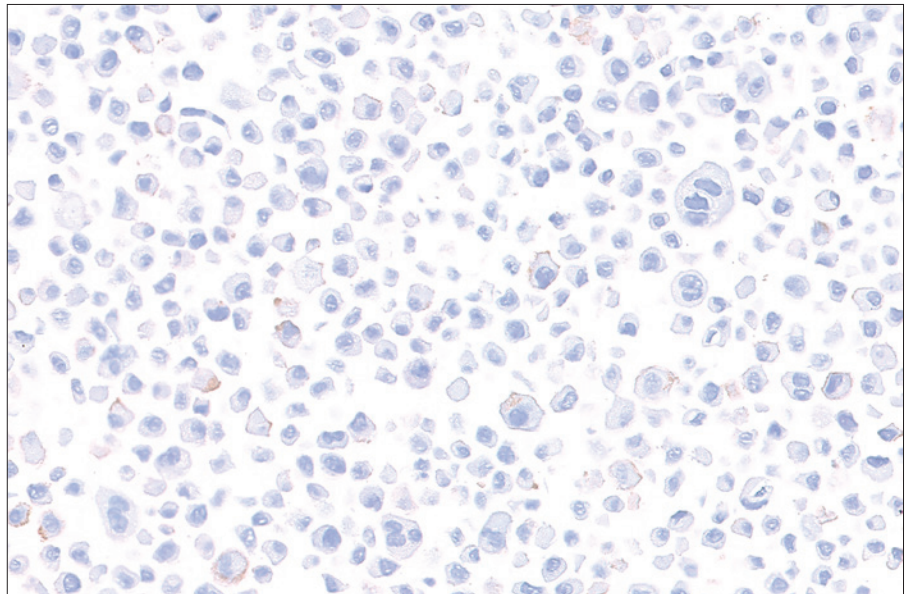


Figure 69: NCI-H226 cell pellet (20x magnification).

## 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.

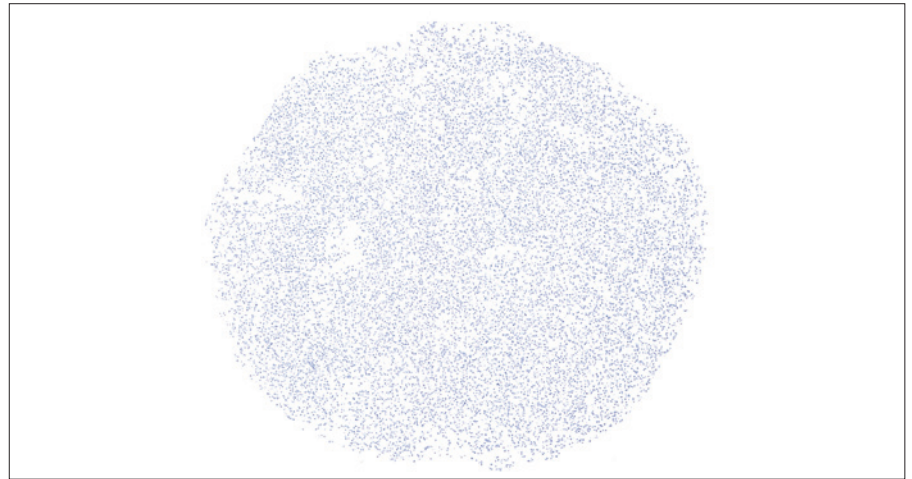


Figure 70: MCF-7 cell pellet (2x magnification).

### 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+

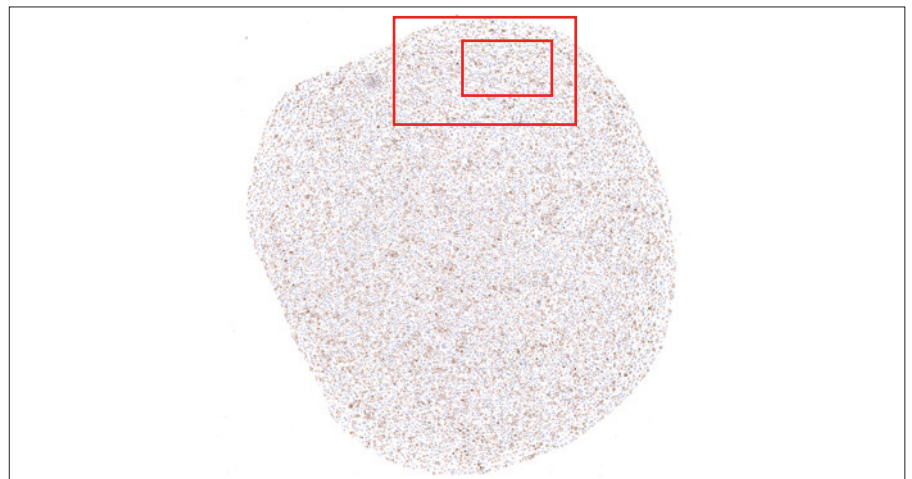


Figure 71: NCI-H226 cell pellet (2x magnification).

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

Failed PD-L1 positive CCL (10×)

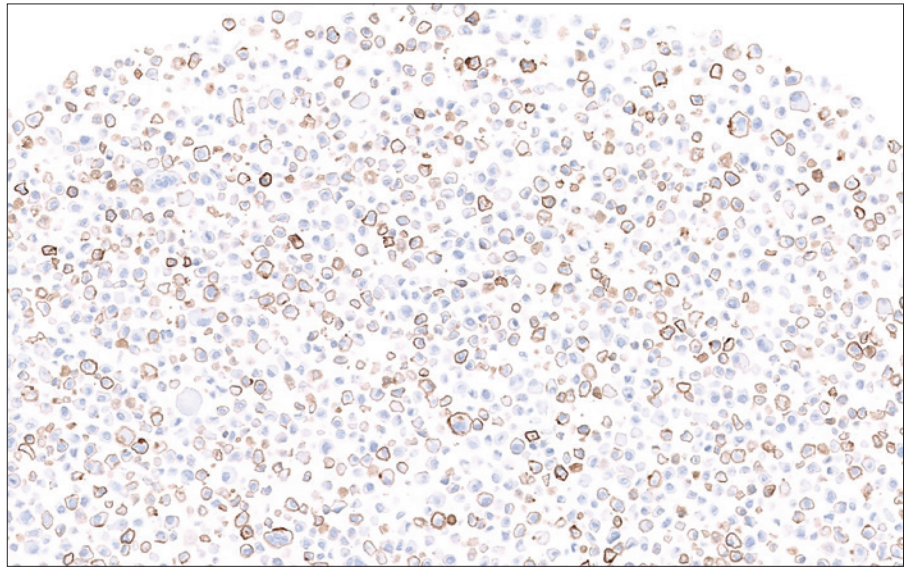


Figure 72: NCI-H226 cell pellet (10× magnification).

Failed PD-L1 positive CCL (20×)

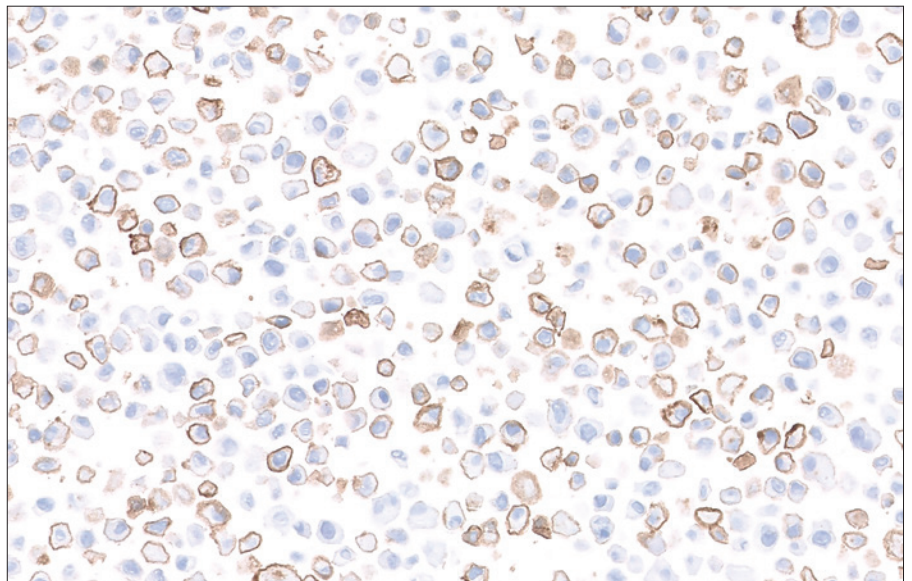


Figure 73: NCI-H226 cell pellet (20× magnification).

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

#### Passing PD-L1 positive CCL

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



Figure 74: 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 or nonspecific nuclear staining that is  $\geq 1+$  intensity

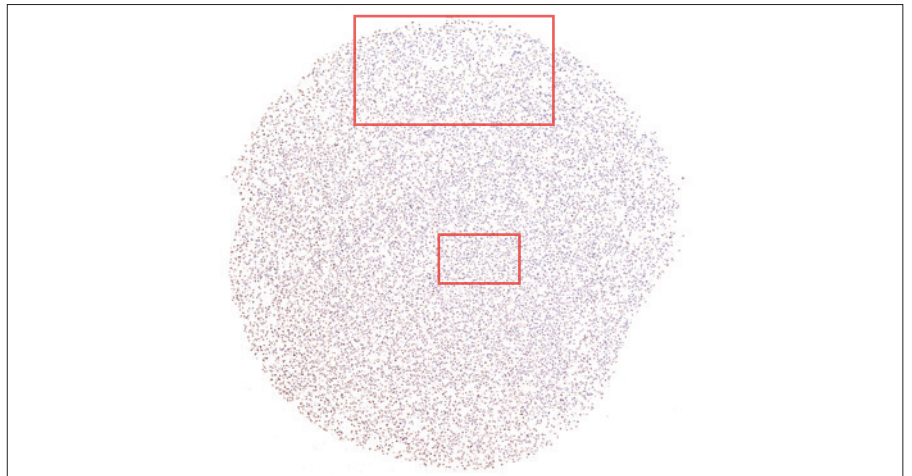


Figure 75: MCF-7 cell pellet (2x magnification).

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

Failed PD-L1 negative CCL (10x)

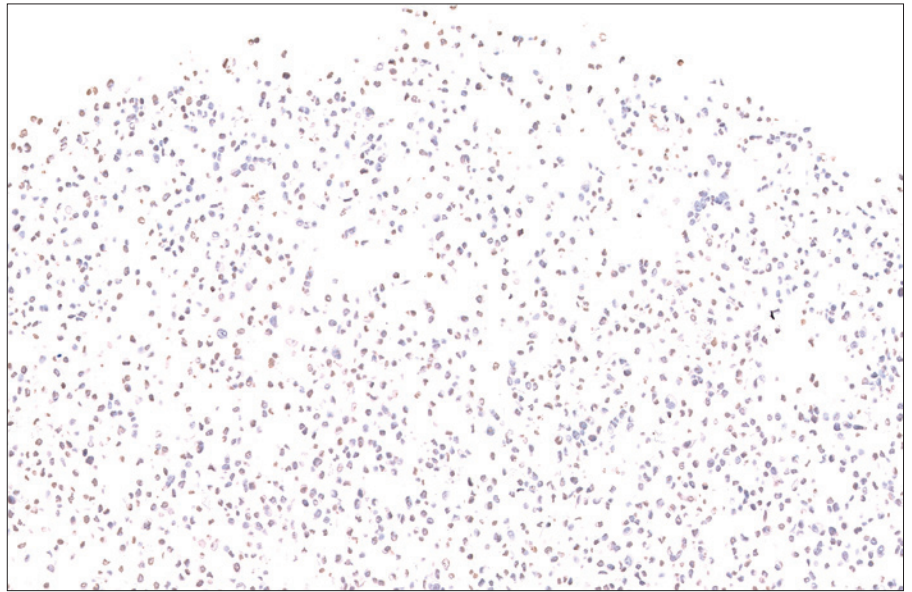


Figure 76: MCF-7 cell pellet (10x magnification).

Failed PD-L1 negative CCL (20x)

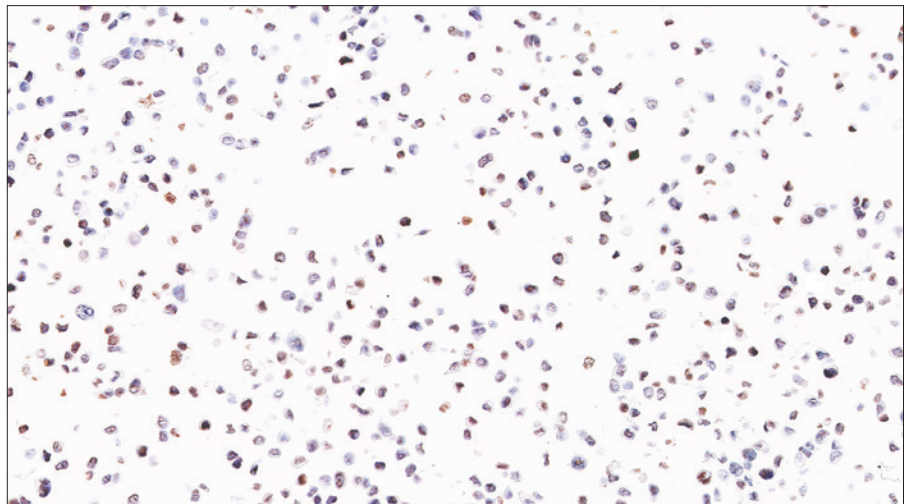


Figure 77: MCF-7 cell pellet (20x magnification).



# 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 program was selected for programming of slides
	Lack of reaction with DAB+ Substrate-Chromogen Solution (DAB)	Verify that DAB+ Substrate-Chromogen Solution was prepared properly
	Sodium azide in wash buffer	Use only Dako Wash Buffer (Code K8007)
	Degradation of Control Slide	Check kit expiration date and kit storage conditions on outside of package
Weak staining of specimen slides	Inappropriate fixation method used	Ensure that only neutral buffered formalin fixative and approved fixation methods are used
	Insufficient reagent volume applied	Check size of tissue section and reagent volume applied
	Inappropriate wash buffer used	Use only Dako Wash Buffer, Code K8007
Weak staining of specimen slides or of the positive cell line pellet on the Control Cell Line Slide provided with the kit	Inadequate target retrieval	Verify that the 3-in-1 pretreatment procedure was correctly performed
	Inappropriate wash buffer used	Use only Dako Wash Buffer, 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 Dako FLEX IHC Microscope Slides, (Code K8020), or Superfrost Plus slides
	Inadequate preparation of specimens	Cut sections should be placed in a 58 ± 2 °C oven for 1 hour prior to staining
Excessively strong specific staining	Inappropriate fixation method used	Ensure that only approved fixatives and fixation methods are used
	Inappropriate wash buffer used	Only use Dako Wash Buffer, Code K8007
Target Retrieval Solution is cloudy in appearance when heated	When heated, the Target Retrieval Solution turns cloudy in appearance	This is normal and does not influence staining

Continued on next page



Problem	Probable Cause	Suggested Action
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 1x Envision FLEX Wash Buffer after Deparaffinization, Rehydration, and Target Retrieval (3-in-1) procedure and after loading on the Autostainer Link 48. Ensure that the Autostainer Link 48 lid is properly closed to prevent reagent evaporation during the staining procedure.
	Improper manual slide rinsing with 1x 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 1x EnVision FLEX Wash Buffer should be manually applied to the slides using a wash bottle. Ensure slides remain wet prior to initiating the Autostainer Link 48 procedure.

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

## Clinical Performance Evaluation

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

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

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

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

Efficacy results are summarized in Table 5.

**Table 5: Efficacy Results in Cohort E of KEYNOTE-158 (CPS  $\geq$  1, n=77\*)**

Objective Response Rate	
ORR (95% CI)	14.3% (7.4, 24.1)
Complete response rate	2.6%
Partial response rate	11.7%
Response Duration	
Median in months (range)	NR (4.1, 18.6 <sup>†</sup> )
% with duration $\geq$ 6 months	91%

\* Median follow-up time of 11.7 months (range 0.6 to 22.7 months)

<sup>†</sup> Based on patients (n=11) with a response by independent review

<sup>‡</sup> Denotes ongoing

NR = Not reached

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