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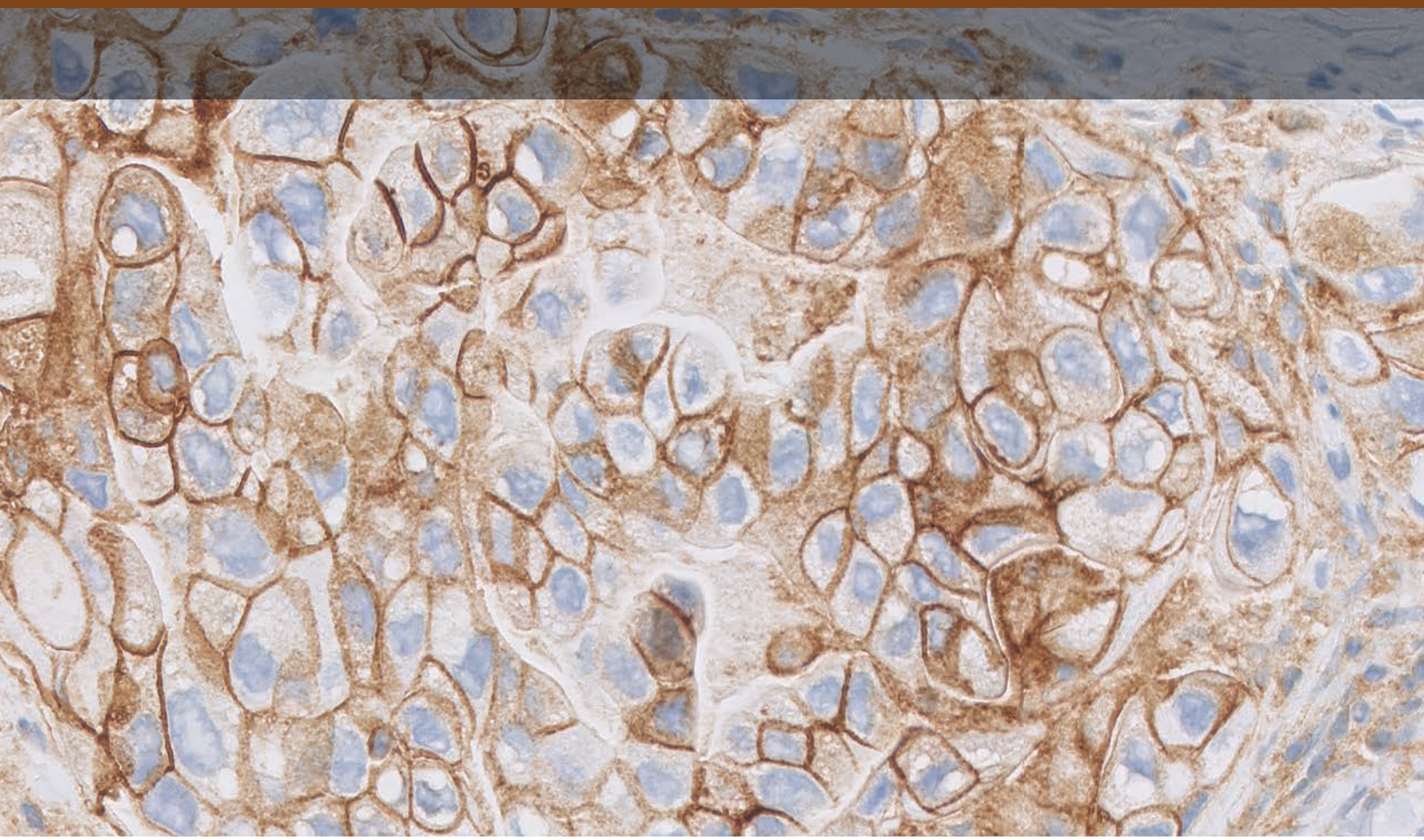
PD-L1 IHC 22C3 pharmDx Interpretation Manual – Urothelial Carcinoma

FDA-approved for in vitro diagnostic use



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Trusted Answers

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

Table of Contents

Intended Use

For in vitro diagnostic use.

PD-L1 IHC 22C3 pharmDx is a qualitative immunohistochemical assay using Monoclonal Mouse Anti-PD-L1, Clone 22C3 intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) non-small cell lung cancer (NSCLC), gastric or gastroesophageal junction (GEJ) adenocarcinoma, cervical cancer and urothelial carcinoma tissues using EnVision FLEX visualization system on Autostainer Link 48.

Non-Small Cell Lung Cancer (NSCLC)

PD-L1 protein expression in NSCLC is determined by using Tumor Proportion Score (TPS), which is the percentage of viable tumor cells showing partial or complete membrane staining at any intensity. The specimen should be considered to have PD-L1 expression if TPS ≥ 1% and high PD-L1 expression if TPS ≥ 50%.

PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying NSCLC patients for treatment with KEYTRUDA® (pembrolizumab). See the KEYTRUDA® product label for expression cutoff values guiding therapy in specific clinical circumstances.

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 ≥ 1.

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

Cervical Cancer

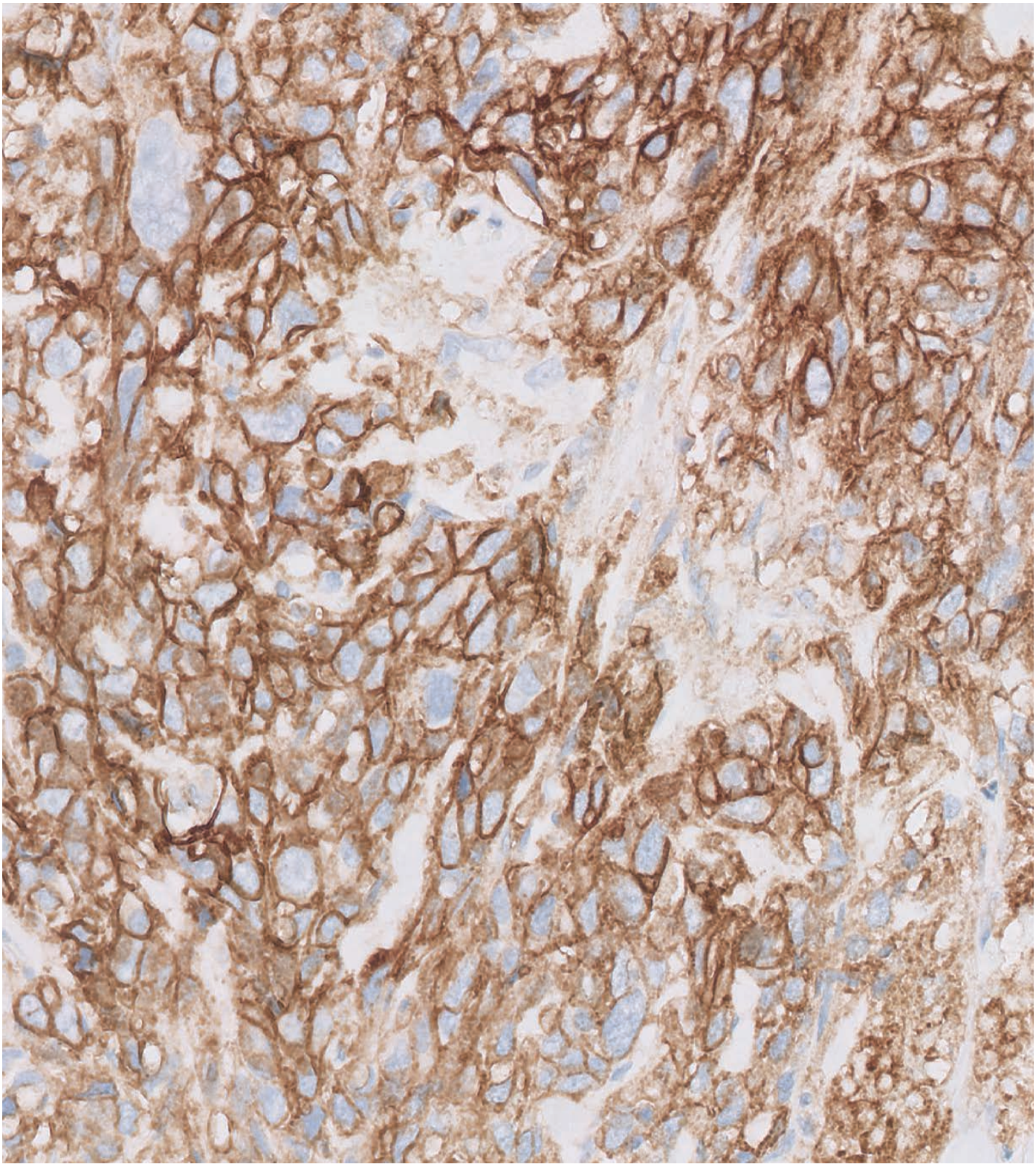
PD-L1 protein expression in cervical cancer is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. The specimen should be considered to have PD-L1 expression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

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

Urothelial Carcinoma

PD-L1 protein expression in urothelial carcinoma 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 ≥ 10.

PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying urothelial carcinoma patients for treatment with KEYTRUDA® (pembrolizumab). See the KEYTRUDA® product label for specific clinical circumstances guiding PD-L1 testing.



Introduction

PD-L1 IHC 22C3 pharmDx is the only companion diagnostic approved by the FDA as an aid in identifying patients with urothelial carcinoma 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 urothelial carcinoma 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 urothelial carcinoma 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 urothelial carcinoma specimens stained with PD-L1 IHC 22C3 pharmDx can help individual labs achieve reproducible and reliable results.

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

Urothelial carcinoma tissue specimens that are tested for PD-L1 expression are scored and divided into two groups based on their Combined Positive Score (CPS):

- CPS < 10
- CPS ≥ 10

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

Assay Interpretation

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

Reporting Results

To help understand what information should be reported to the treating physician, please refer to the Reporting Results section of this manual on page 33.

Photomicrographs

The included photomicrographs are of urothelial carcinoma 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 Asterand Bioscience.

Tissue samples were provided by the Cooperative Human Tissue Network which is funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects.

Data and tissue used in this project were provided by Tumorgenetics Ltd., Budapest, Hungary with appropriate ethics approval and through Trans-Hit Biomarkers Inc.

PD-L1 Overview

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

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

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

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

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

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

PD-L1 IHC 22C3 pharmDx Detects PD-L1 in Urothelial Carcinoma Specimens

Detection of PD-L1 upregulation in urothelial carcinoma 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-052) to evaluate the relationship between PD-L1 expression and clinical efficacy. KEYTRUDA is a humanized monoclonal PD-1-blocking antibody.

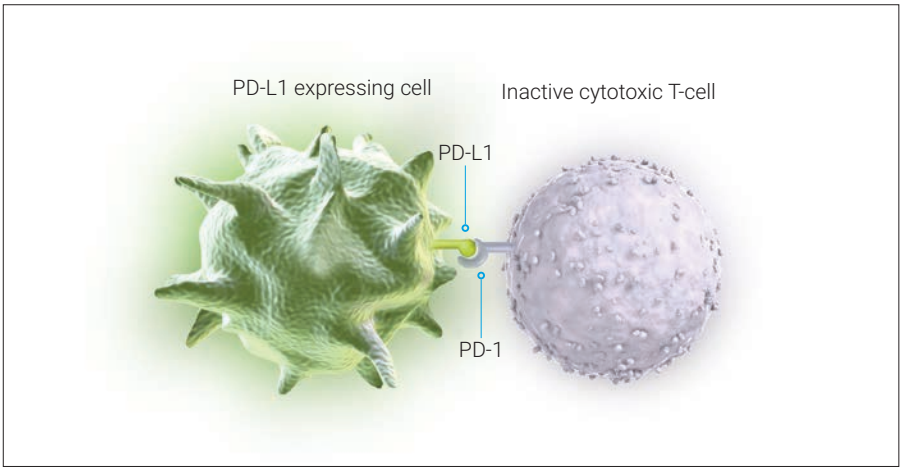


Figure 1: Inactivation of T-cells limits damage to normal tissue.

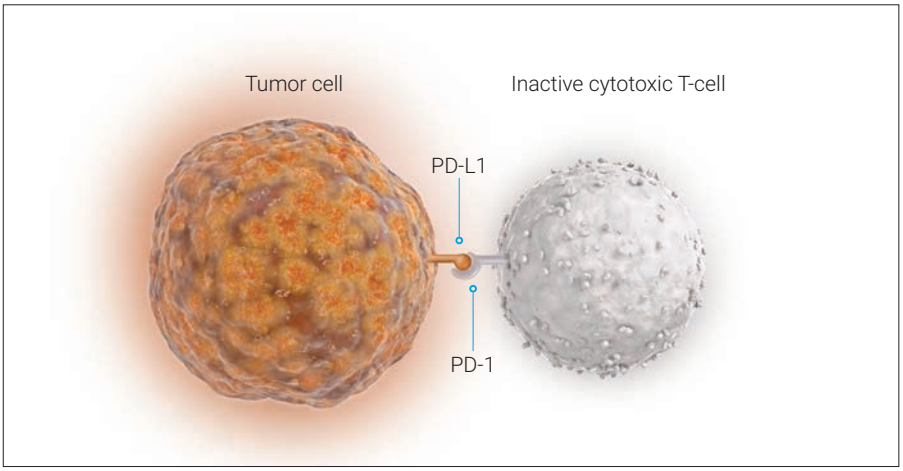


Figure 2: Inactivation of T-cells reduces tumor cell death and elimination.

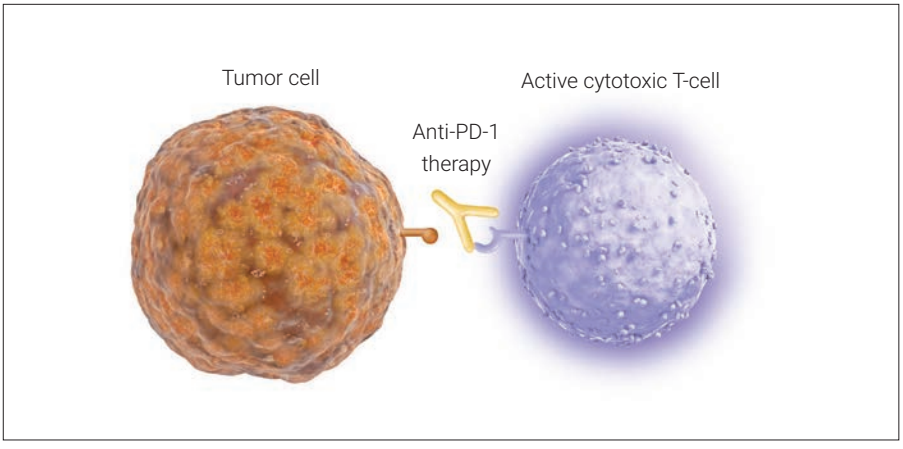


Figure 3: Blocking the PD-1/PD-L1 interaction helps to enable active T-cells and tumor cell death and elimination.

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 urothelial carcinoma 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) urothelial carcinoma 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 of diagnostic quality.

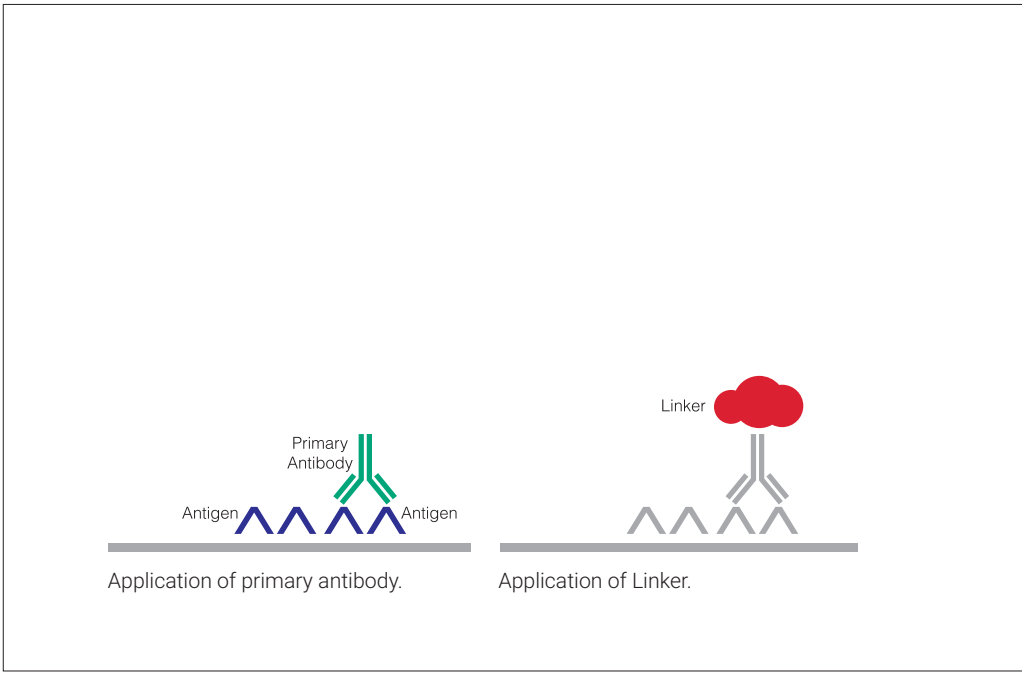


Figure 4: PD-L1 IHC 22C3 pharmDx staining procedure.

Kit Configuration

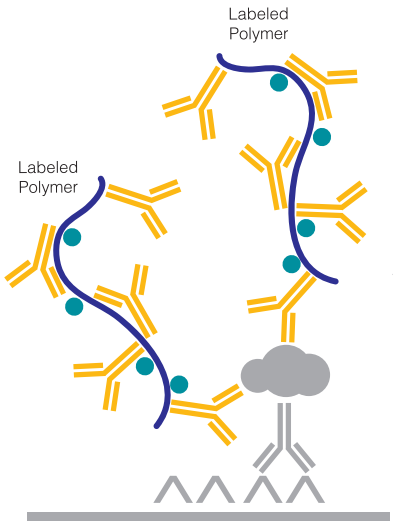
Figure 5: PD-L1 IHC 22C3 pharmDx components.

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

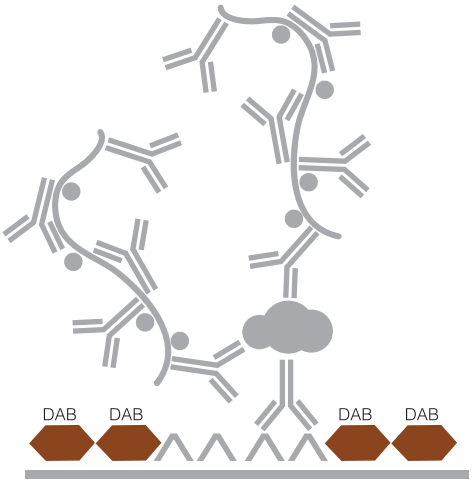
1. EnVision FLEX Target Retrieval Solution, Low pH (50×)
2. Peroxidase-Blocking Reagent
3. Primary antibody: Monoclonal Mouse Anti-PD-L1, Clone 22C3
4. Negative Control Reagent
5. Mouse LINKER
6. Visualization Reagent-HRP
7. DAB+ Substrate Buffer
8. DAB+ Chromogen
9. DAB Enhancer
10. PD-L1 IHC 22C3 pharmDx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

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

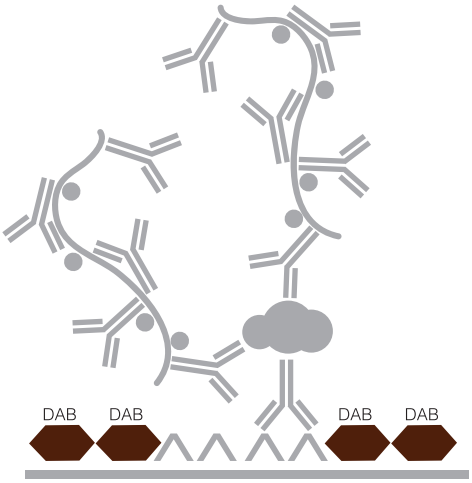
** Dr. AF Gazdar and Dr. JD Minna at NIH are acknowledged for their contribution in developing NCI-H226 (ATCC Number: CRL-5826™)*



Application of Visualization Reagent.



Application of DAB+ Substrate Chromogen Solution.



Application of DAB Enhancer.

Technical Considerations

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

Specimen Preparation

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

In-house Control Tissue

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

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

The ideal positive control tissue provides a complete dynamic representation of weak-to-moderate staining of tumor cells and tumor-associated mononuclear inflammatory cells (MICs: lymphocytes and macrophages). The negative control tissue should demonstrate no staining in tumor cells and only a few staining immune cells.

Optional Additional In-house Control: Tonsil Tissue

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

DAB+ Substrate-Chromogen Solution

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

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

Controls to Assess Staining Quality

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

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

Deparaffinization, Rehydration, and Target Retrieval

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

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

Staining and Counterstaining

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

Mounting

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

Technical Checklist

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

Customer Name/Institution _____

Name and Title _____

Autostainer Link 48 Serial Number _____ Software Version _____

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

Additional observations or comments:

_____

_____

Slide Evaluation

General Considerations

PD-L1 IHC 22C3 pharmDx evaluation should be performed by a qualified pathologist using a light microscope of diagnostic quality. 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. In-house control tissue slides should also be assessed with every IHC run.

Specimen Adequacy

Confirm the Presence of at Least 100 Viable Tumor Cells

A hematoxylin and eosin (H&E) 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.

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

Instructions for Patient Specimens With Less Than 100 Viable Tumor Cells

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

Evaluating Controls

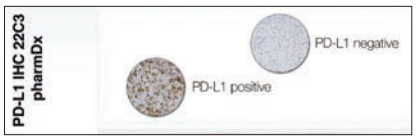


Figure 6: Each Control Cell Line Slide contains sections of cell pellets with positive and negative PD-L1 expression.

PD-L1 IHC 22C3 pharmDx Control Cell Line Slide

Examine the PD-L1 IHC 22C3 pharmDx Control Cell Line Slide to determine that reagents are functioning properly. Each slide contains sections of cell pellets with positive and negative PD-L1 expression (Figure 6). Assess the percentage of positive cells 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 ≥ 70% of cells
- ≥ 2+ average staining intensity
- Non-specific staining < 1+ intensity

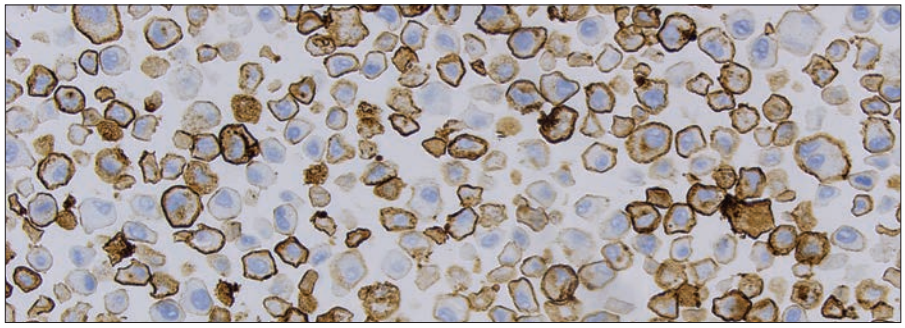


Figure 7: Positive cell pellet with acceptable staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20× magnification).

Negative Control Cell Pellet

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

- The majority of cells should demonstrate no staining. **Note:** The presence of 10 or fewer cells with distinct cell membrane staining is acceptable
- Any background staining is less than 1+ staining intensity

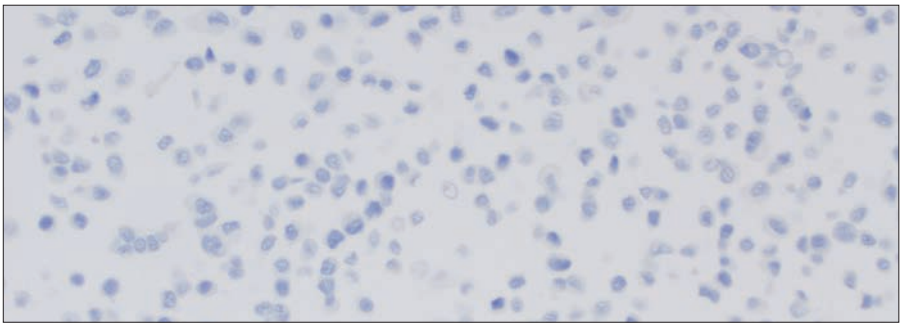


Figure 8: Negative cell pellet with no staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20× magnification).

Positive and Negative In-house Control Tissue (Urothelial Carcinoma)

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

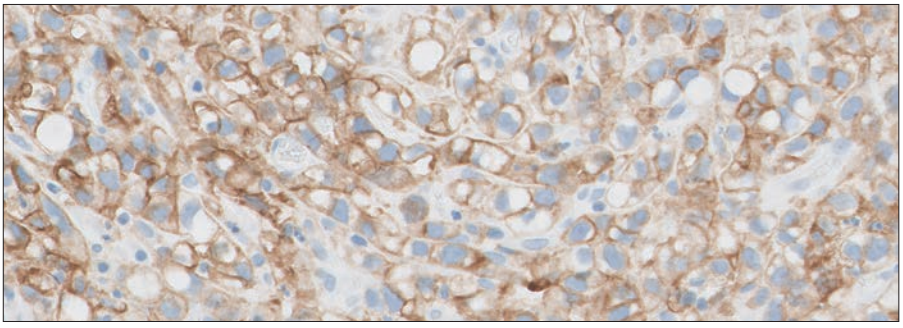


Figure 9: Ideal positive in-house control tissue (20× magnification).

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, staining immune cells are acceptable. Examine the negative in-house control tissue to determine the expected staining. The variety of different cell types present in most tissue sections offers internal negative control sites; this should be verified by the user.

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

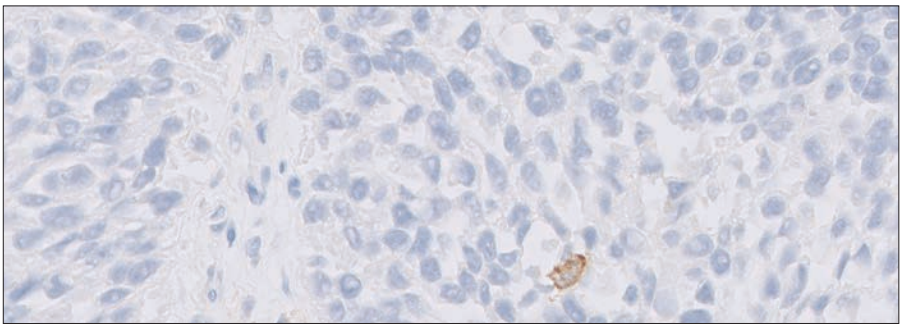


Figure 10: Ideal negative in-house control tissue demonstrating PD-L1 positive expression of CPS < 1 (20× magnification).

Optional Control Tissue

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

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

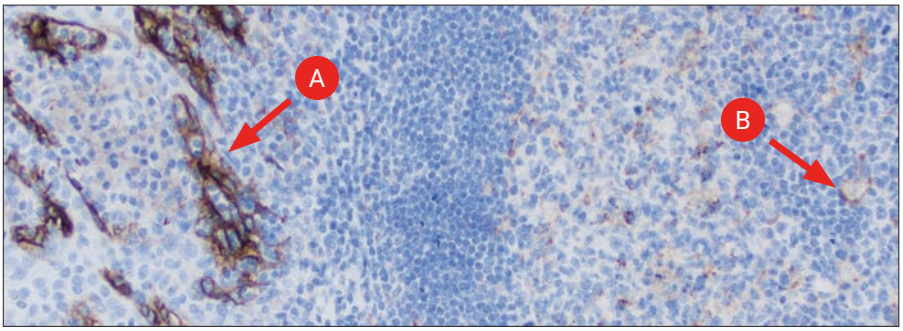


Figure 11: Tonsil stained with PD-L1 primary antibody exhibiting strong membrane staining in portions of the crypt epithelium (A) and weak-to-moderate membrane staining of follicular macrophages in the germinal centers (B) (10× magnification).

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

Negative Control Reagent (NCR)

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

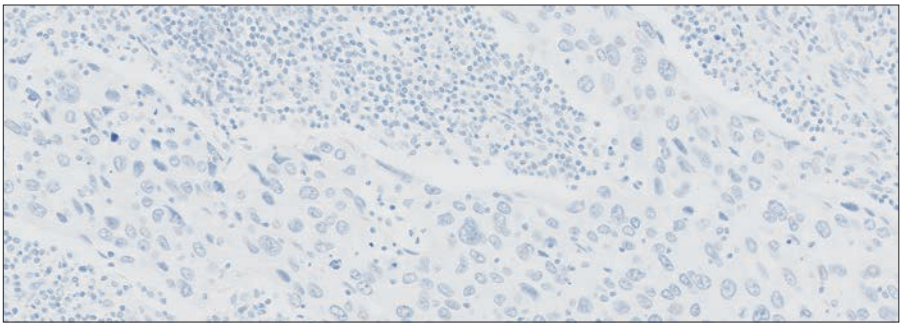


Figure 12: Ideal negative in-house control tissue stained with Negative Control Reagent showing no specific staining (20× magnification).

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

Slide Evaluation Flowchart

Figure 13: Recommended order of slide evaluation.

Combined Positive Score

Definition of Combined Positive Score (CPS)

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

See Table 1 on page 26 for additional CPS numerator inclusion/ exclusion criteria.

Determining Combined Positive Score

- At lower magnifications (4×, 10×), examine all well-preserved tumor areas. Evaluate overall areas of PD-L1 staining and non-staining tumor cells, keeping in mind that partial membrane staining or 1+ membrane staining may be difficult to see at low magnifications. Ensure there are at least 100 viable tumor cells in the sample
  - A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide (biopsy and resection) for the specimen to be considered adequate for evaluation
- For specimens with less than 100 viable tumor cells, tissue from a deeper level of the block or potentially another block could have a sufficient number of tumor cells for evaluation of PD-L1 expression
- At higher magnification (20×), evaluate PD-L1 expression and calculate CPS:
  - Determine the total number of viable tumor cells, both PD-L1 staining and non-staining (CPS denominator)
  - Determine the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) (CPS numerator; see Table 1 on page 26 for additional CPS numerator inclusion/exclusion criteria)
  - Calculate CPS
- Evaluation of membrane staining should be performed at no higher than 20× magnification. Slide reviewer should not perform the CPS calculation at 40× magnification

Table 1: CPS numerator inclusion/exclusion criteria

Tissue Elements	Included in the Numerator	Excluded from the Numerator
Tumor Cells	Convincing partial or complete linear membrane staining (at any intensity) of viable urothelial carcinoma tumor cells including: – High grade papillary carcinoma – Carcinoma in situ (CIS) – Any lamina propria, muscularis, or serosal invasion – Metastatic carcinoma	– Non-staining tumor cells – Tumor cells with only cytoplasmic staining – Low grade papillary carcinoma§
Immune Cells	Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma†: – Lymphocytes (including lymphocyte aggregates) – Macrophages‡ Only MICs directly associated with the response to the tumor are scored	– Non-staining MICs – MICs (including lymphoid aggregates) associated with ulcers, chronic cystitis, and other processes not associated with the tumor – MICs associated with normal structures – Neutrophils, eosinophils, and plasma cells – BCG**-induced granulomas
Other Cells	Not included	– Normal cells – Stromal cells (including fibroblasts) – Necrotic cells and/or cellular debris

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

Table 2: CPS denominator inclusion/exclusion criteria

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

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

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.

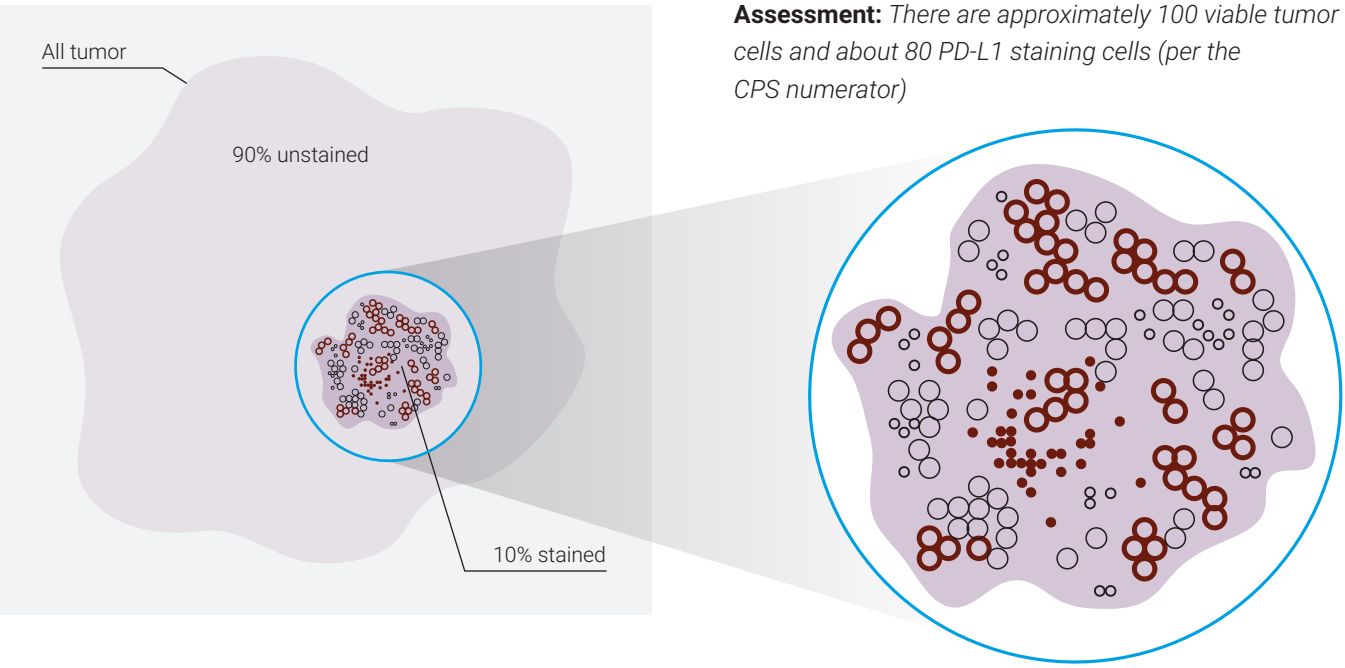
Example 1: Calculation of Combined Positive Score in a Small Tumor Area With Staining

At lower magnifications (4×, 10×): 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 (20×): 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 non-staining 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% × 80 = ~CPS 8

Clinical Interpretation: CPS < 10

** Including tumor cells, lymphocytes, macrophages*

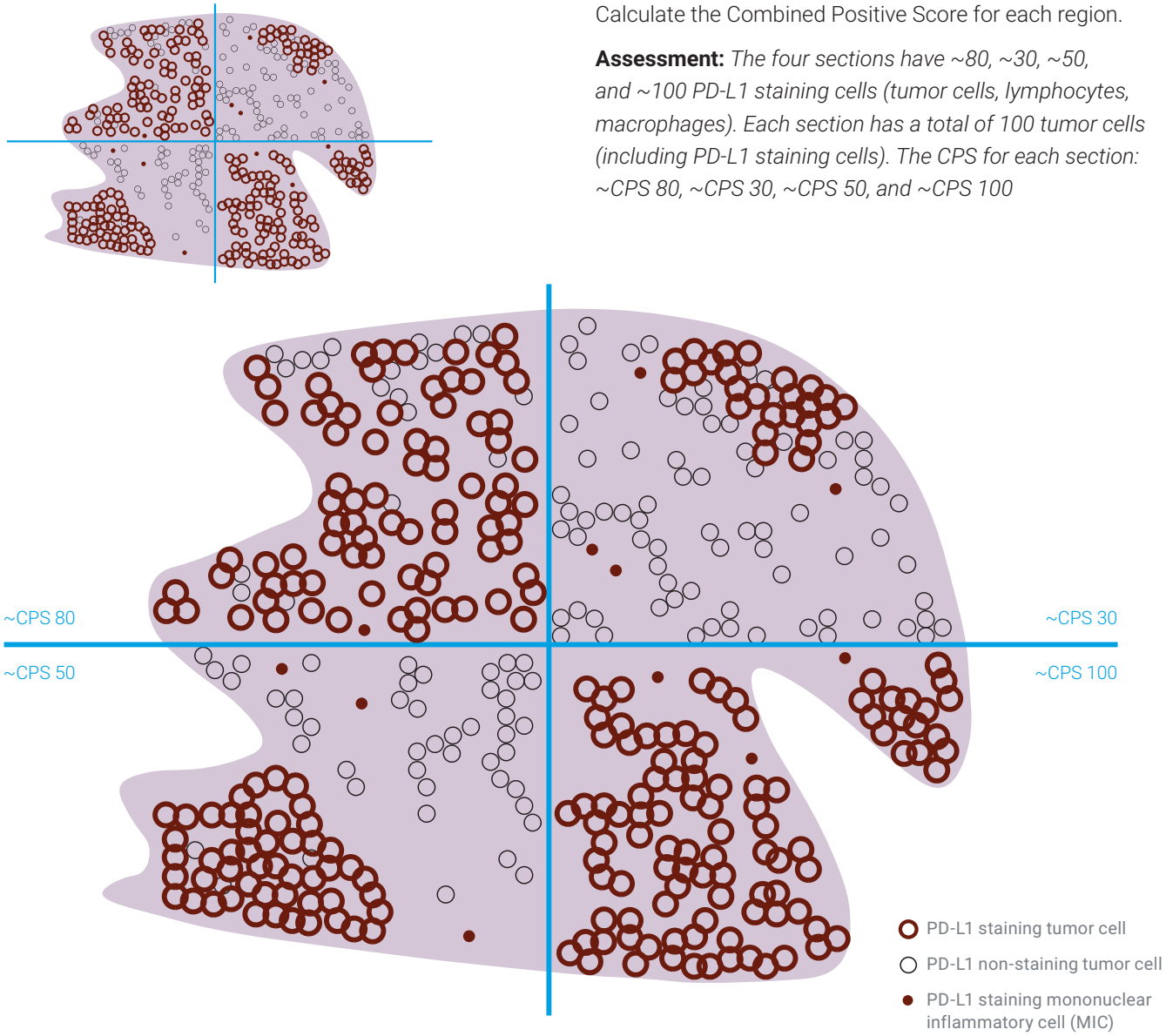
Figure 14: Example of tumor with small staining area.

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

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

At higher magnification (20×): Observe each region and estimate the total number of viable tumor cells and PD-L1 staining cells (tumor cells, lymphocytes, macrophages). Calculate the Combined Positive Score for each region.

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



Calculate the Combined Positive Score of the entire tumor area:

Assessment:

Combined Positive Score:

(80 + 30 + 50 + 100) / 4 = ~CPS 65

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

Clinical Interpretation: CPS ≥ 10

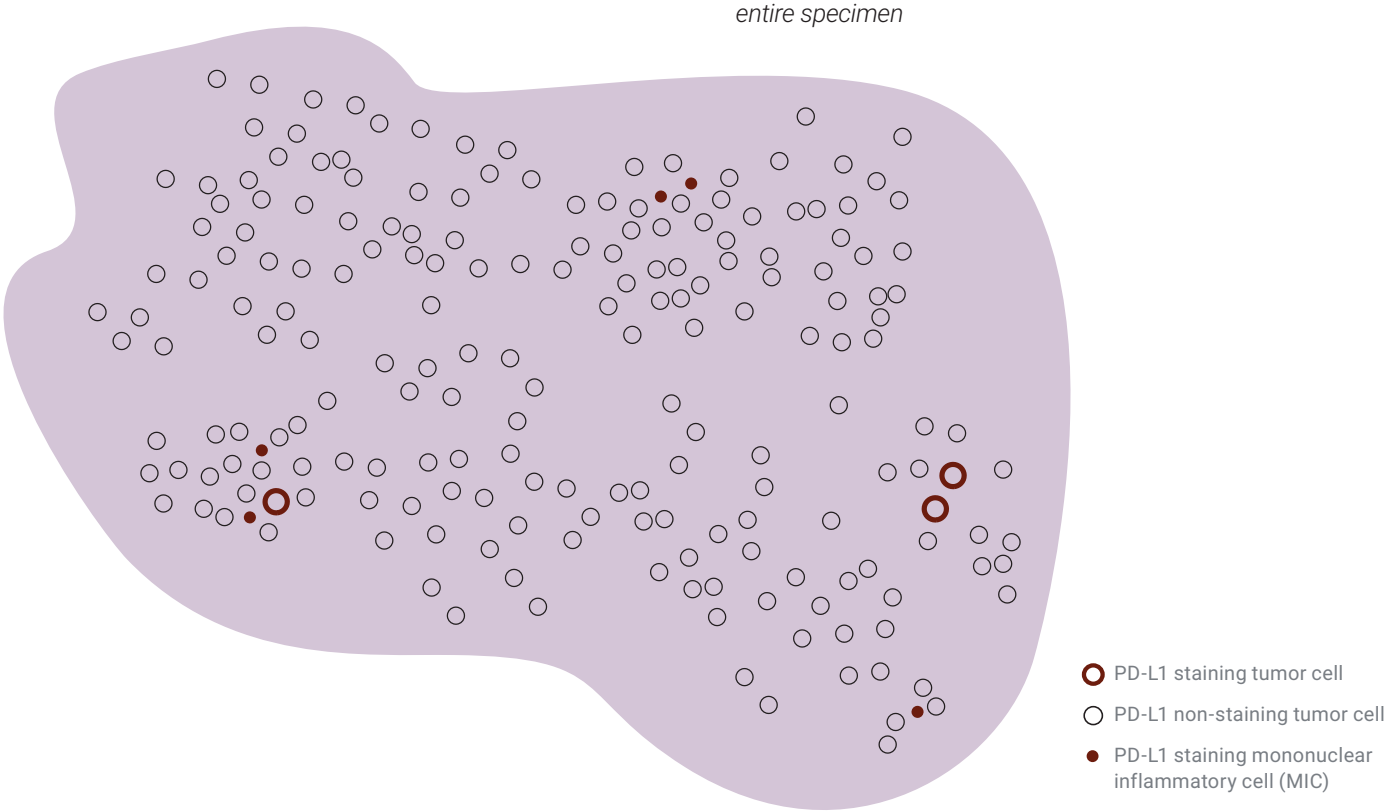
Figure 15: Example with heterogeneous tumor area.

Example 3: Calculation of Combined Positive Score for a Near Cut-off Specimen (CPS 1–20)

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

At higher magnification (20×): Confirm that there is no staining in areas that appeared void of staining at lower magnifications. Evaluate all staining areas and estimate the total number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages). Then re-evaluate the entire specimen (staining and non-staining areas) and estimate the total number of viable tumor cells (PD-L1 staining and non-staining tumor cells). Calculate the Combined Positive Score.

Assessment: *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:

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

Clinical Interpretation: CPS < 10

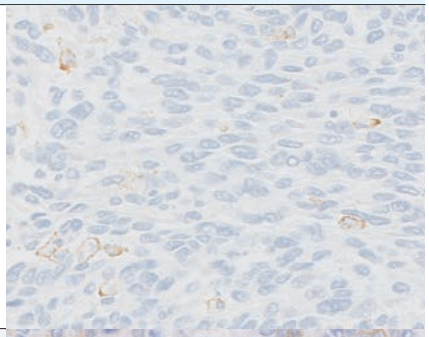
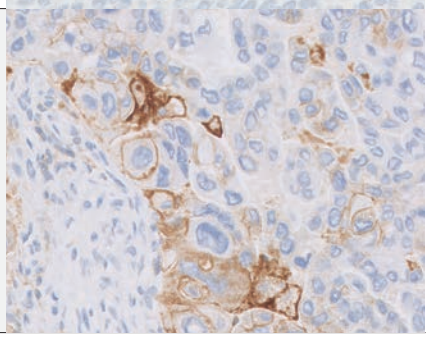
** Including tumor cells, lymphocytes, macrophages*

Figure 16: Example of near cut-off specimen (CPS 1–20).

Interpretation of CPS

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

Table 3: CPS and PD-L1 expression

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

Identifying Patients With Urothelial Carcinoma for Treatment

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

Clinical Validation of PD-L1 IHC 22C3 pharmDx in Locally Advanced or Metastatic Urothelial Carcinoma Patients Not Eligible for Cisplatin-containing Chemotherapy

The clinical validity of PD-L1 IHC 22C3 pharmDx in evaluating PD-L1 expression in patients not eligible for cisplatin-containing chemotherapy with locally advanced or metastatic urothelial carcinoma is based on the KEYTRUDA KEYNOTE-052 study sponsored by Merck Sharp & Dohme Corp. Specimens from patients were tested for PD-L1 expression using PD-L1 IHC 22C3 pharmDx. Thirty percent of enrolled patients had tumors that expressed PD-L1 with a Combined Positive Score (CPS) of greater than or equal to 10 (CPS ≥ 10).

Clinical efficacy of KEYTRUDA treatment is presented in the Clinical Performance Evaluation section on pages 72–73.

Table 4: PD-L1 prevalence in patients with urothelial carcinoma enrolled in KEYNOTE-052*

PD-L1 Expression	CPS < 10	CPS ≥ 10
Prevalence (n)†	69.5% (251)	30.5% (110)

** Based on all enrolled subjects into KEYNOTE-052 (n=370)*
† 9 patients had unknown PD-L1 status

PD-L1 IHC 22C3 pharmDx Testing Scheme

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

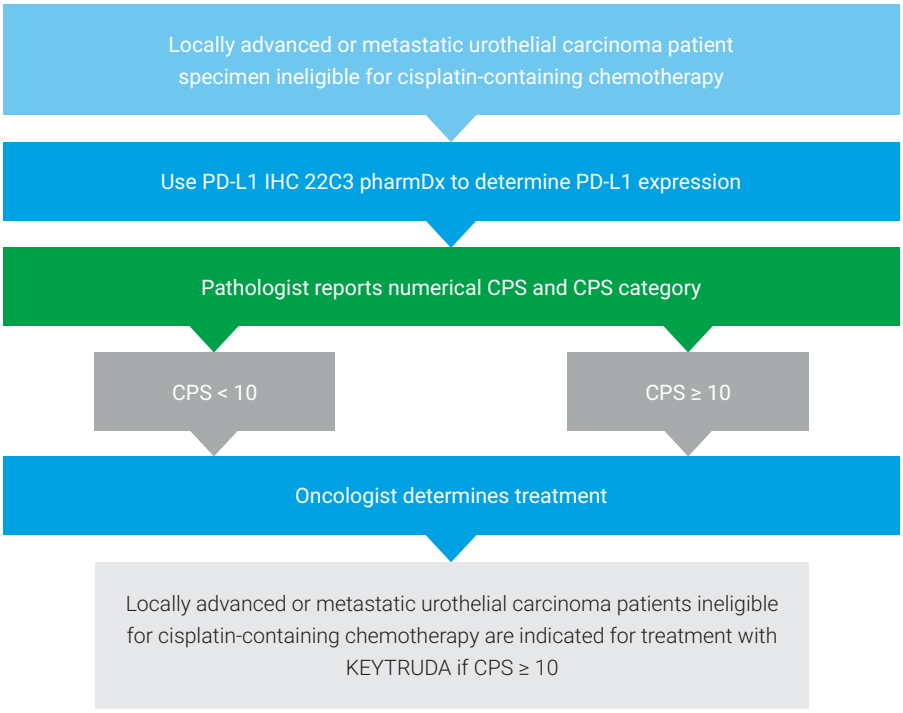


Figure 17: Testing algorithm for PD-L1 IHC 22C3 pharmDx.

Reporting Results

Suggested information to include when reporting results for urothelial carcinoma 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 Urothelial Carcinoma Comprehensive Panel: Yes: ☐ No: ☐

PD-L1 Testing Results

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

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

PD-L1 IHC 22C3 pharmDx Result to Treating Physician

Combined Positive Score: _____

CPS ≥ 10: ☐ CPS < 10: ☐

Other Comments to Treating Physician:

_____

_____

_____

Combined Positive Score Summary and Examples

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

By definition, PD-L1 staining cells in urothelial carcinoma 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 immediately adjacent supporting stroma with convincing membrane and/or cytoplasmic staining (at any intensity). Mononuclear inflammatory cells (MICs) must be directly associated with the response against the tumor

PD-L1 expression status in urothelial carcinoma 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 urothelial carcinoma unless otherwise noted in the figure caption.

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

PD-L1 Staining Cells Included in the CPS Numerator

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 Table 1 on page 26 for additional CPS numerator inclusion/exclusion criteria). Below are common staining characteristics of PD-L1 staining cells that must be included in the CPS numerator. All images are urothelial carcinoma unless otherwise noted in the figure caption.

Linear Membrane Staining: Tumor Cells

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

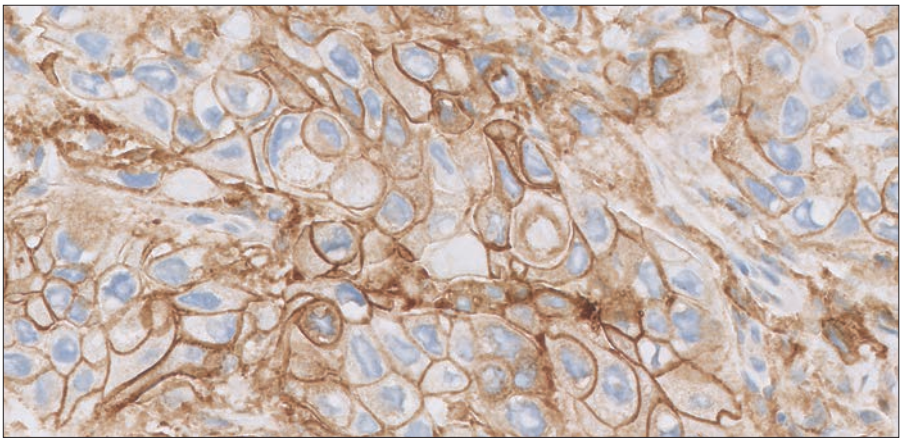


Figure 18: PD-L1 primary antibody exhibiting linear membrane staining of tumor cells (20× magnification).

Key point

Convincing linear membrane staining of tumor cells should be included in the CPS numerator

Partial and Complete Linear Membrane Staining: Tumor Cells

Tumor cells commonly exhibit partial and/or complete linear membrane staining. Any partial or complete linear membrane staining observed at any intensity and convincing at no higher than 20× magnification must be included in the CPS numerator.

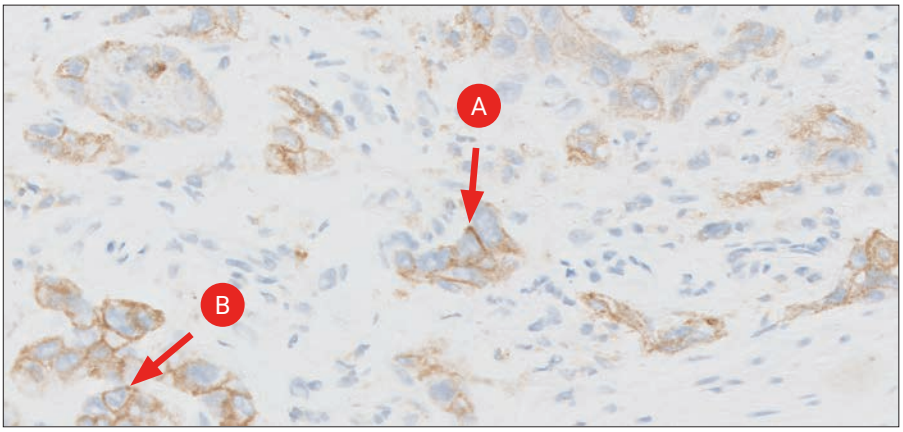


Figure 19: PD-L1 primary antibody exhibiting partial (A) and complete (B) linear membrane staining of tumor cells (20× magnification).

Key point

Convincing partial and/or complete membrane staining of tumor cells should be included in the CPS numerator

Weak Linear Membrane Staining: Tumor Cells

Tumor cells must exhibit convincing membrane staining at any intensity, including weak 1+ intensity, at no higher than 20× magnification.

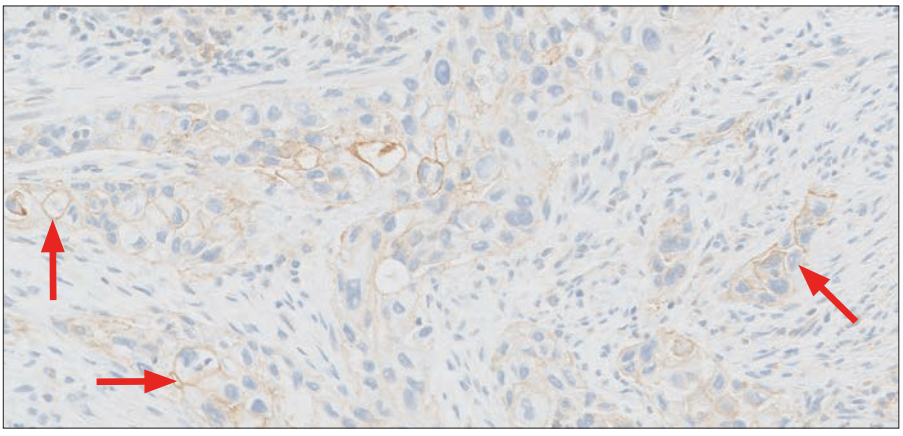


Figure 20: PD-L1 primary antibody exhibiting weak but perceptible and convincing membrane staining of tumor cells (arrows) (20× magnification).

Key point

Weak 1+ convincing membrane staining of tumor cells should be included in the CPS numerator

Linear Membrane and Cytoplasmic Staining: Tumor Cells

Tumor cells with both convincing linear membrane staining (≥ 1+ intensity) and cytoplasmic staining at no higher than 20× magnification should be included in the CPS numerator.

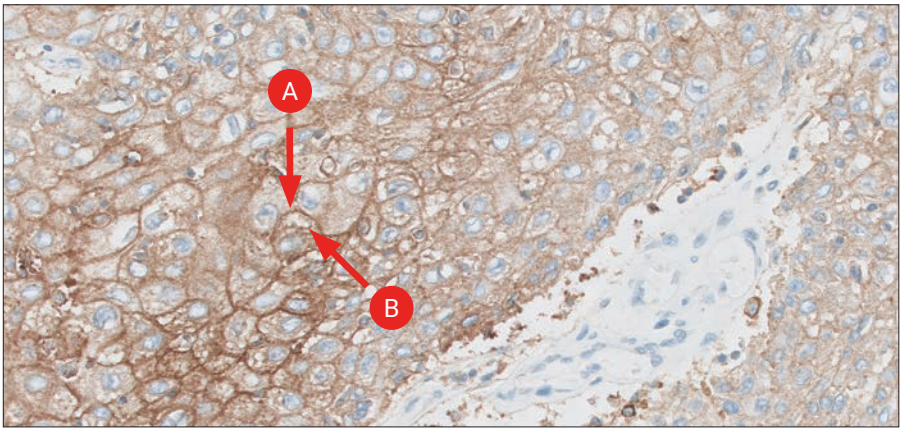


Figure 21: PD-L1 primary antibody exhibiting linear membrane (A) staining distinct from cytoplasmic (B) staining (20× magnification).

Key point

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

Membrane and Cytoplasmic Staining: Tumor-associated Lymphocytes and Macrophages

Tumor-associated lymphocytes and macrophages (mononuclear inflammatory cells, MICs) exhibiting convincing membrane and/or cytoplasmic staining at no higher than 20× magnification (≥ 1+ intensity) are considered PD-L1 staining cells and should be included in the CPS numerator. Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor nests and/or immediately adjacent supporting stroma, and are directly associated with the response against the tumor.

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 mononuclear inflammatory cells (MICs) should be included in the CPS numerator.

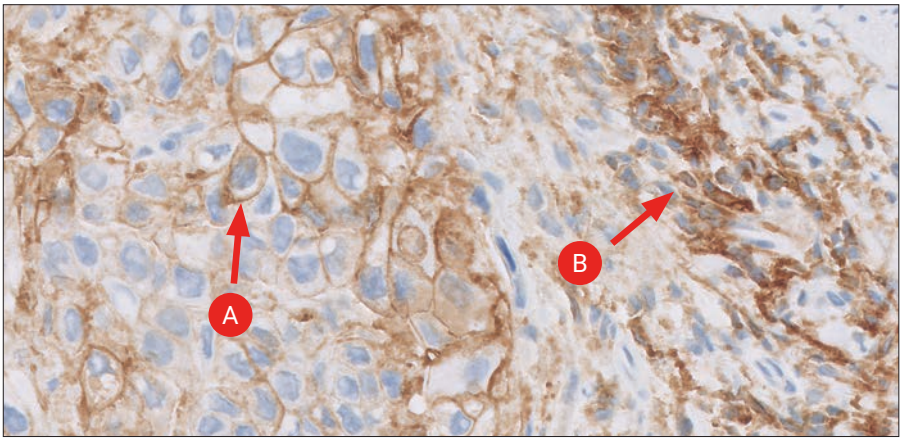


Figure 22a: PD-L1 primary antibody exhibiting staining of tumor (A) and tumor-associated mononuclear inflammatory cells (MICs) (B) (20× magnification).

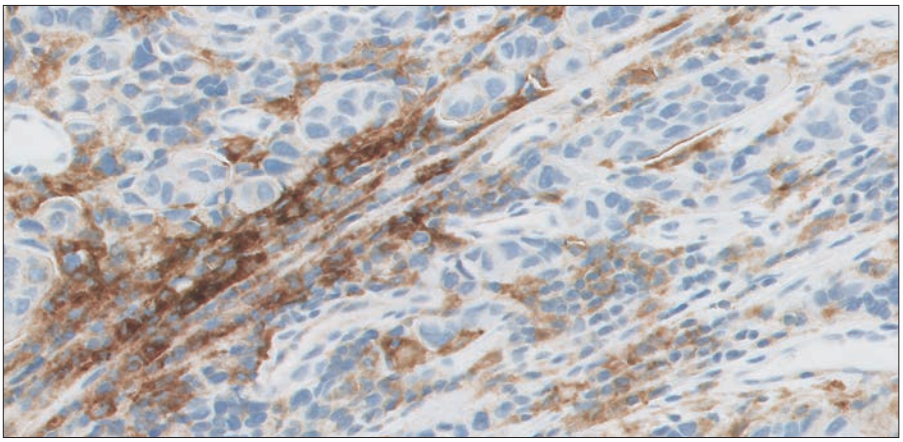


Figure 22b: PD-L1 primary antibody exhibiting staining of tumor-associated lymphocytes (20× magnification). **Note:** Tumor-associated lymphocytes are present within the tumor nests and/or immediately adjacent supporting stroma, and are directly associated with the response against the tumor.

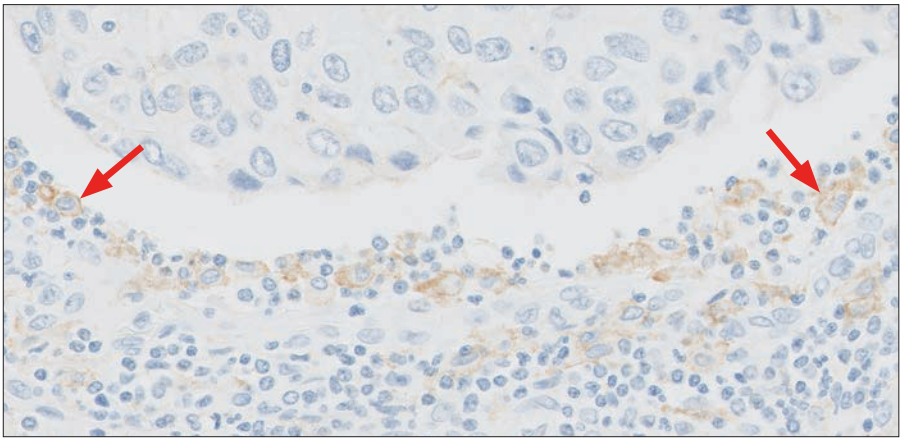


Figure 22c: PD-L1 primary antibody exhibiting moderate staining of tumor-associated macrophages (arrows) (20× magnification). **Note:** Tumor-associated macrophages are present within the tumor nests and/or immediately adjacent supporting stroma, and are directly associated with the response against the tumor.

Key point

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

Weak Staining: Tumor-associated Mononuclear Inflammatory Cells (MICs)

Tumor-associated lymphocytes and macrophages (mononuclear inflammatory cells, MICs) exhibiting weak membrane and/or cytoplasmic staining at no higher than 20× magnification (≥ 1+ intensity) are considered PD-L1 staining cells and should be included in the CPS numerator.

Note: Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor nests and/or immediately adjacent supporting stroma, and are directly associated with the response against the tumor.

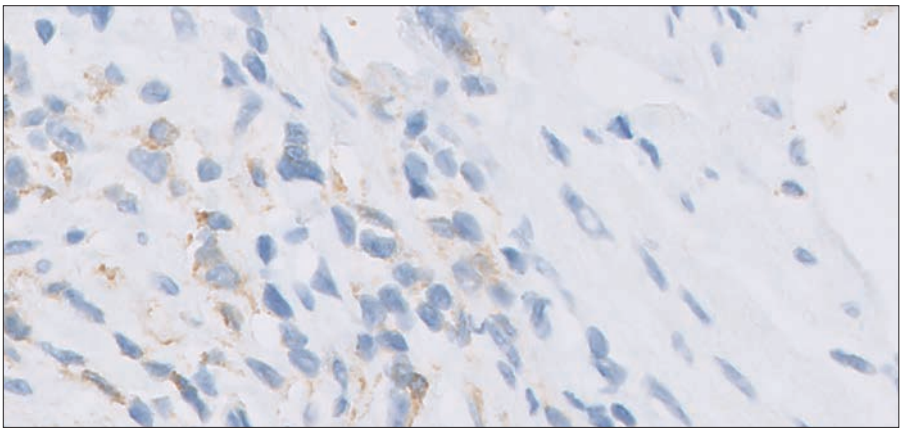


Figure 23a: PD-L1 primary antibody exhibiting weak staining of tumor-associated mononuclear inflammatory cells (MICs) (20× magnification). **Note:** Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor nests and/or immediately adjacent supporting stroma, and are directly associated with the response against the tumor.

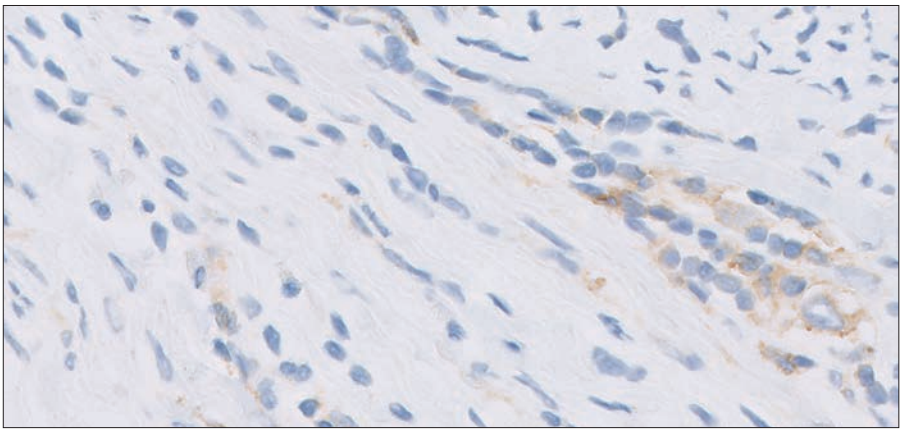


Figure 23b: PD-L1 primary antibody exhibiting weak staining of tumor-associated mononuclear inflammatory cells (MICs) (20× magnification). **Note:** Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor nests and/or immediately adjacent supporting stroma, and are directly associated with the response against the tumor.

Key point

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

Strong Staining: Tumor-associated Lymphocytes and Macrophages

Tumor-associated lymphocytes and macrophages (mononuclear inflammatory cells, MICs) exhibiting strong membrane and/or cytoplasmic staining at no higher than 20× magnification (≥ 1+ intensity) are considered PD-L1 staining cells and should be included in the CPS numerator.

Note: Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor cells and/or immediately adjacent supporting stroma, and are directly associated with the response against the tumor.

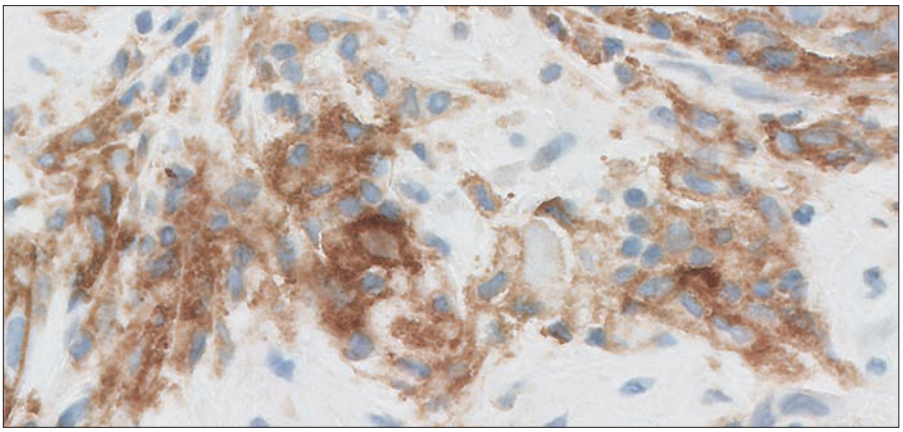


Figure 24: PD-L1 primary antibody exhibiting strong staining of tumor-associated mononuclear inflammatory cells (MICs) (20× magnification). **Note:** Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor nests and/or immediately adjacent supporting stroma, and are directly associated with the response against the tumor.

Key point

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

Heterogeneous Staining Intensities

Convincing staining of tumor cells (linear membrane) and tumor-associated lymphocytes and macrophages (membrane and/or cytoplasmic) is often heterogeneous, with various staining intensities present. At no higher than 20× magnification, any convincing staining of tumor cells and tumor-associated lymphocytes and macrophages at any intensity should be included in the CPS numerator.

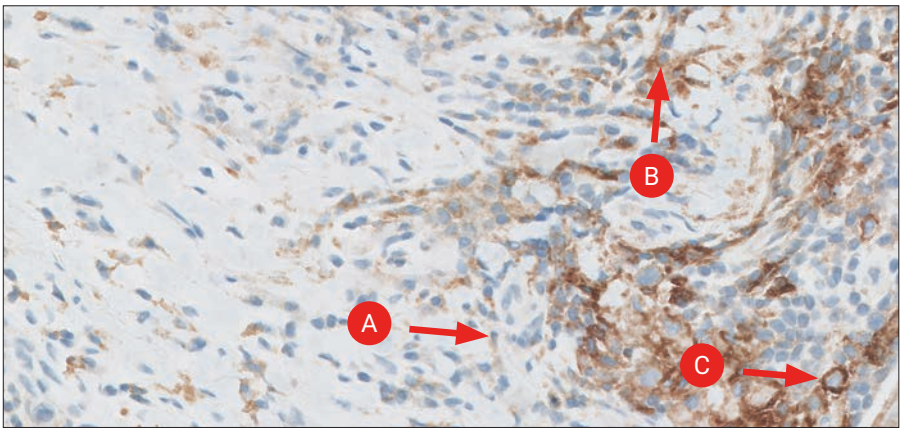


Figure 25a: PD-L1 primary antibody exhibiting heterogeneous staining intensities of mononuclear inflammatory cells (MICs) (A: 1+ intensity, B: 2+ intensity, C: 3+ intensity) (20× magnification).
Note: Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor nests and/or immediately adjacent supporting stroma, and are directly associated with the response against the tumor.

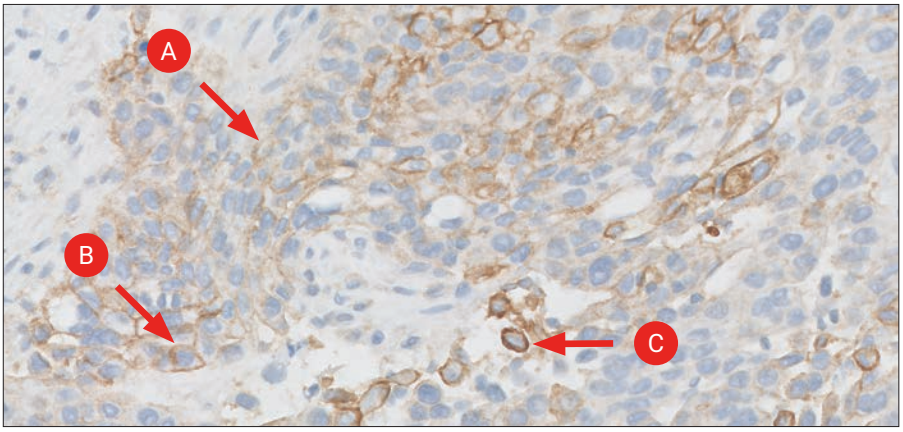


Figure 25b: PD-L1 primary antibody exhibiting heterogeneous staining intensities of tumor cells (A: 1+ intensity, B: 2+ intensity, C: 3+ intensity) (20× magnification).

Key point

Convincing staining of tumor cells and tumor-associated lymphocytes and macrophages at all intensities should be included in the CPS numerator

Granular Staining

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

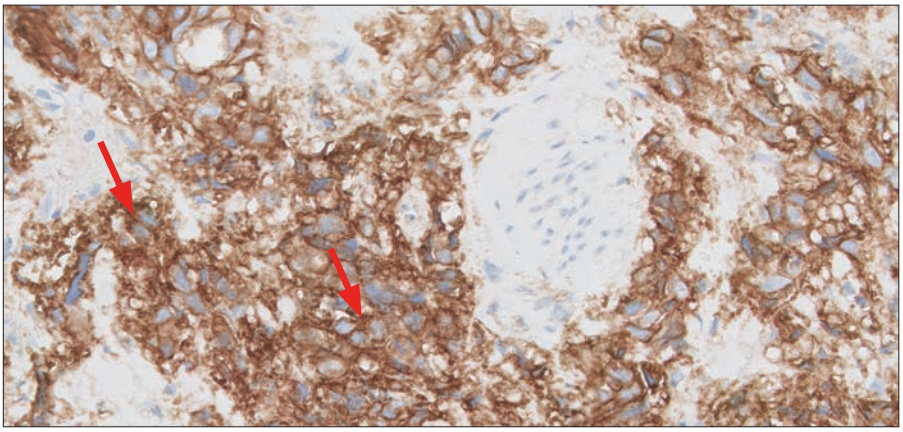


Figure 26: PD-L1 primary antibody exhibiting granular membrane staining pattern (arrows) (20× magnification).

Key point

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

Indistinguishable Tumor and Immune Cells

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

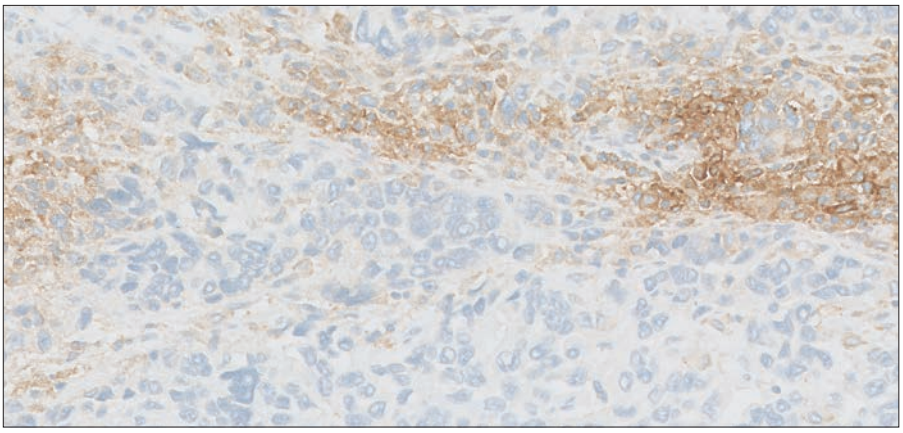


Figure 27a: Tumor and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other and exhibit PD-L1 primary antibody staining (20× magnification).

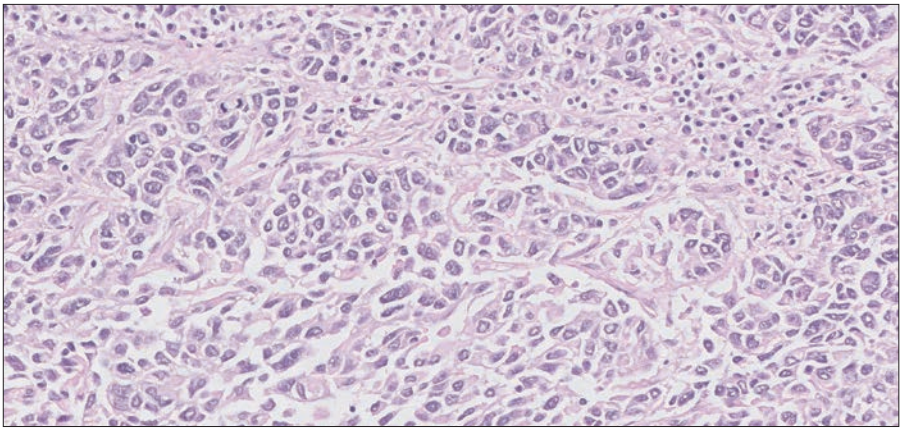


Figure 27b: Corresponding H&E to reference when tumor and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other (20× magnification).

Key point

Use the H&E slide to determine cell morphology when tumor cells and tumor-associated lymphocytes and macrophages are indistinguishable from each other in the PD-L1 slide

Morphological Patterns Included in CPS

Lamina Propria Invasion

In addition to muscle invasion, lamina propria invasion is also commonly demonstrated in urothelial carcinoma. Any tumor cells and/or PD-L1 staining tumor-associated mononuclear inflammatory cells (MICs) in the lamina propria should be included in the CPS calculation.

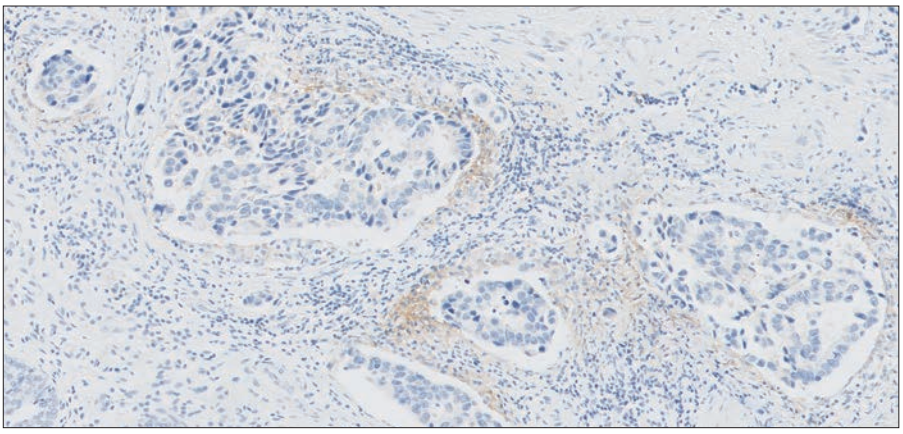


Figure 28: PD-L1 primary antibody exhibiting staining of tumor-associated mononuclear inflammatory cells (MICs) in lamina propria invasion (10× magnification).

Key point

All invasive tumor cells and staining tumor-associated mononuclear inflammatory cells (MICs) in the lamina propria should be included in the CPS calculation

What to Exclude from CPS

Only tumor cells exhibiting PD-L1 membrane staining and mononuclear inflammatory cells (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).

Tumor Cells With Only Cytoplasmic Staining

Tumor cells exhibiting only cytoplasmic staining and/or membrane staining that is only convincing at 40× magnification should not be included in the CPS numerator, as this is considered non-specific staining. They should, however, still be included in the CPS denominator.

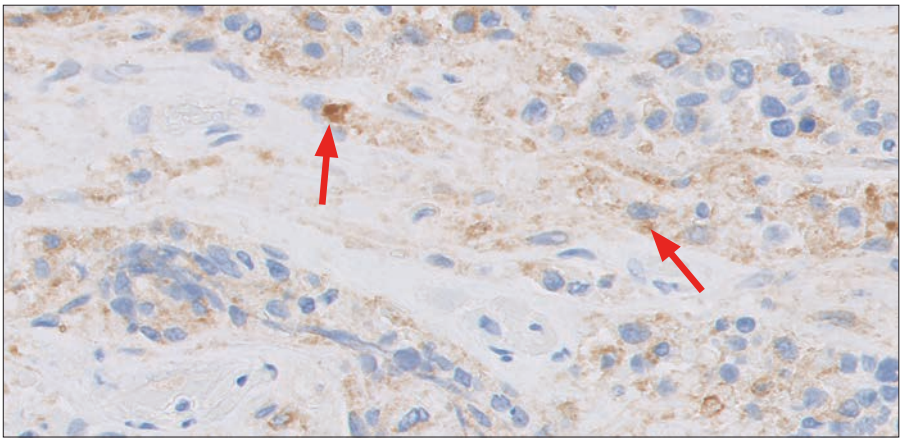


Figure 29: PD-L1 primary antibody exhibiting staining of tumor cytoplasm only (arrows) (20× magnification).

Key point

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

Non-tumor-associated Mononuclear Inflammatory Cells (MICs)

Mononuclear inflammatory cells (MICs, lymphocytes and macrophages) commonly exhibit PD-L1 staining in urothelial carcinoma specimens. Only PD-L1 staining mononuclear inflammatory cells (MICs) that are tumor-associated (present within tumor nests and/or immediately adjacent supportive stroma; directly associated with the response against the tumor) should be included in the CPS calculation.

PD-L1 staining mononuclear inflammatory cells (MICs) that are not tumor-associated must be excluded from the CPS calculation. Examples of non-tumor-associated mononuclear inflammatory cells (MICs) include those associated with papilloma, ulceration, normal cells, and other processes not associated with the tumor.

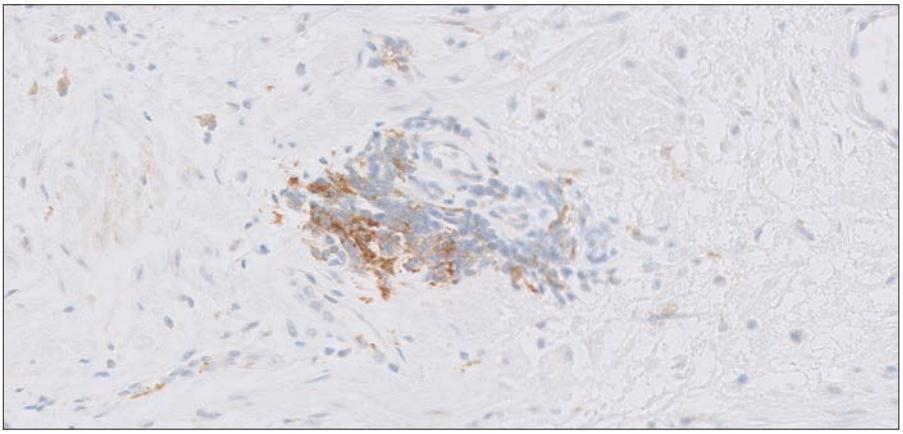


Figure 30a: PD-L1 primary antibody exhibiting staining of non-tumor associated mononuclear inflammatory cells (MICs) (20× magnification). This cluster of partially staining lymphocytes does not appear to be associated with any tumor and therefore should be excluded from the CPS calculation.

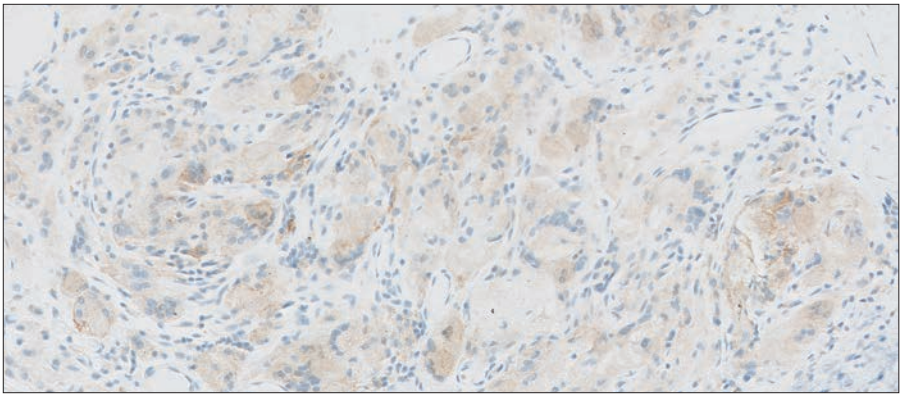


Figure 30b: PD-L1 primary antibody exhibiting staining of cells associated with BCG-induced granulomas (20× magnification).

Key point

Non-tumor-associated mononuclear inflammatory cells (MICs) should be excluded from the CPS calculation

Stromal Cells

Various tissue elements can exhibit PD-L1 staining, but only PD-L1 staining tumor cells and tumor-associated lymphocytes and macrophages should be included in the CPS numerator. Normal cells, stromal cells (including fibroblasts), eosinophils, and plasma cells should be excluded from the CPS calculation.

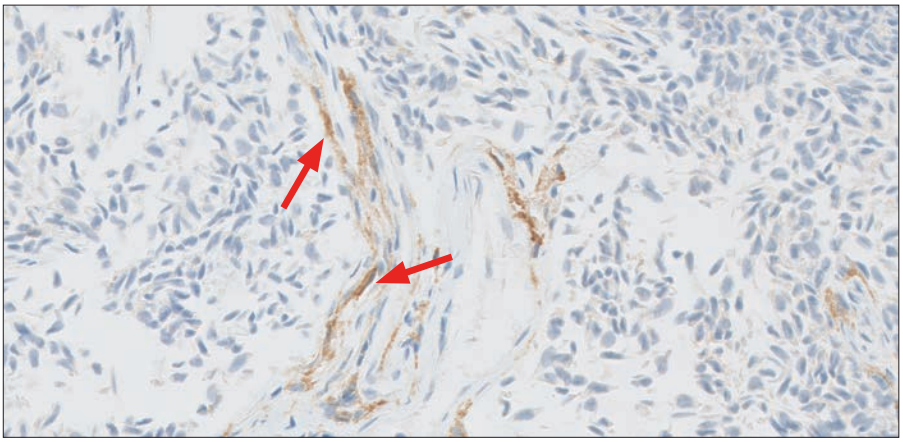


Figure 31a: PD-L1 primary antibody exhibiting staining of stromal cells (arrows) (20× magnification).

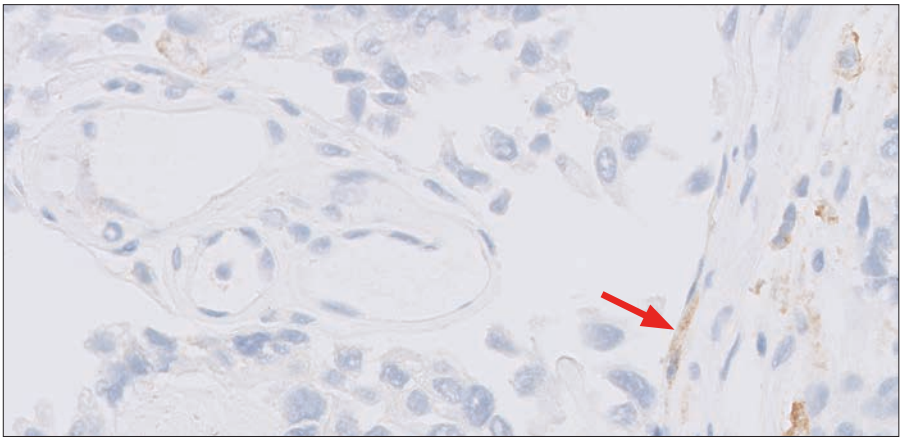


Figure 31b: PD-L1 primary antibody exhibiting staining of a fibroblast (arrow) (20× magnification).

Key point

PD-L1 staining normal cells, stromal cells, eosinophils, and plasma cells should be excluded from the CPS calculation

CPS < 10 Case Examples

Case 1: CPS < 10

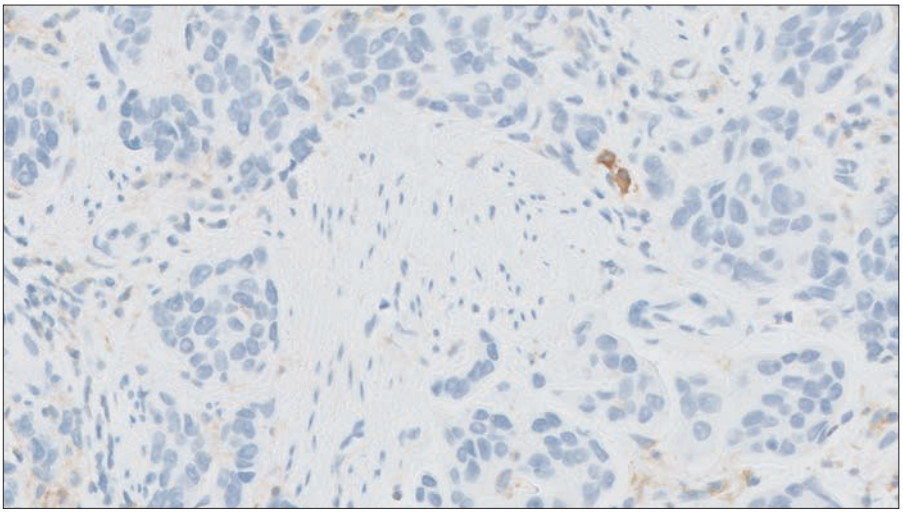


Figure 32a: 10× magnification.

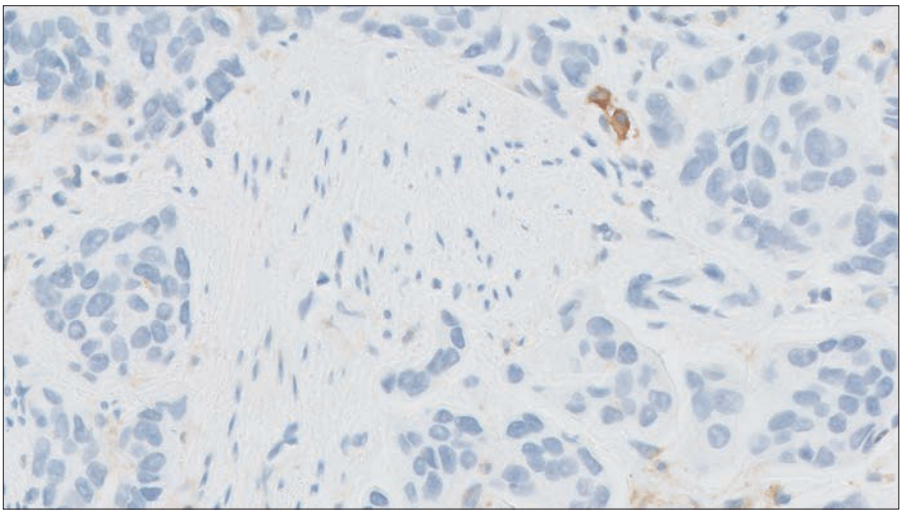


Figure 32b: 20× magnification.

Figure 32a–32b: PD-L1 antibody exhibiting CPS < 10 at 20× magnification.

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

Case 2: CPS < 10

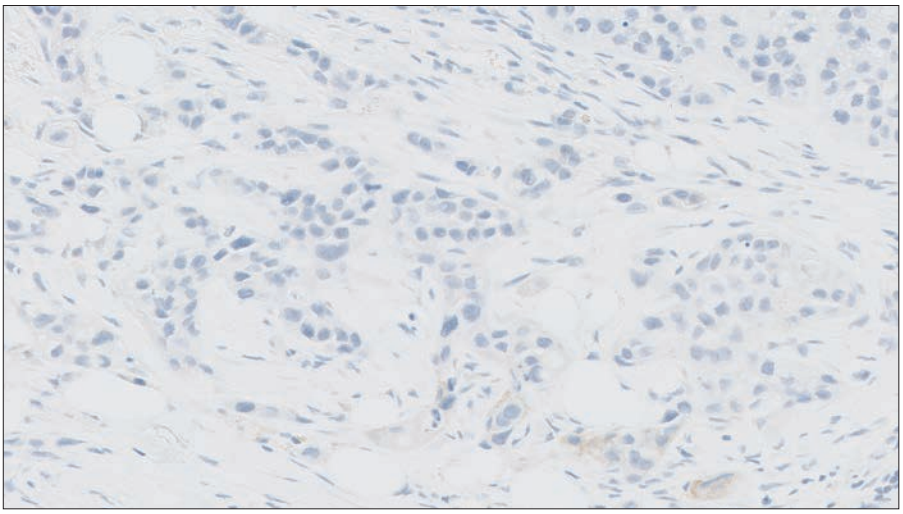


Figure 33a: 10× magnification.

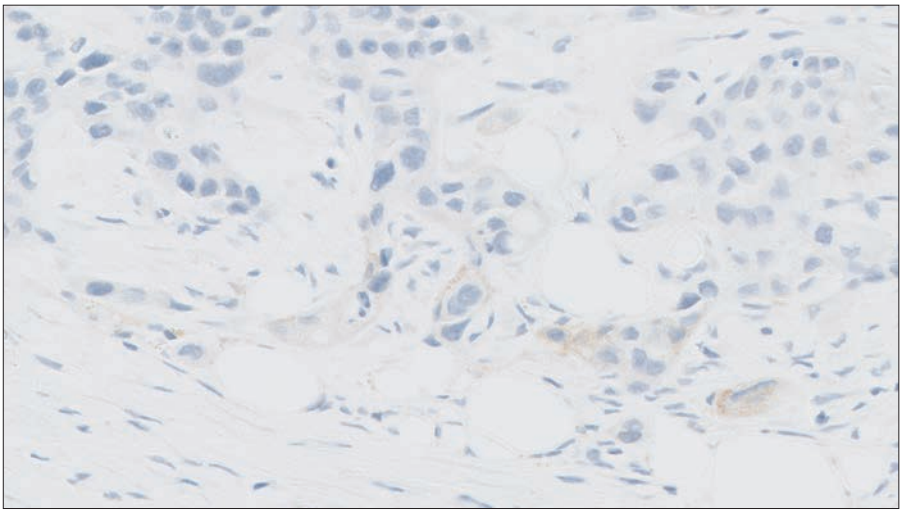


Figure 33b: 20× magnification.

Figure 33a–33b: PD-L1 antibody exhibiting CPS < 10 at 20× magnification.

Case 3: CPS < 10

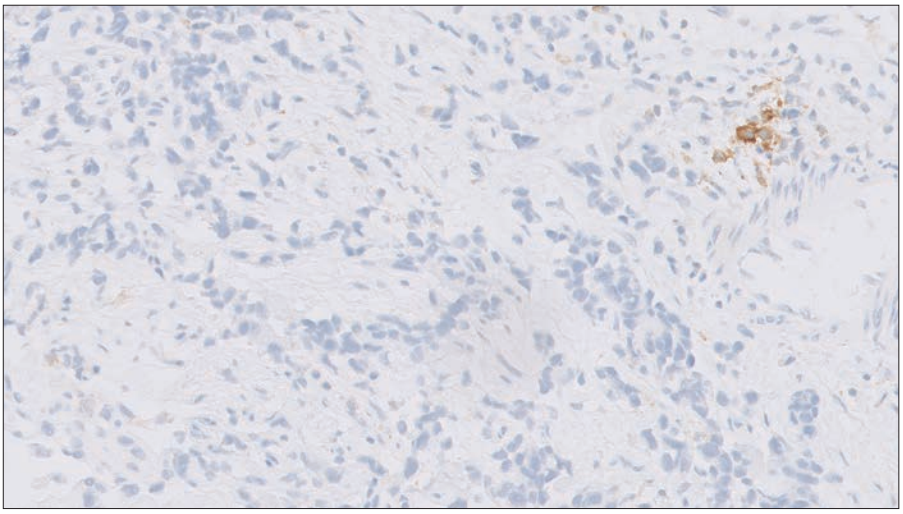


Figure 34a: 10× magnification.

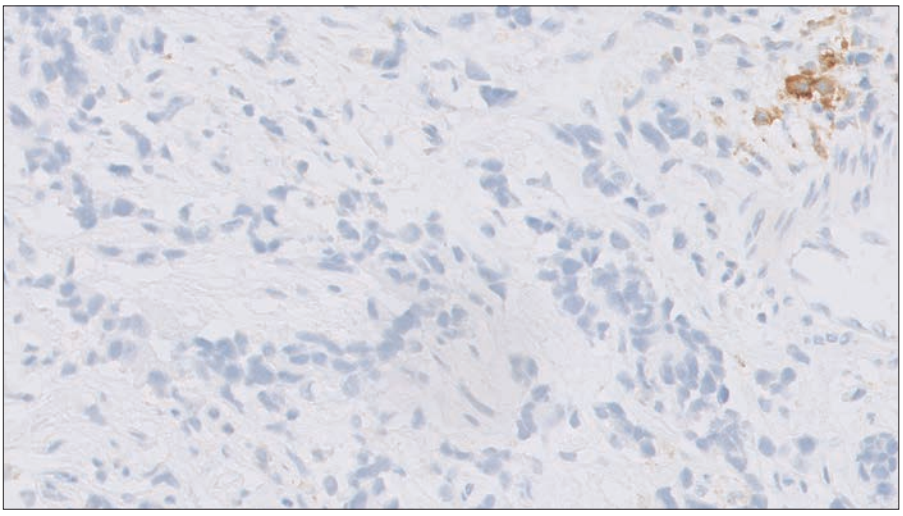


Figure 34b: 20× magnification.

Figure 34a–34b: PD-L1 antibody exhibiting CPS < 10 at 20× magnification.

Case 4: CPS < 10

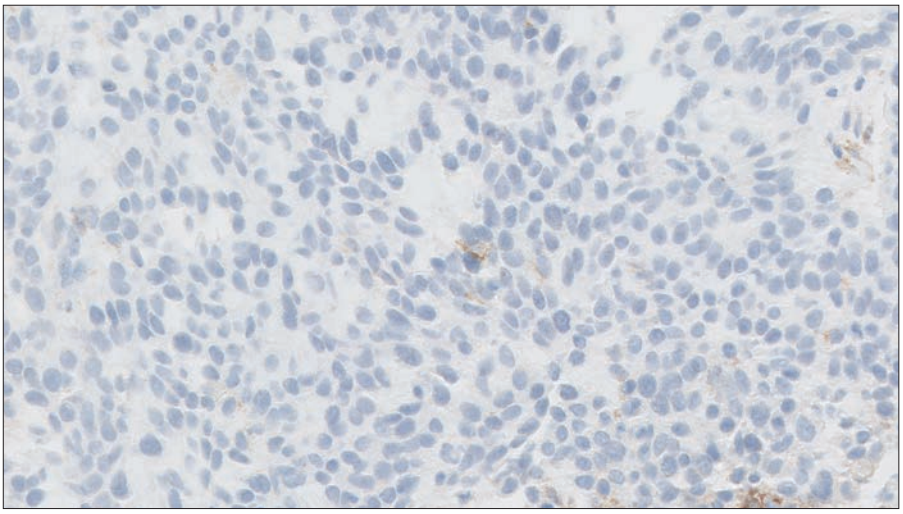


Figure 35a: 10× magnification.

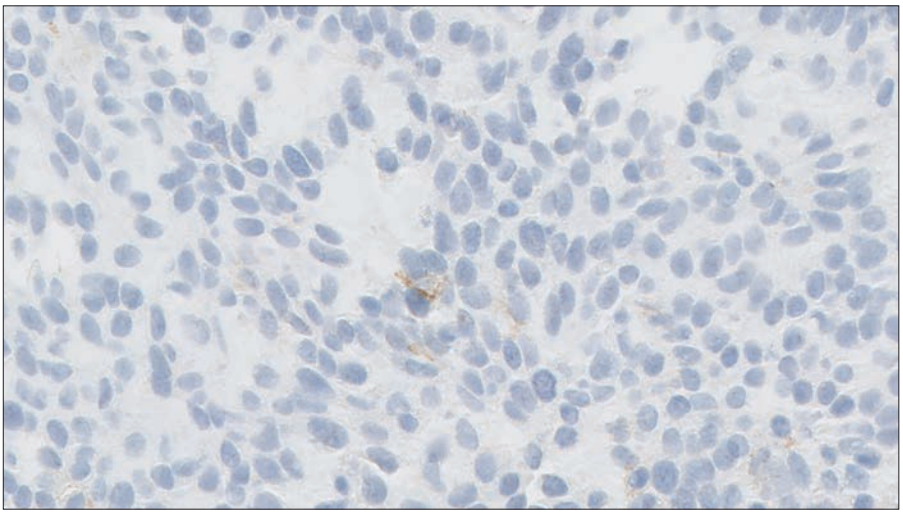


Figure 35b: 20× magnification.

Figure 35a–35b: PD-L1 antibody exhibiting CPS < 10 at 20× magnification.

CPS ≥ 10 Case Examples

Case 5: CPS ≥ 10

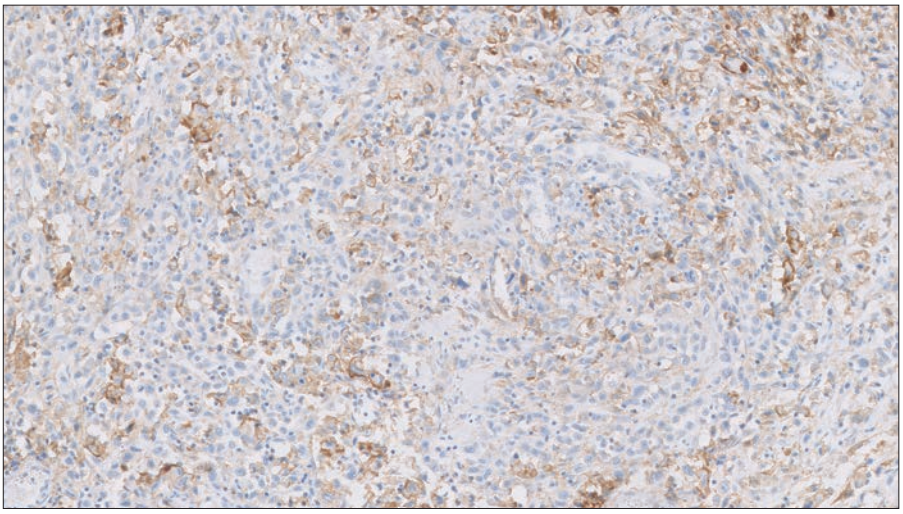


Figure 36a: 10× magnification.

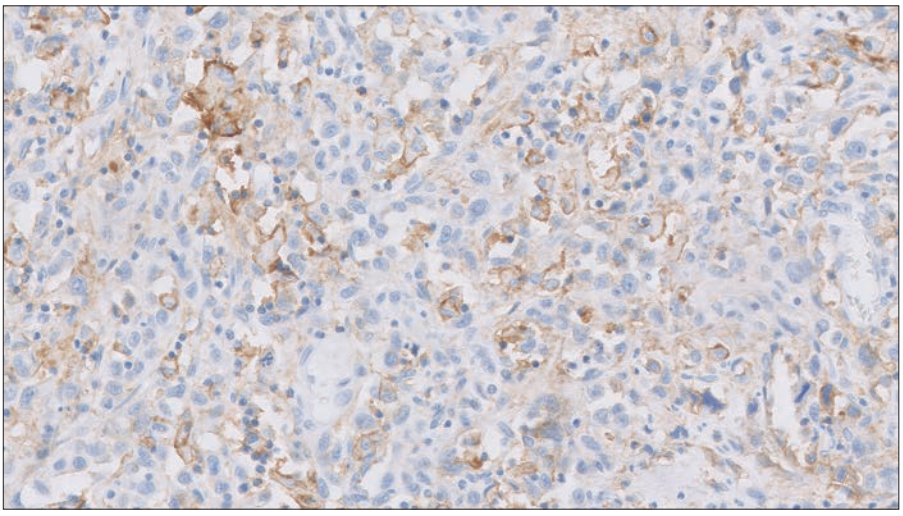


Figure 36b: 20× magnification.

Figure 36a–36b: PD-L1 antibody exhibiting CPS ≥ 10 at 20× magnification.

Case 6: CPS ≥ 10

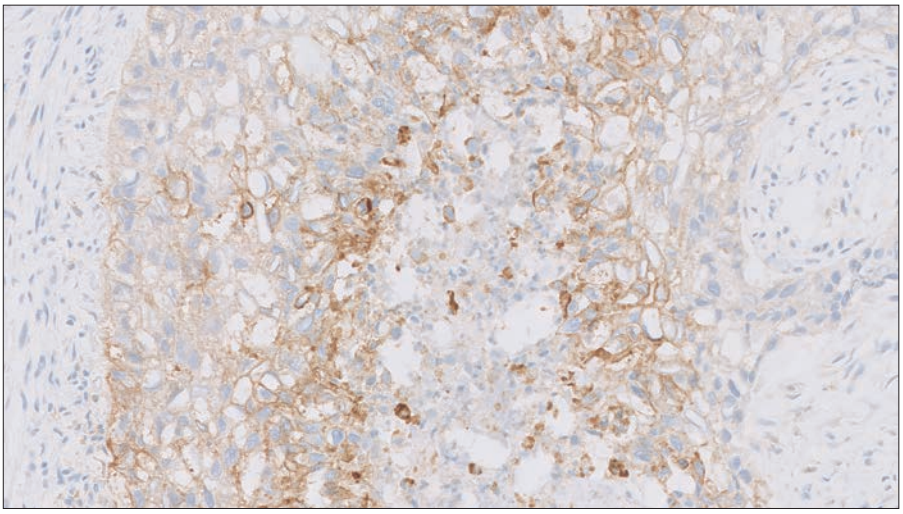


Figure 37a: 10× magnification.

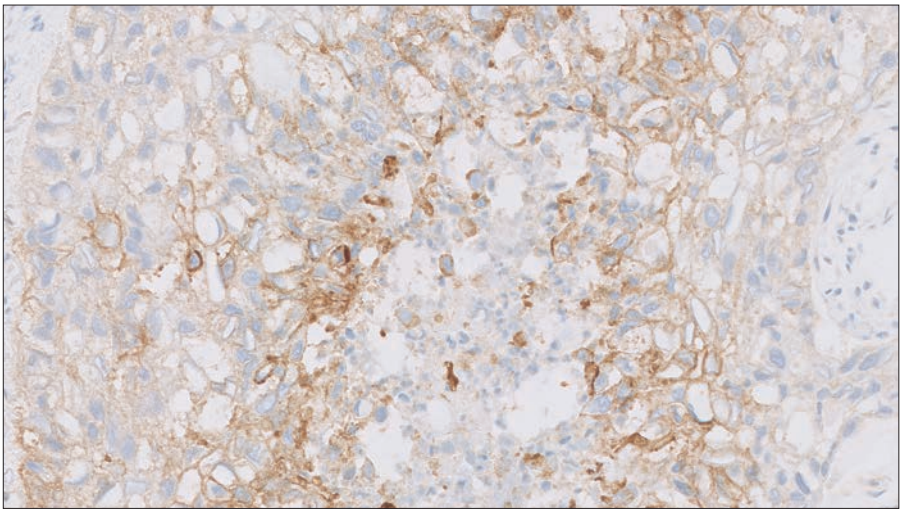


Figure 37b: 20× magnification.

Figure 37a–37b: PD-L1 antibody exhibiting CPS ≥ 10 at 20× magnification.

Case 7: CPS ≥ 10

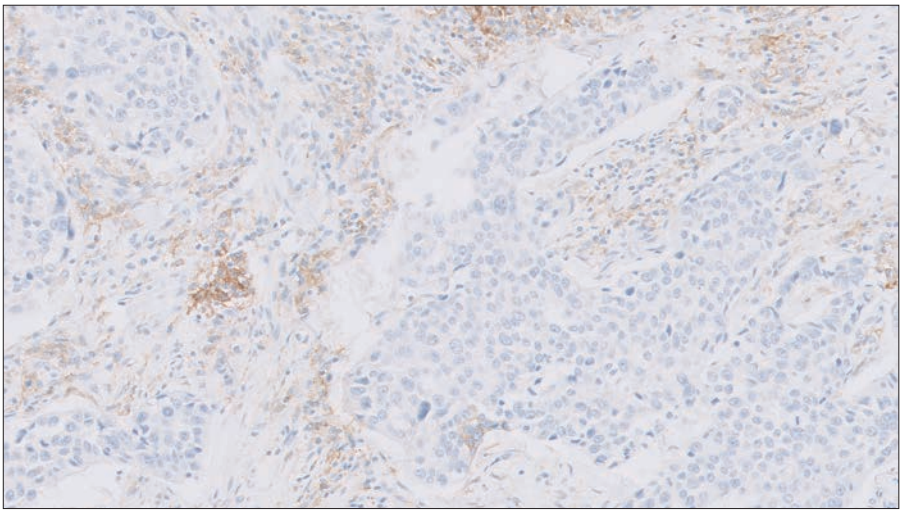


Figure 38a: 10× magnification.

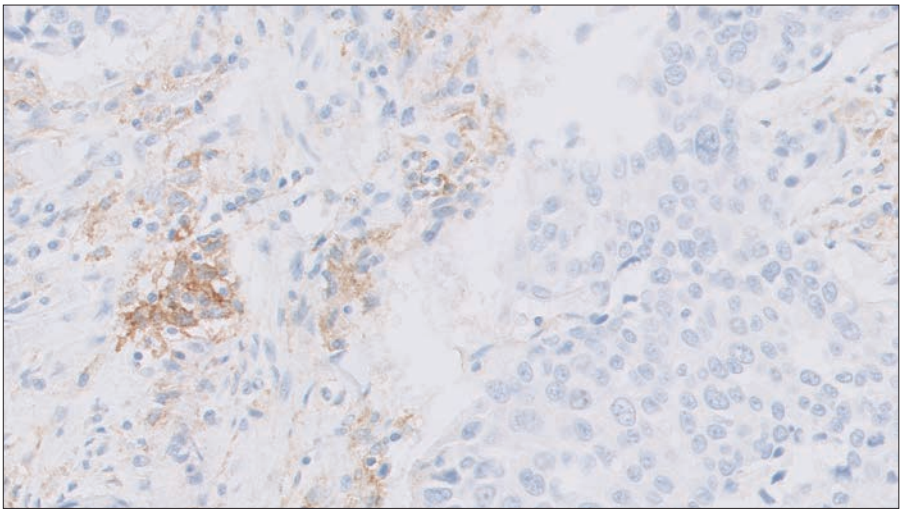


Figure 38b: 20× magnification.

Figure 38a–38b: PD-L1 antibody exhibiting CPS ≥ 10 at 20× magnification.

Case 8: CPS ≥ 10

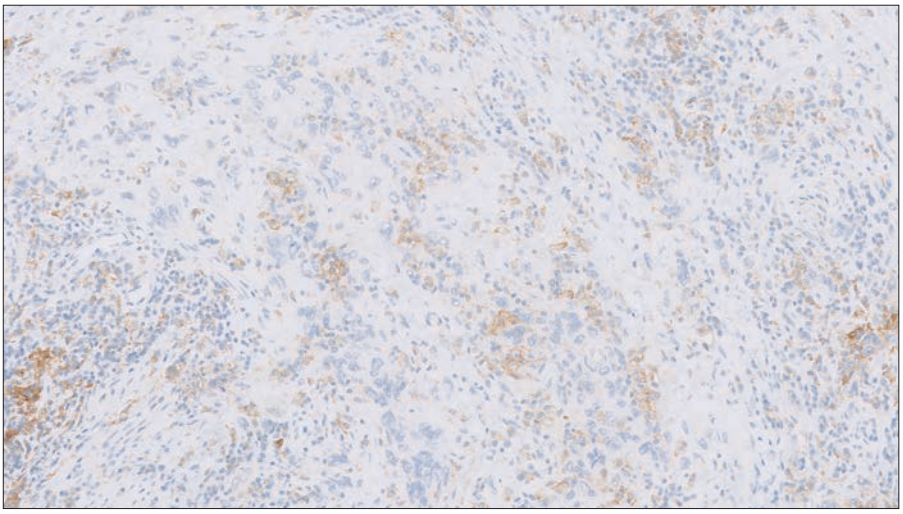


Figure 39a: 10× magnification.

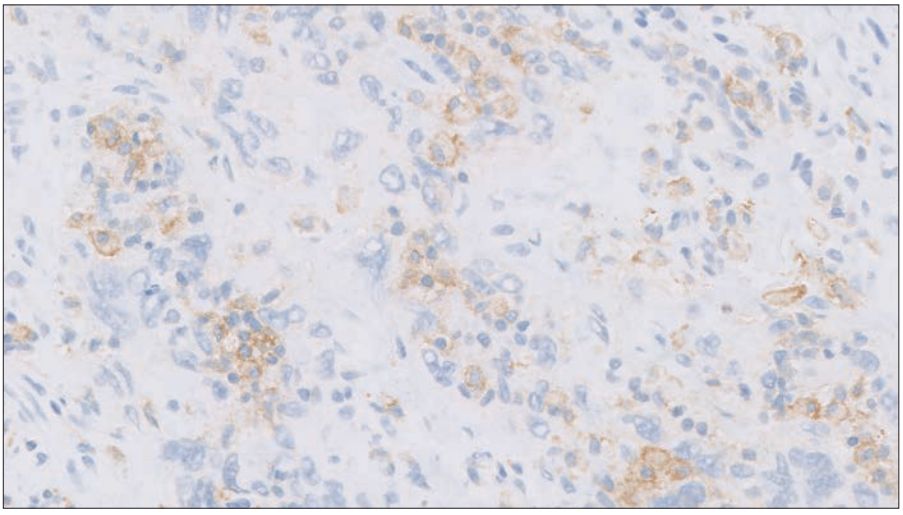


Figure 39b: 20× magnification.

Figure 39a–39b: PD-L1 antibody exhibiting CPS ≥ 10 at 20× magnification.

Case 9: CPS ≥ 10

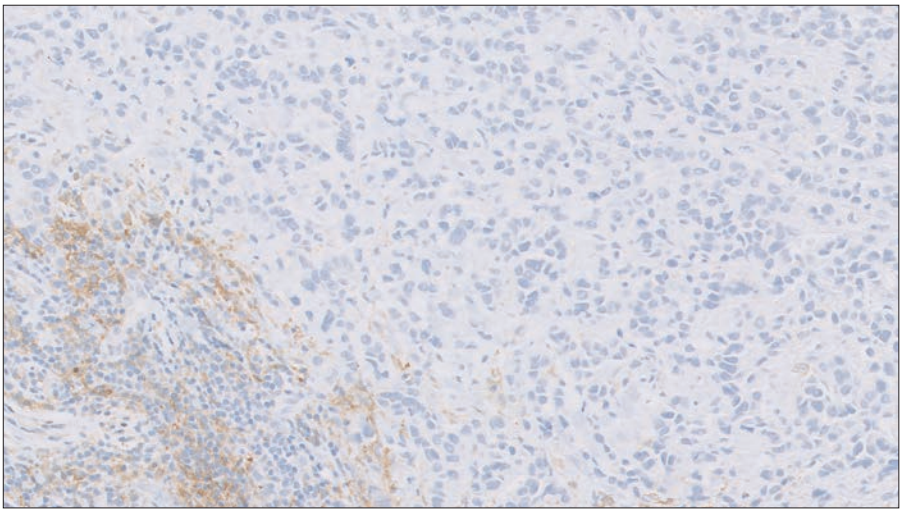


Figure 40a: 10× magnification.

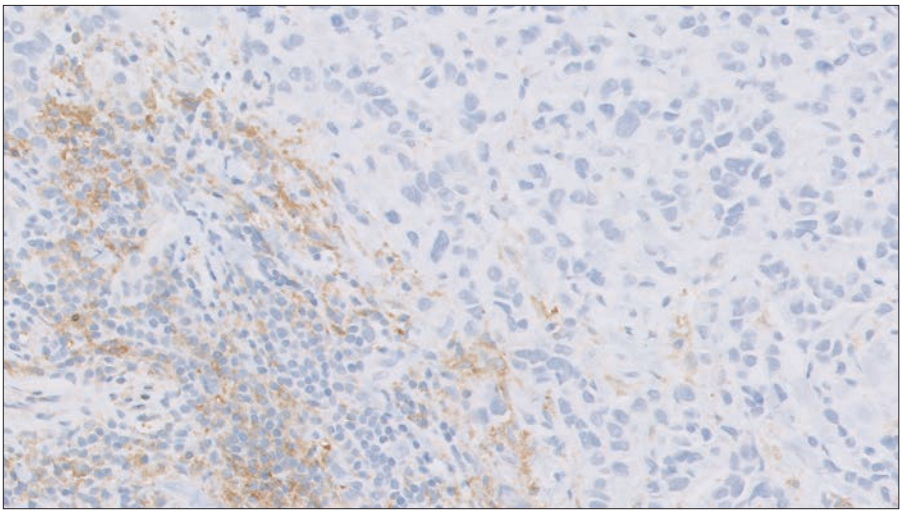


Figure 40b: 20× magnification.

Figure 40a–40b: PD-L1 antibody exhibiting CPS ≥ 10 at 20× magnification.

Case 10: CPS ≥ 10

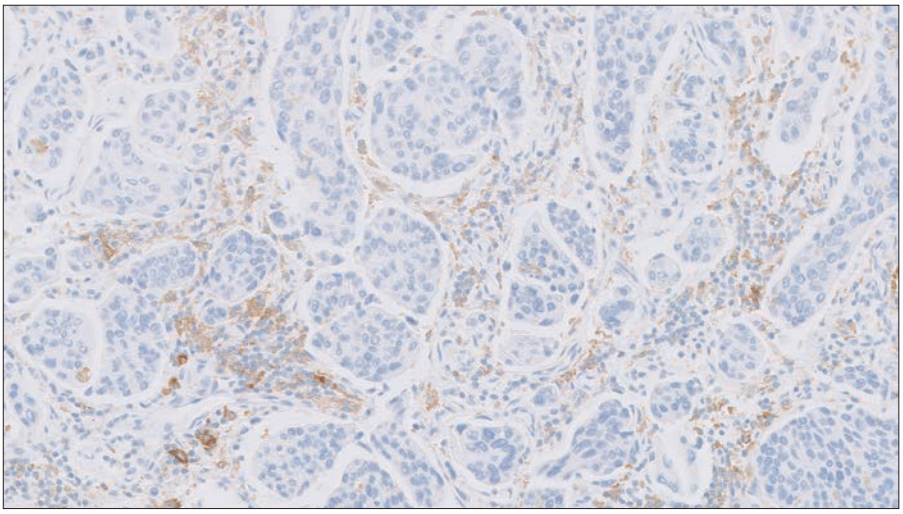


Figure 41a: 10× magnification.

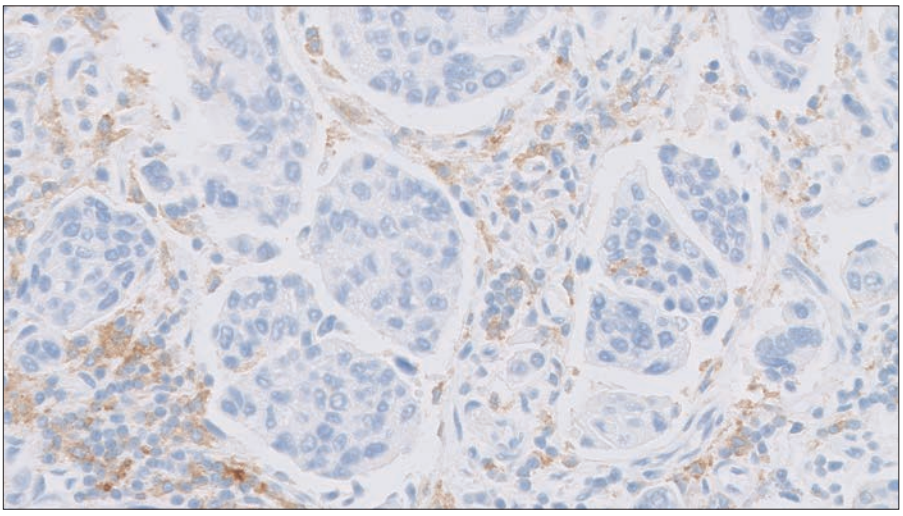


Figure 41b: 20× magnification.

Figure 41a–41b: PD-L1 antibody exhibiting CPS ≥ 10 at 20× magnification.

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

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

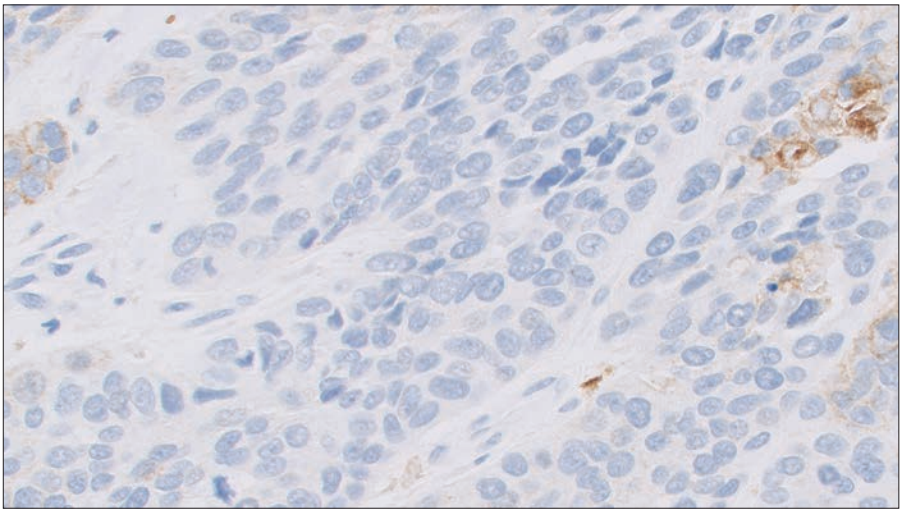


Figure 42: PD-L1 antibody exhibiting CPS 8 at 20× magnification.

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

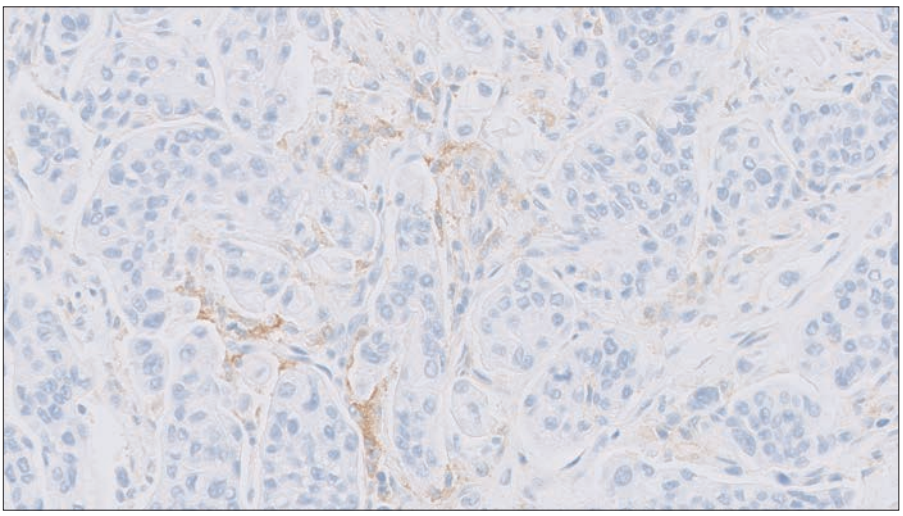


Figure 43: PD-L1 antibody exhibiting CPS 8 at 20× magnification.

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

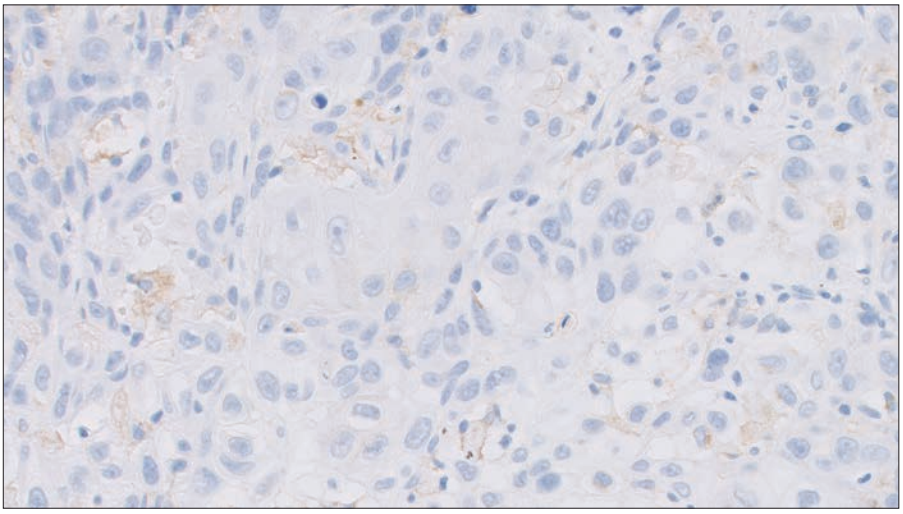


Figure 44: PD-L1 antibody exhibiting CPS 5 at 20× magnification.

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

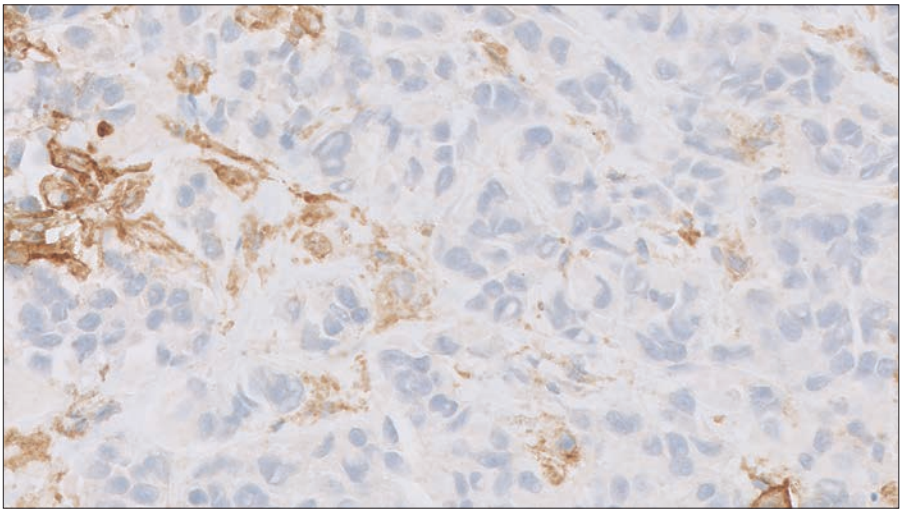


Figure 45: PD-L1 antibody exhibiting CPS 7 at 20× magnification.

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

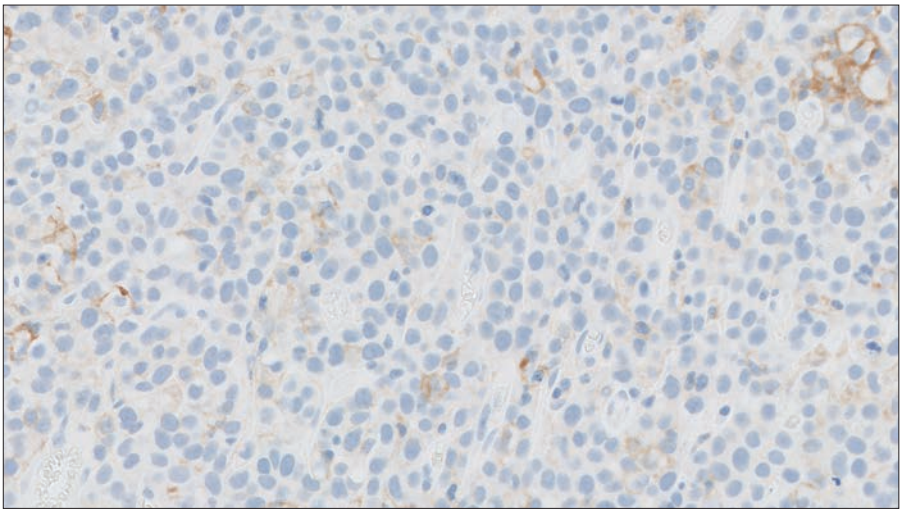


Figure 46: PD-L1 antibody exhibiting CPS 7 at 20× magnification.

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

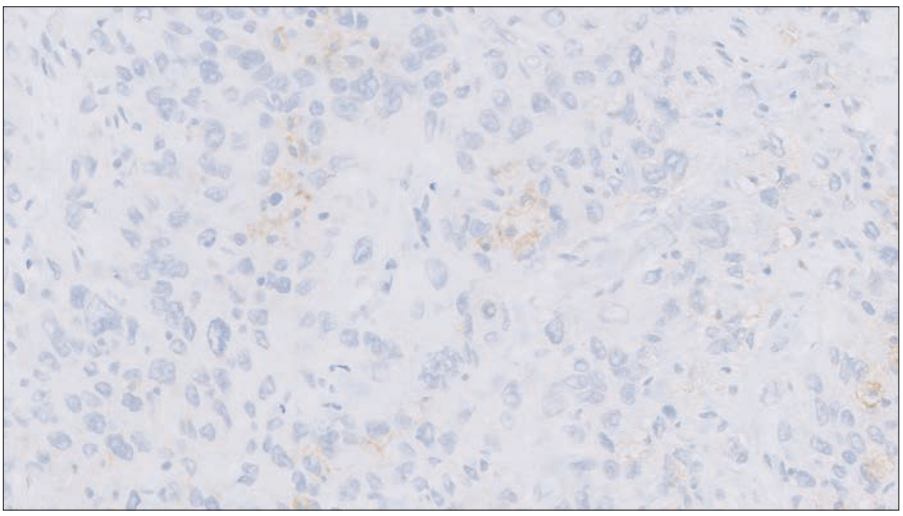


Figure 47: PD-L1 antibody exhibiting CPS 7 at 20× magnification.

Challenging Case 7: Near Cut-off (CPS Range of Greater Than 1 but Less Than 10)

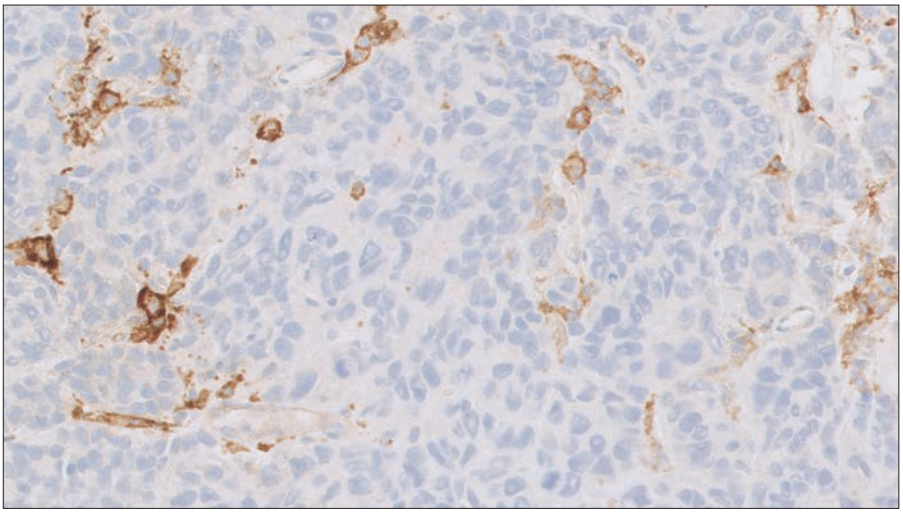


Figure 48: PD-L1 antibody exhibiting CPS 6 at 20× magnification.

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

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

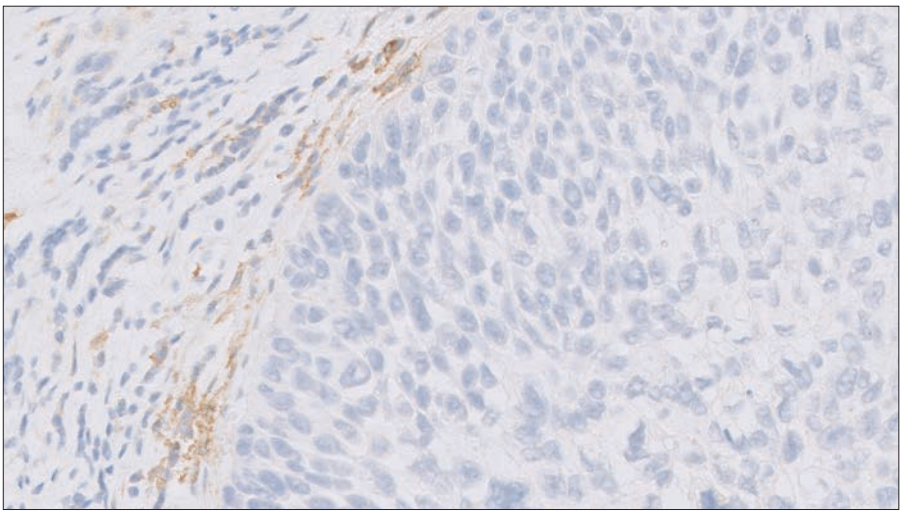


Figure 49: PD-L1 antibody exhibiting CPS 10 at 20× magnification.

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

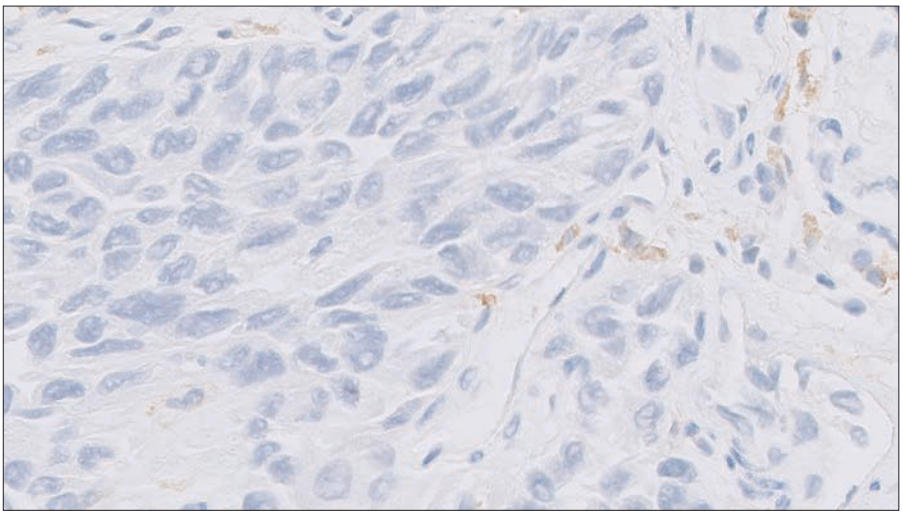


Figure 50: PD-L1 antibody exhibiting CPS 10 at 20× magnification.

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

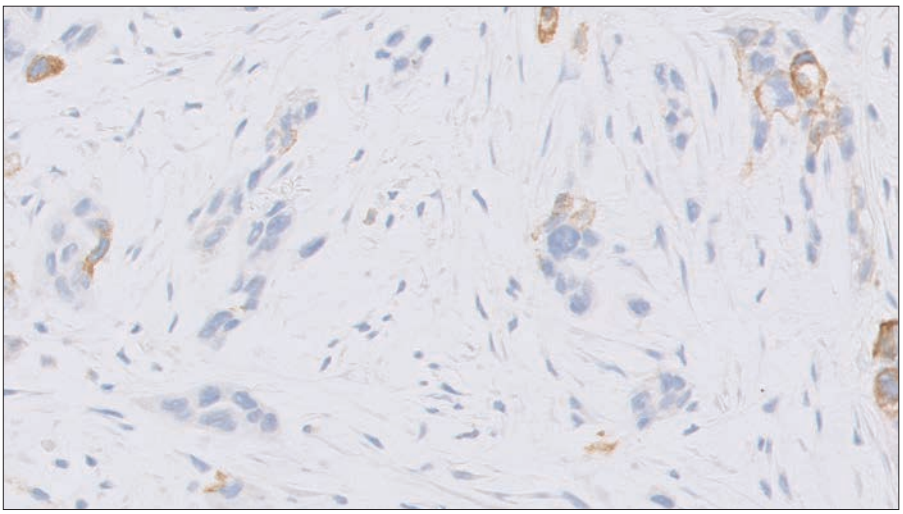


Figure 51: PD-L1 antibody exhibiting CPS 13 at 20× magnification.

Challenging Case 11: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

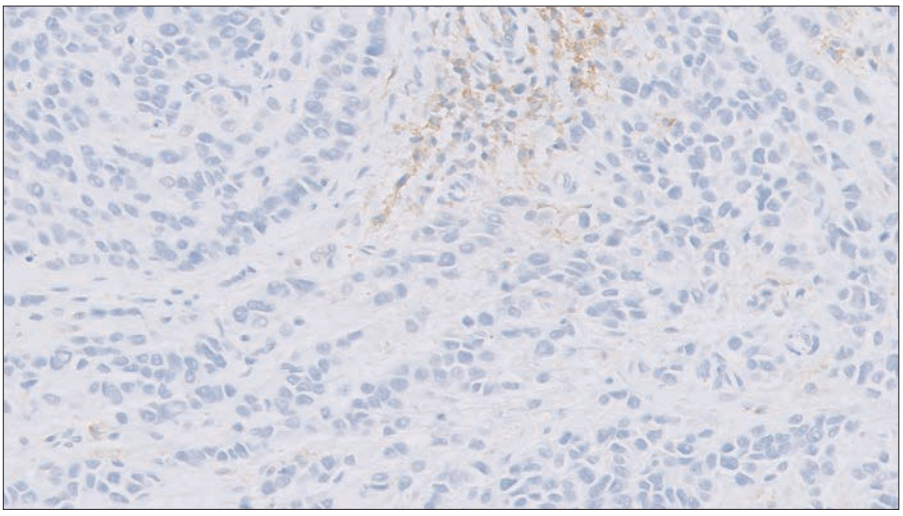


Figure 52: PD-L1 antibody exhibiting CPS 10 at 20× magnification.

Challenging Case 12: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

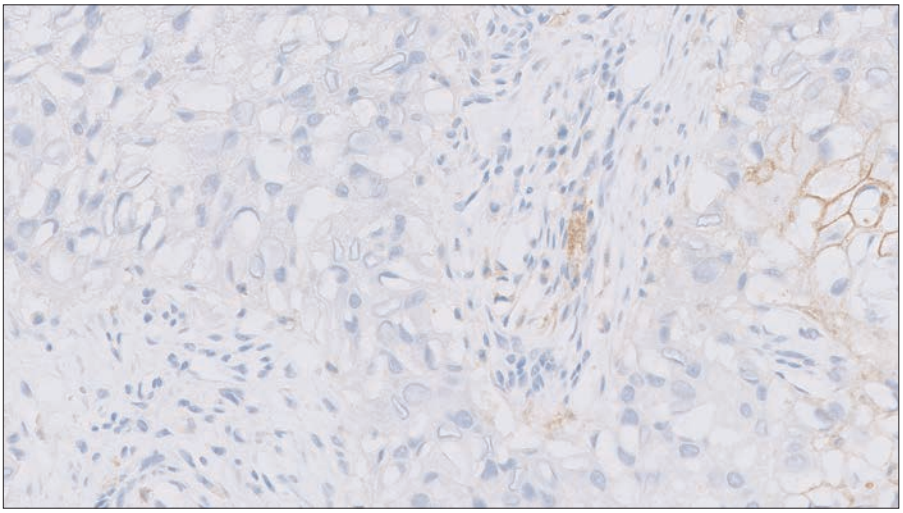


Figure 53: PD-L1 antibody exhibiting CPS 15 at 20× magnification.

Challenging Case 13: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

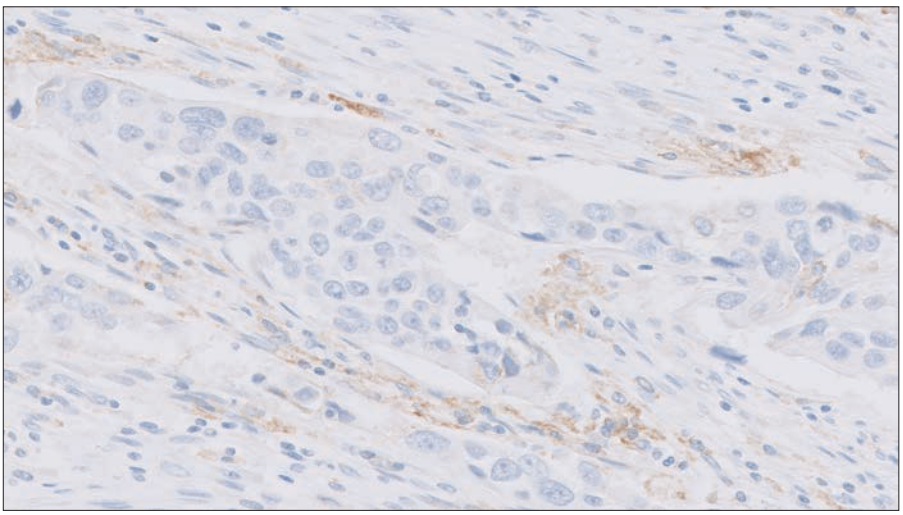


Figure 54: PD-L1 antibody exhibiting CPS 20 at 20× magnification.

Artifacts

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

Non-specific Background Staining

Background staining is defined as diffuse, non-specific staining of a specimen. It is caused by several factors. These factors include, but are not limited to, pre-analytic fixation and processing of the specimen, incomplete removal of paraffin from sections, and incomplete rinsing of slides during staining.

The use of fixatives other than neutral buffered formalin may be a source of background staining. Background staining with PD-L1 IHC 22C3 pharmDx is rare.

Possible Causes of Background

- Improper drying of slides; ensure slides remain wet with buffer while loading onto Autostainer Link 48 and prior to initiating run
- Improper deparaffinization procedure
- Incomplete rinsing of reagents from slides

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

Key point

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

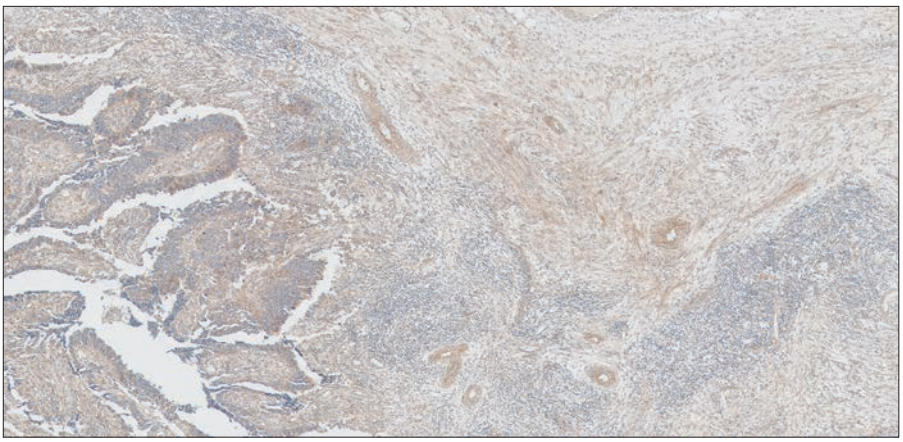


Figure 55a: Negative Control Reagent (NCR) exhibiting unacceptable non-specific background staining (5× magnification).

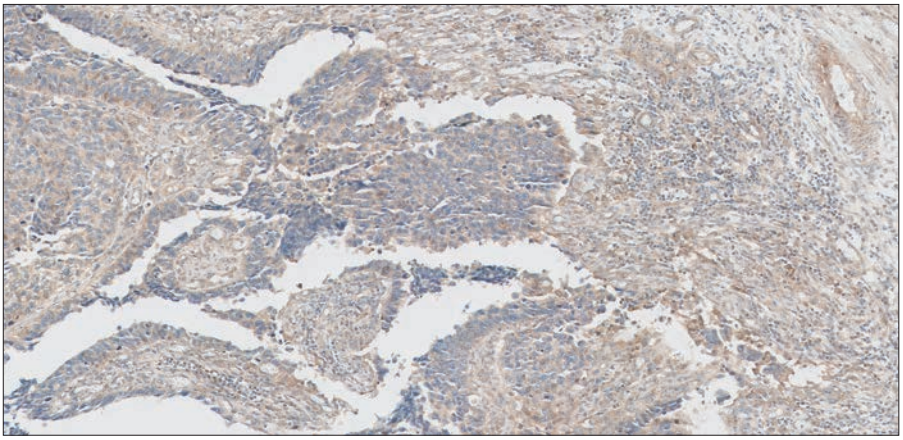


Figure 55b: PD-L1 primary antibody exhibiting blush staining; blush should be excluded from scoring (10× magnification).

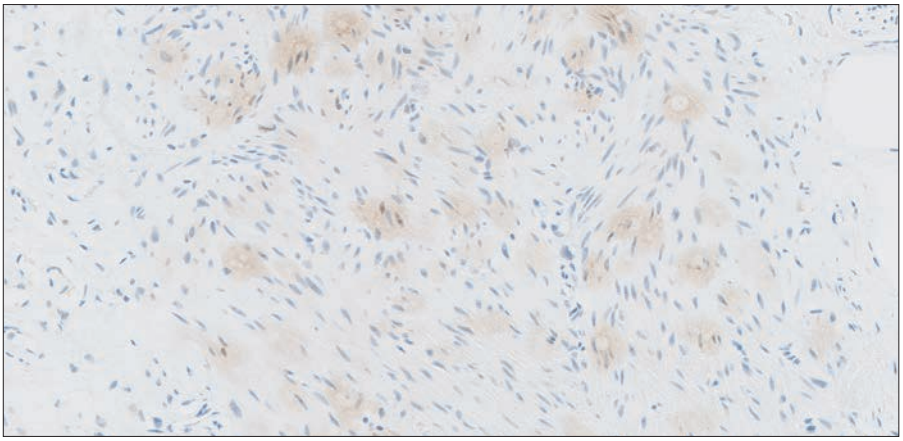


Figure 55c: Staining caused by DAB droplets should be excluded from scoring (20× magnification).

Edge Artifact

Commonly, edge artifacts are linked to the pre-analytic handling of the tissue.

- 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 non-staining as optimal fixation is only present at the periphery
- Increased staining is observed around the periphery of the tissue specimen, known as the "edge artifact"
- Edge artifacts can be due to drying of the tissue specimen prior to fixation or during the staining procedure
- If the positive reaction 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

Key point

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

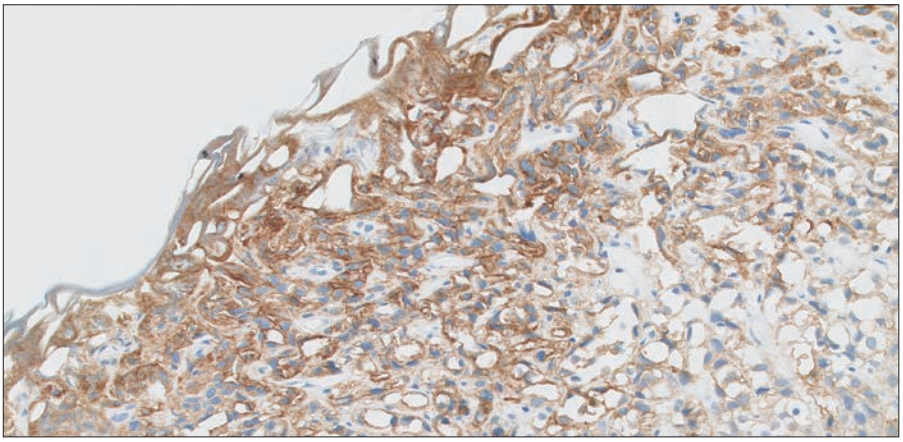


Figure 56: PD-L1 primary antibody exhibiting edge artifact staining; edge staining should be excluded from scoring (10× magnification).

Poor Fixation

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

Key point

Proper fixation is important for accurate diagnosis

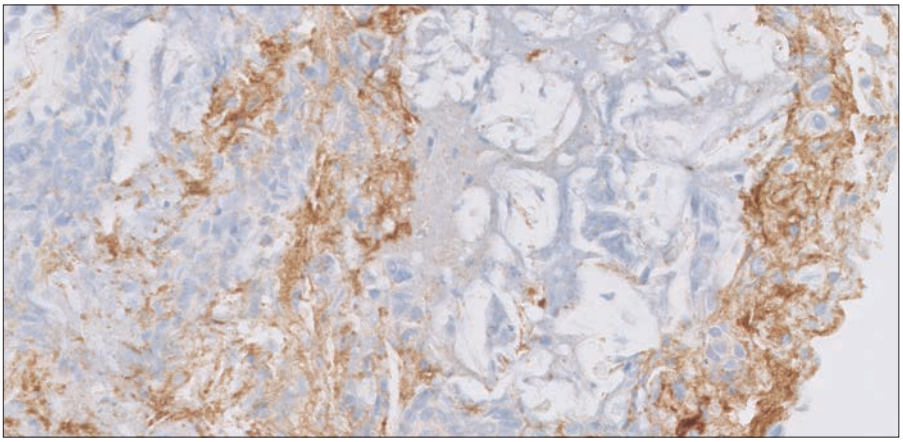


Figure 57: PD-L1 primary antibody exhibiting poor tissue fixation (20× magnification).

Necrosis

Necrosis can be described as morphological changes indicative of cell death with undefined cellular detail. Necrosis is often present in urothelial carcinoma specimens and should be excluded from scoring.

Key point

Scoring of necrotic areas should be excluded from the CPS calculation

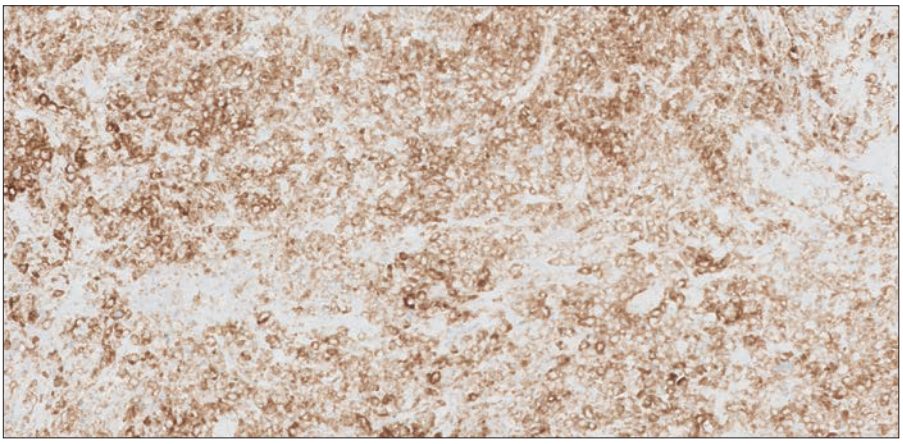


Figure 58: PD-L1 primary antibody exhibiting staining of necrosis; necrosis should not be scored (20× magnification).

Troubleshooting Guide

Troubleshooting Guidelines for PD-L1 IHC 22C3 pharmDx

For further troubleshooting help, contact your local Agilent representative.

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

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

Clinical Performance Evaluation

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

Patients received KEYTRUDA 200 mg every 3 weeks until unacceptable toxicity or disease progression. Patients with initial radiographic disease progression could receive additional doses of treatment during confirmation of progression unless disease progression was symptomatic, was rapidly progressive, required urgent intervention, or occurred with a decline in performance status. Patients without disease progression could be treated for up to 24 months. Tumor response assessments were performed at 9 weeks after the first dose, then every 6 weeks for the first year, and then every 12 weeks thereafter. The major efficacy outcome measures were ORR according to RECIST 1.1 as assessed by independent radiology review and duration of response.

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

Among the 370 patients, 30% (n = 110) had tumors that expressed PD-L1 with CPS ≥ 10 and PD-L1 status was unknown for 9 patients. Baseline characteristics of these patients were: median age 73 years, 68% male, and 88% White. Eighty-two percent had M1 disease, and 18% had M0 disease. Eighty-one percent had a primary tumor in the lower tract, and 18% of patients had a primary tumor in the upper tract. Seventy-six percent of patients had visceral metastases, including 11% with liver metastases. Reasons for cisplatin ineligibility included: 45% with baseline creatinine clearance of <60 mL/min, 37% with ECOG performance status of 2, 10% with ECOG 2 and baseline creatinine clearance of <60 mL/min, and 8% with other reasons (Class III heart failure, Grade 2 or greater peripheral neuropathy, and Grade 2 or greater hearing loss). Ninety percent of patients were treatment naïve, and 10% received prior adjuvant or neoadjuvant platinum based chemotherapy.

Among the 270 patients in the validation set, 30% (n = 80) had tumors that expressed PD-L1 with CPS ≥ 10. Baseline characteristics of these patients were: median age 72 years, 68% male, and 86% White. Seventy-one percent had M1 disease, and 26% had M0 disease. Seventy-nine percent had a primary tumor in the lower tract, and 20% of patients had a primary tumor in the upper tract. Seventy-eight percent of patients had visceral metastases, including 8% with liver metastases. Reasons for cisplatin ineligibility included: 41% with baseline creatinine clearance of <60 mL/min, 43% with ECOG performance status of 2, 11% with ECOG 2 and baseline creatinine clearance of <60 mL/min, and 5% with other reasons (Class III heart failure, Grade 2 or greater peripheral neuropathy, and Grade 2 or greater hearing loss). Ninety percent of patients were treatment naïve, and 10% received prior adjuvant or neoadjuvant platinum-based chemotherapy.

Efficacy results are summarized in Table 5.

Table 5: Efficacy Results in KN052

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

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

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

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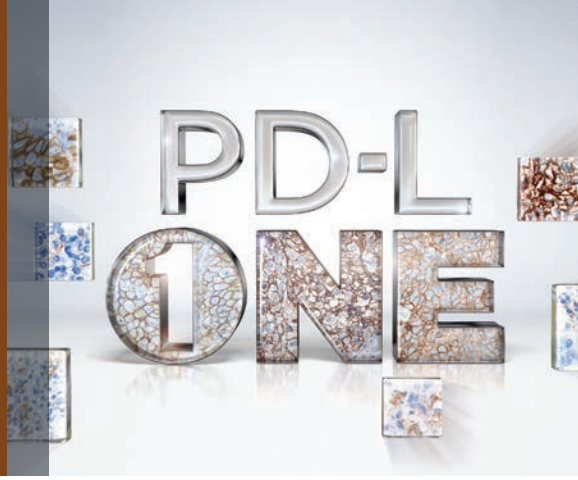

KEYNOTE

The **ONE** assay used to assess PD-L1 across KEYTRUDA clinical trials

The **ONE** assay established with KEYTRUDA in the pivotal NSCLC studies

FDA

The **ONE** assay FDA-approved as an aid in identifying patients for treatment with KEYTRUDA

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