

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

CE-IVD–marked for in vitro diagnostic use

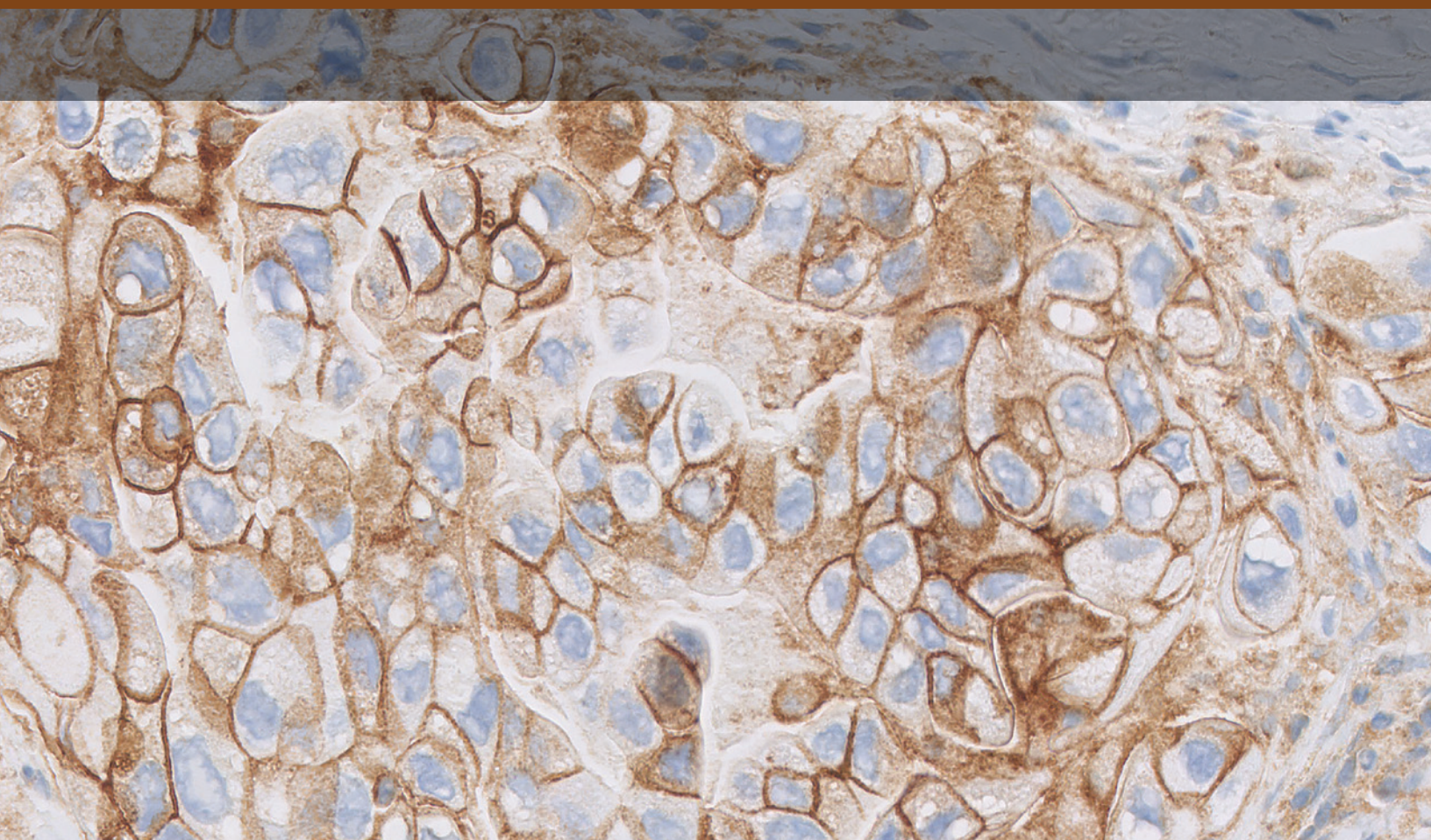


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Intended Use

For in vitro diagnostic use.

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

Non-Small Cell Lung Cancer (NSCLC)

PD-L1 protein expression is determined by using Tumor Proportion Score (TPS), which is the percentage of viable tumor cells showing partial or complete membrane staining at any intensity.

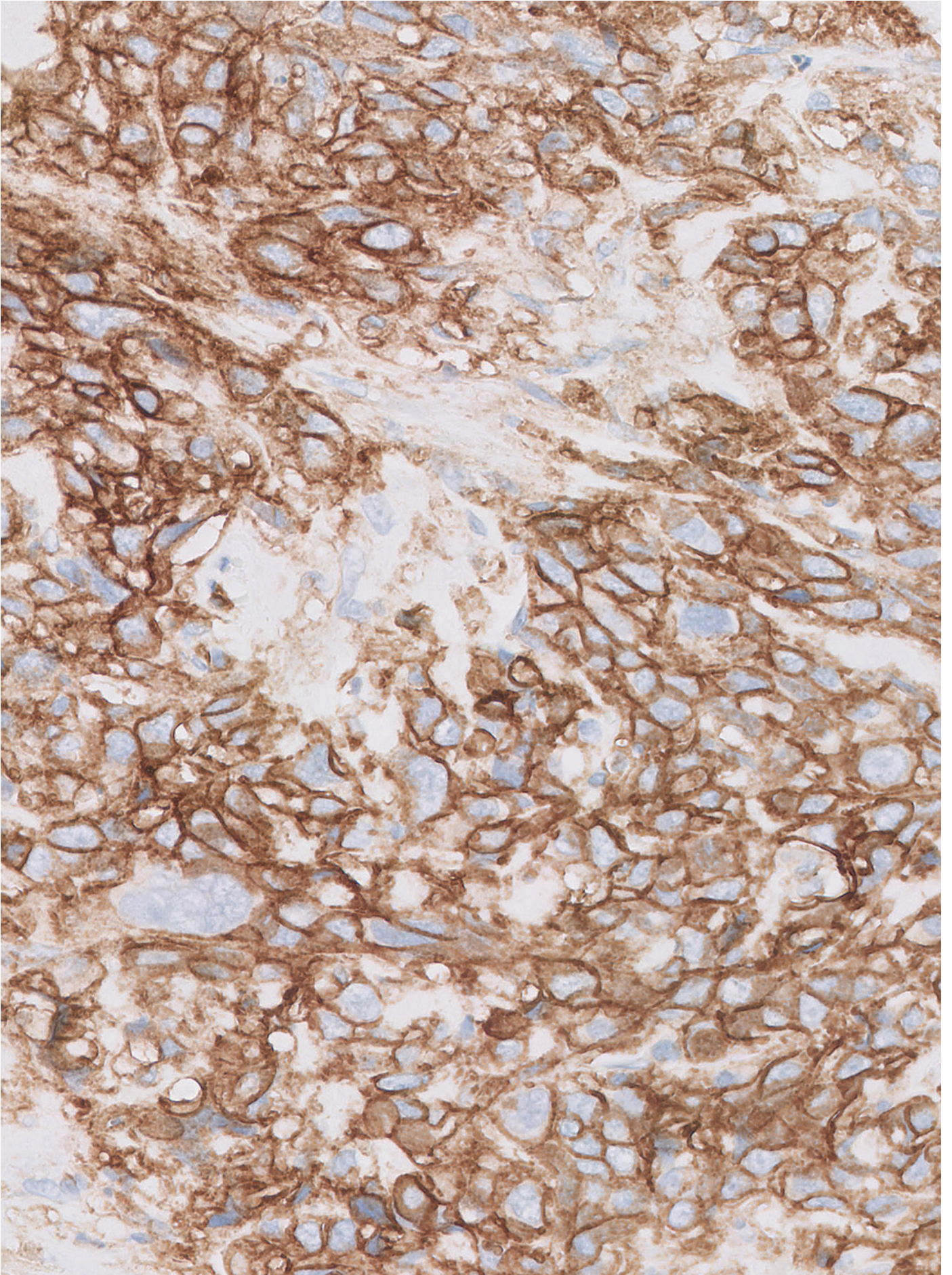
PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying NSCLC patients for treatment with KEYTRUDA® (pembrolizumab). See the KEYTRUDA product label for expression cutoff values guiding therapy in specific clinical circumstances.

Urothelial Carcinoma

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

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

KEYTRUDA is a registered trademark of Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc.



Introduction

PD-L1 IHC 22C3 pharmDx is the only companion diagnostic CE-IVD–marked 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 considered in two categories:

- CPS < 10
- CPS ≥ 10

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

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 31.

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

PD-L1 upregulation in urothelial carcinoma is a biomarker for response to anti-PD-1 therapy. PD-L1 IHC 22C3 pharmDx is the only CE-IVD–marked assay used in the KEYTRUDA® (pembrolizumab) clinical trials (KEYNOTE-052 and KEYNOTE-045) to evaluate the relationship between PD-L1 expression and clinical efficacy. KEYTRUDA is a humanized monoclonal PD-1-blocking antibody.

The PD-1/PD-L1 Pathway

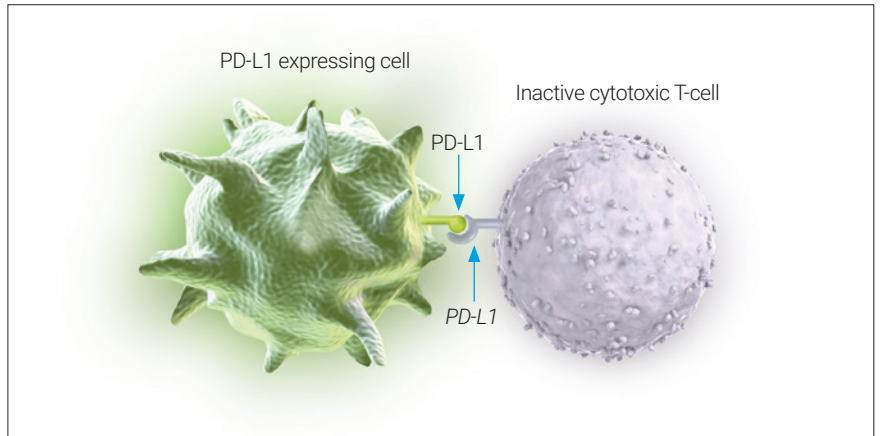


Figure 1: Inactivation of T-cells limits damage to normal tissue.

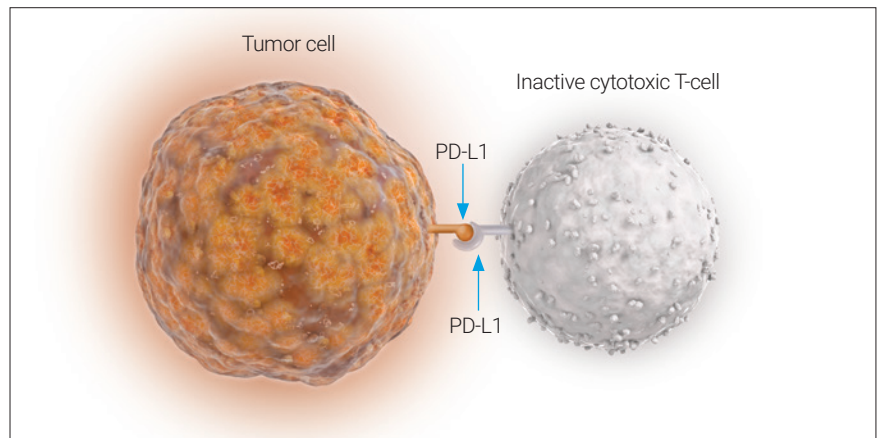


Figure 2: Inactivation of T-cells reduces tumor cell death and elimination.

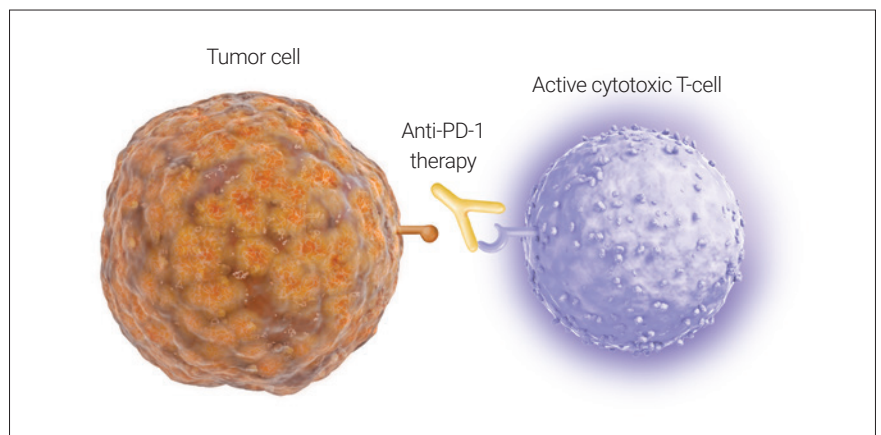


Figure 3: Blocking the PD-1/PD-L1 interaction helps to enable active T-cells and tumor cell death and elimination.

PD-L1 IHC 22C3 pharmDx Overview

What is PD-L1 IHC 22C3 pharmDx?

PD-L1 IHC 22C3 pharmDx is the only companion diagnostic indicated as an aid in identifying patients with 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.

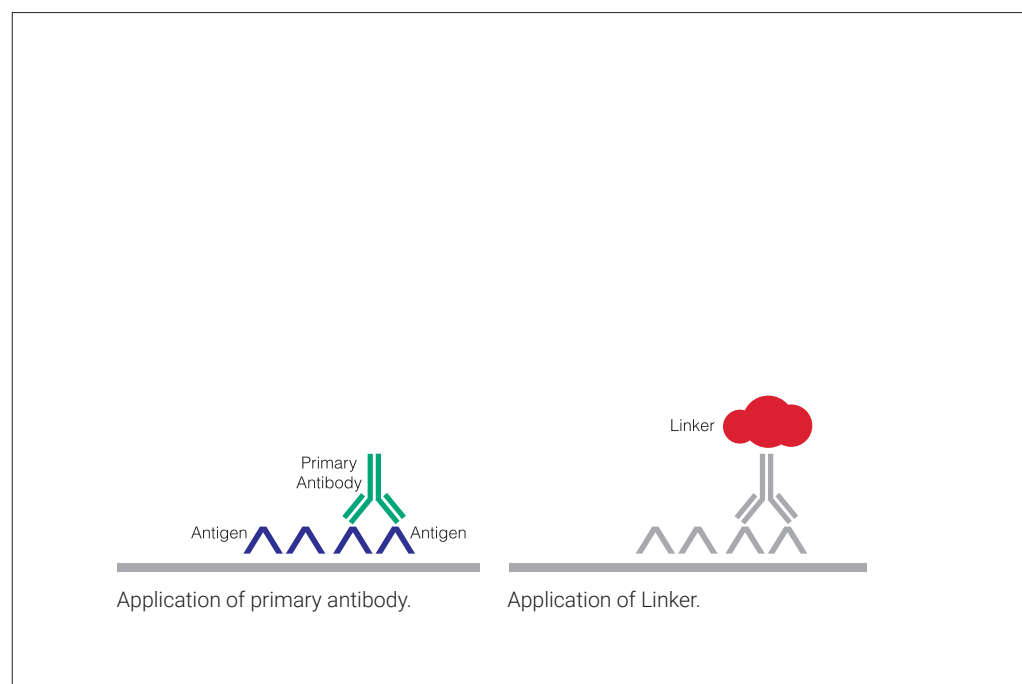


Figure 4: PD-L1 IHC 22C3 pharmDx staining procedure.

Kit Configuration



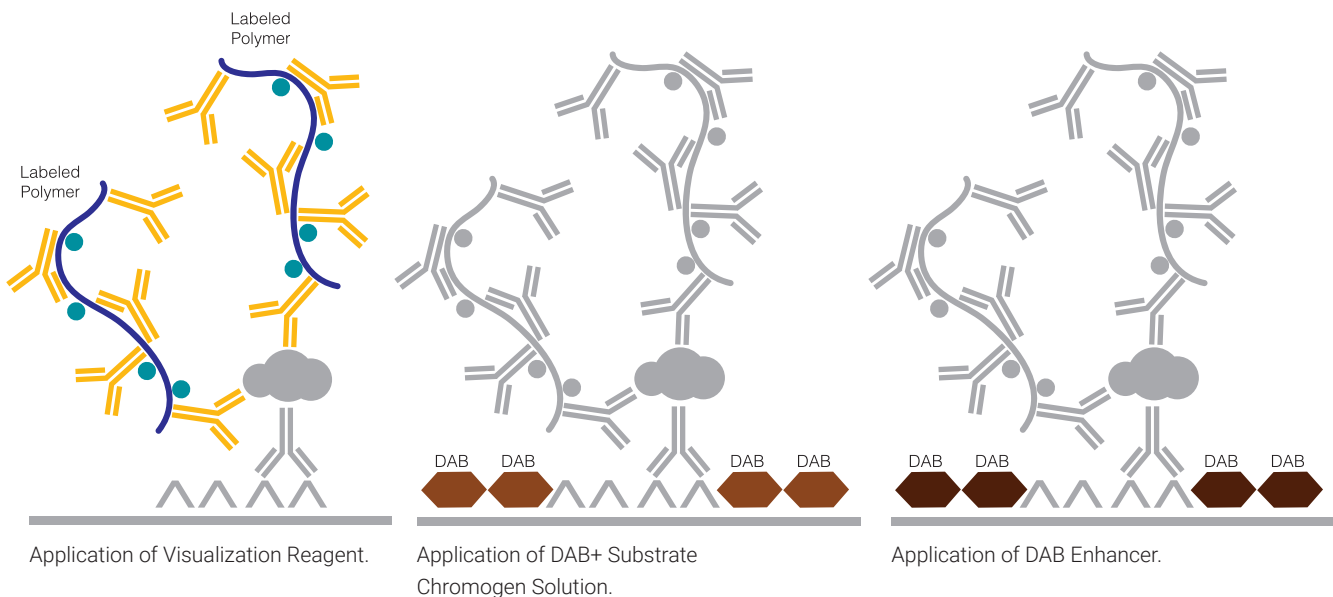
Figure 5: PD-L1 IHC 22C3 pharmDx components.

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*

* 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, (20×) (Code K8007) and EnVision FLEX Hematoxylin (Code K8008) 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.

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 a CPS less than 1 ($CPS < 1$), with 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 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 6 months of sectioning.

PD-L1 IHC 22C3 pharmDx Staining Procedure

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

Reagent Storage

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

Reagent Preparation

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

EnVision FLEX Target Retrieval Solution, Low pH

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

EnVision FLEX Wash Buffer

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

DAB+ Substrate-Chromogen Solution

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

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

Controls to Assess Staining Quality

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

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

Deparaffinization, Rehydration, and Target Retrieval

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

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

Staining and Counterstaining

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

Mounting

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

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?	<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 from urothelial carcinoma 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 Fisherbrand Superfrost Plus charged slides?	<input type="checkbox"/>	<input type="checkbox"/>
Specimens are oven-dried at 58 ± 2 °C for 1 hour?	<input type="checkbox"/>	<input type="checkbox"/>
Specimens are stained within 6 months of sectioning when stored in the dark at 2–8 °C (preferred) or at room temperature in the dark up to 25 °C?	<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"/>
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"/>
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:

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 28. 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

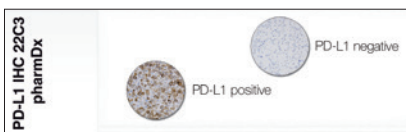


Figure 6: Each Control Cell Line Slide contains sections of cell pellets with positive and negative PD-L1 expression.

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 $\geq 70\%$ of cells
- $\geq 2+$ average staining intensity
- Non-specific staining $< 1+$ intensity

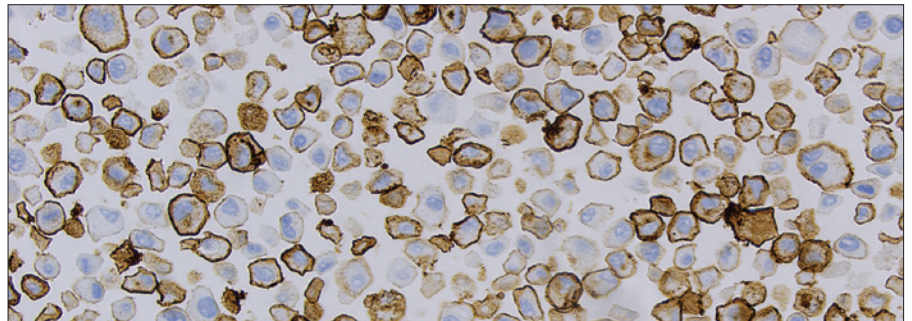


Figure 7: Positive cell pellet with acceptable staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20x magnification).

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

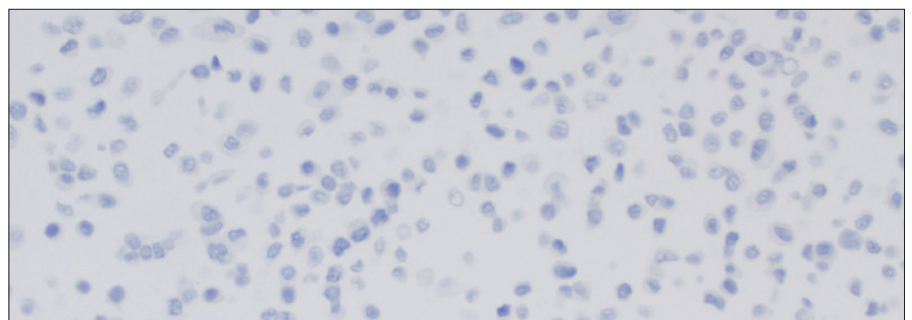


Figure 8: Negative cell pellet with no staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20x magnification).

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.

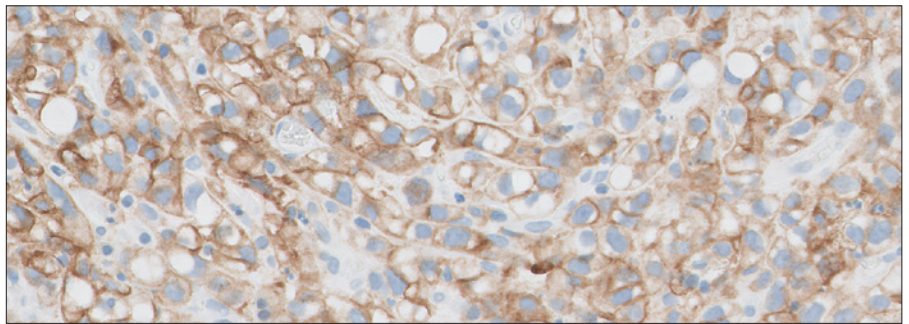


Figure 9: Ideal positive in-house control tissue (20× magnification).

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.

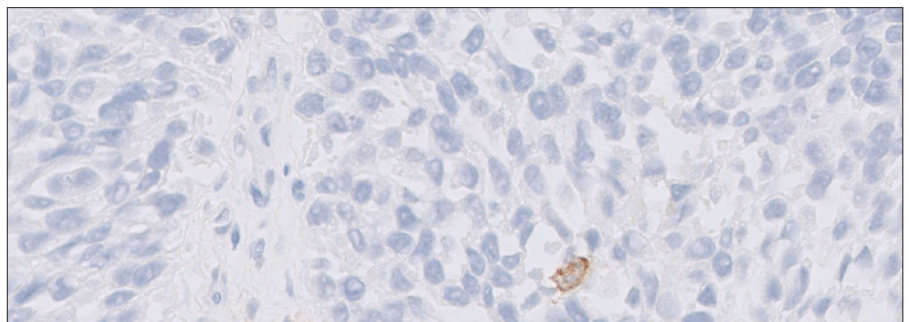


Figure 10: Ideal negative in-house control tissue demonstrating PD-L1 positive expression of CPS < 1 (20× magnification).

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.

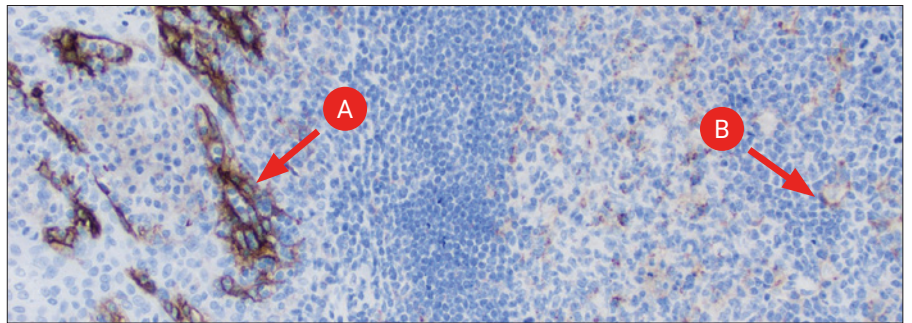


Figure 11: Tonsil stained with PD-L1 primary antibody exhibiting strong membrane staining in portions of the crypt epithelium (A) and weak-to-moderate membrane staining of follicular macrophages in the germinal centers (B) (10× magnification).

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).

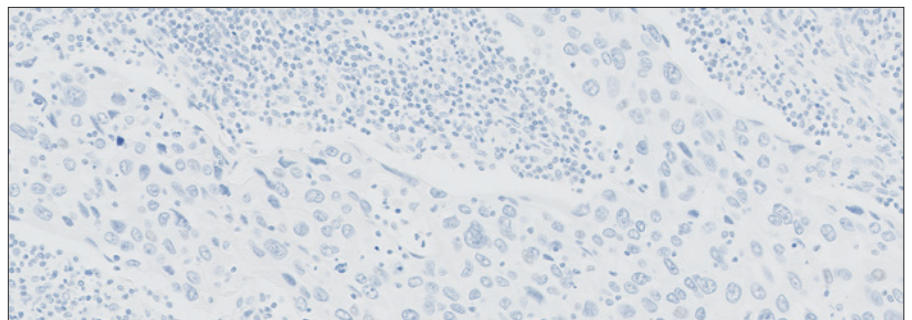


Figure 12: Ideal negative in-house control tissue stained with Negative Control Reagent showing no specific staining (20× magnification).

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

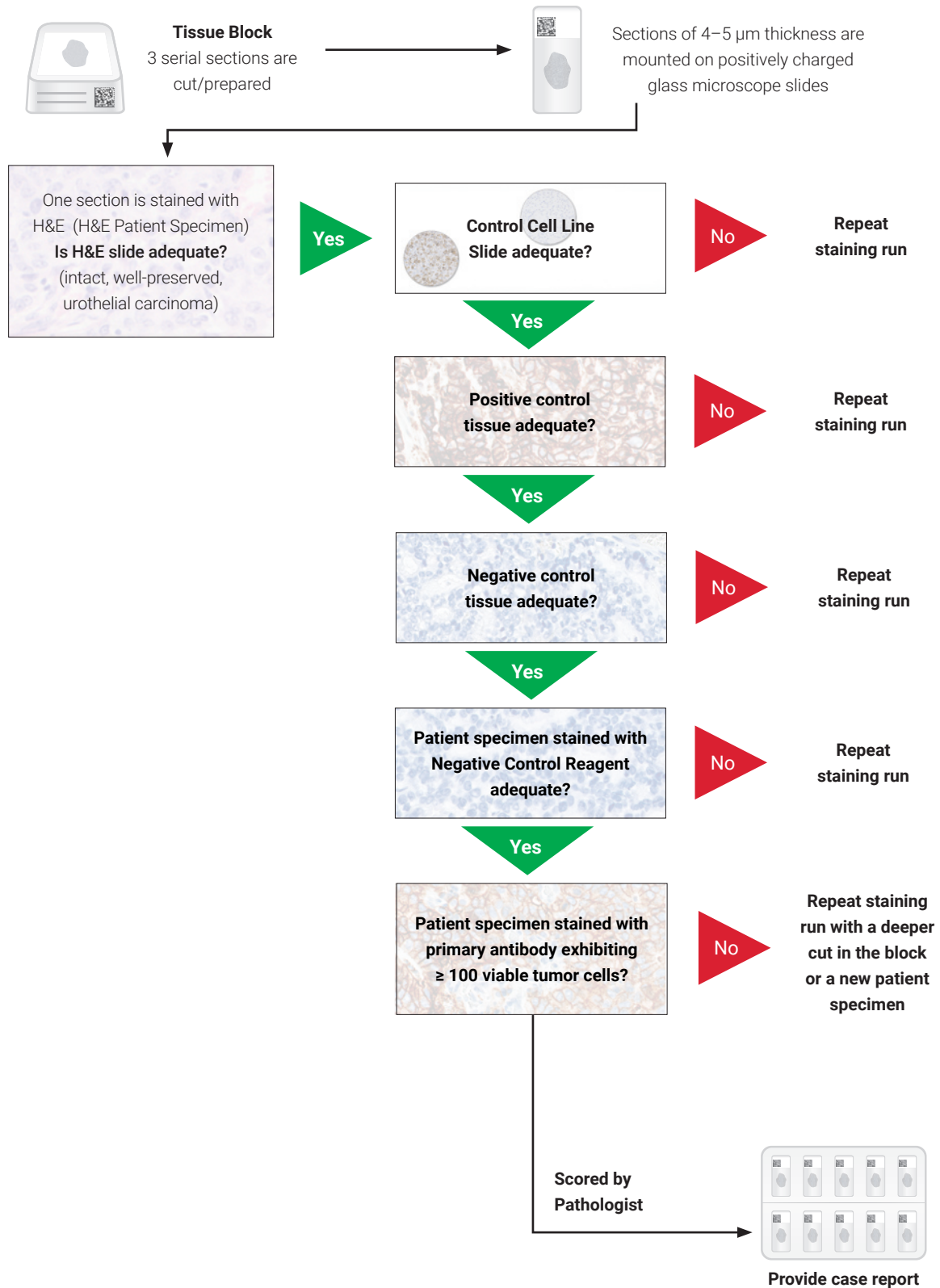


Figure 13: Recommended order of slide evaluation.

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:

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

** 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 24 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 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 (20x), evaluate PD-L1 expression and calculate CPS:
 - Determine the total number of viable tumor cells, both PD-L1 staining and non-staining (CPS denominator)
 - Determine the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) (CPS numerator; see Tables 1 and 2 on page 24 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	<p>Convincing partial or complete linear membrane staining (at any intensity) of viable urothelial carcinoma tumor cells including:</p> <ul style="list-style-type: none"> - High grade papillary carcinoma - Carcinoma in situ (CIS) - Any lamina propria, muscularis, or serosal invasion - Metastatic carcinoma 	<ul style="list-style-type: none"> - Non-staining tumor cells - Tumor cells with only cytoplasmic staining - Low grade papillary carcinoma[§]
Immune Cells	<p>Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma[†]:</p> <ul style="list-style-type: none"> - Lymphocytes (including lymphocyte aggregates) - Macrophages[‡] <p>Only MICs directly associated with the response to the tumor are scored</p>	<ul style="list-style-type: none"> - 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	<ul style="list-style-type: none"> - Normal cells - Stromal cells (including fibroblasts) - Necrotic cells and/or cellular debris

* In MICs, membrane and cytoplasmic staining are often indistinguishable due to high nuclear to cytoplasmic ratio. Therefore, membrane and/or cytoplasmic staining of MICs are included in the score

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

‡ Macrophages and histiocytes are considered the same cells

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

** Bacillus Calmette-Guérin

Table 2: CPS denominator inclusion/exclusion criteria

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	<p>All viable tumor cells including:</p> <ul style="list-style-type: none"> - High grade papillary carcinoma - Carcinoma in situ (CIS) - Any lamina propria, muscularis, or serosal invasion - Metastatic carcinoma 	<ul style="list-style-type: none"> - 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	<ul style="list-style-type: none"> - 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.

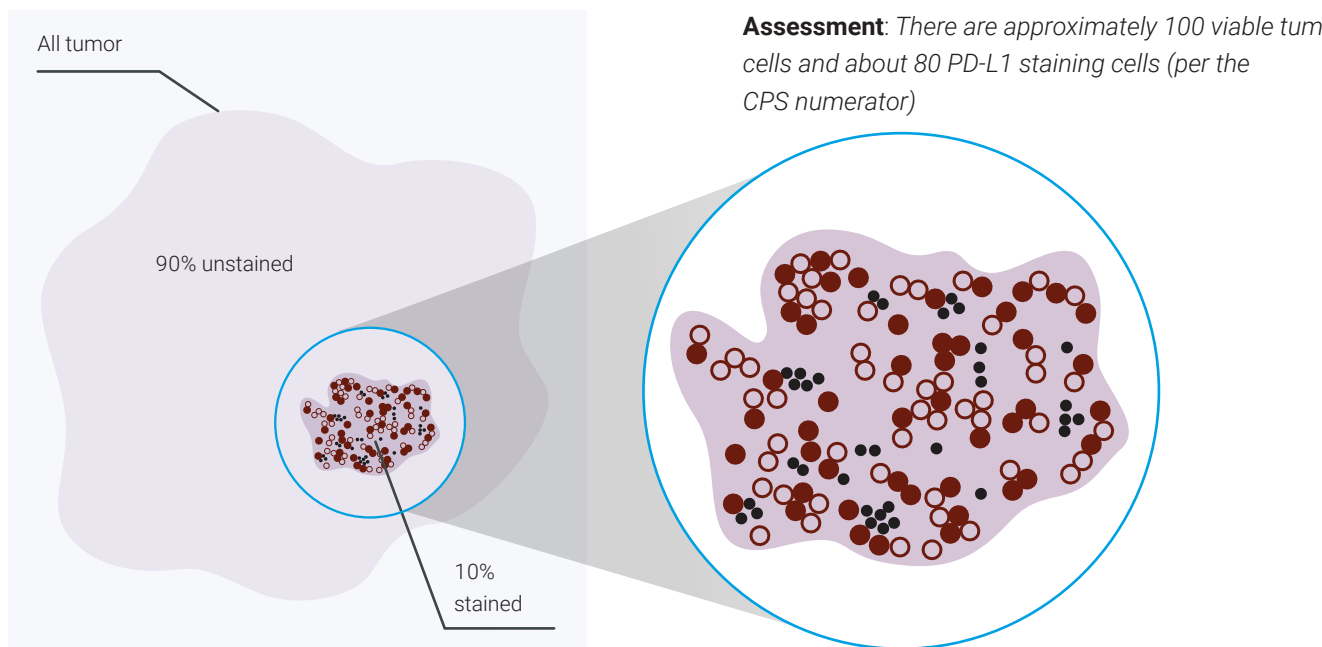
Example 1: Calculation of Combined Positive Score in a Small Tumor Area With Staining

At lower magnifications (4x, 10x): Evaluate the tumor area for convincing staining as described in “Determining Combined Positive Score” on page 23.

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 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

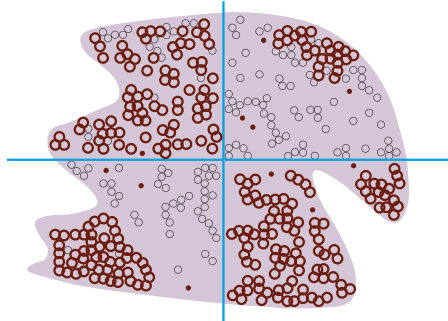
PD-L1 Protein Expression Category: CPS < 10

* Including tumor cells, lymphocytes, macrophages

Figure 14: Example of tumor with small staining area.

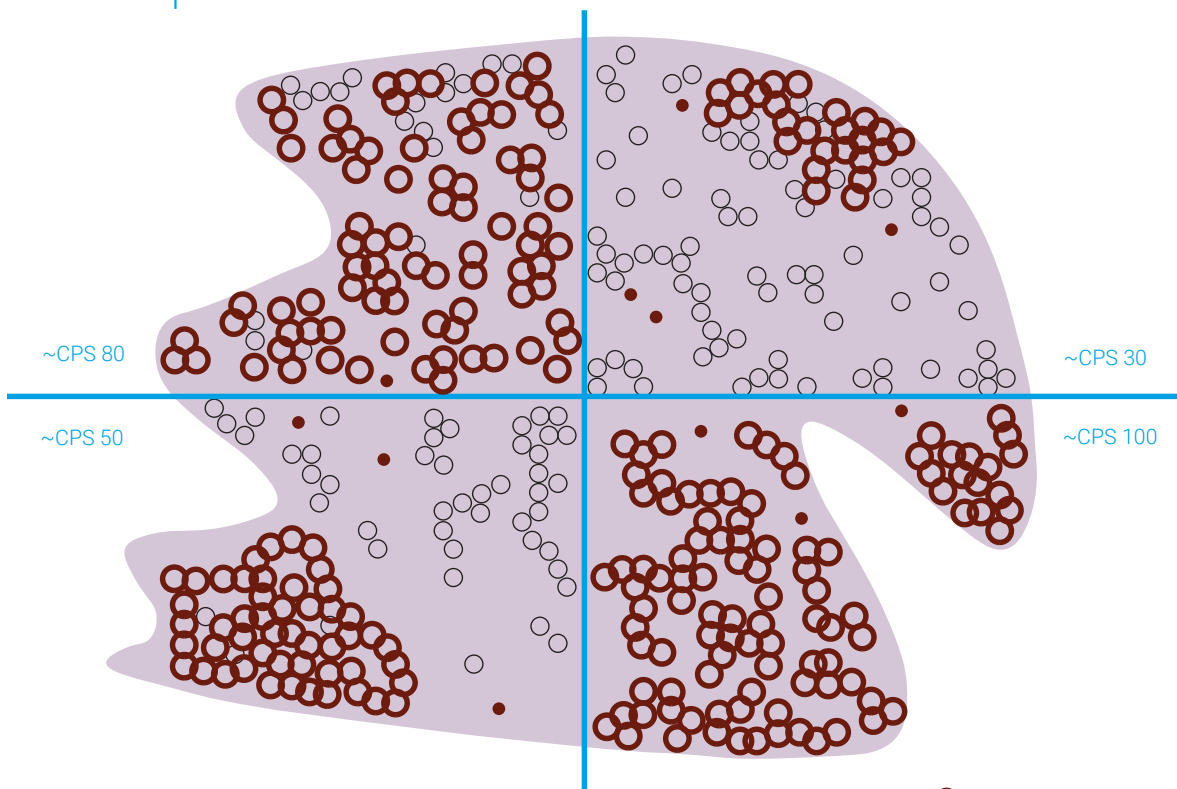
Example 2: Calculation of Combined Positive Score in a Heterogeneous Tumor Area

At lower magnifications (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



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

Calculate the Combined Positive Score of the entire tumor area:

Assessment:

Combined Positive Score:

$$(80 + 30 + 50 + 100) / 4 = \sim\text{CPS } 65$$

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

PD-L1 Protein Expression Category: CPS ≥ 10

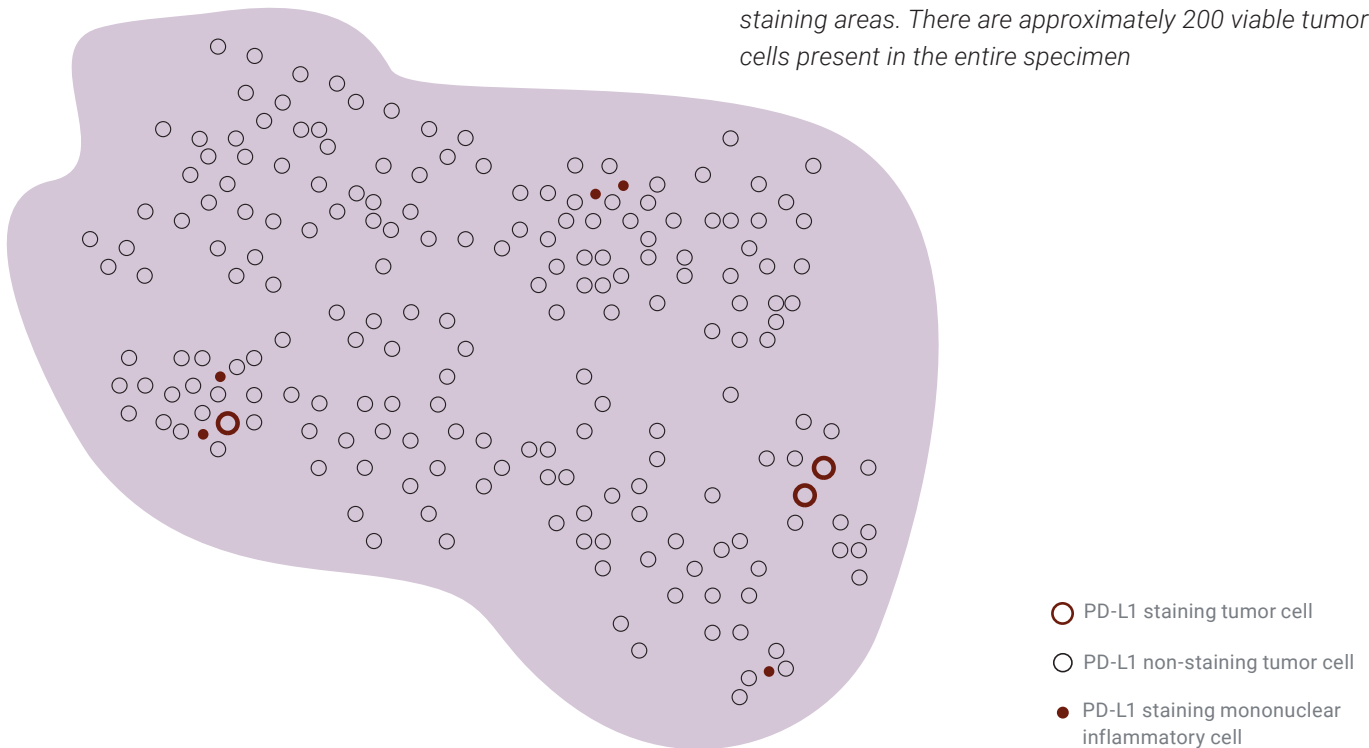
Figure 15: Example with heterogeneous tumor area.

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

At lower magnifications (4x, 10x): Evaluate the specimen for convincing staining as described in “Determining Combined Positive Score” on page 23.

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 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:

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

PD-L1 Protein Expression Category: CPS < 10

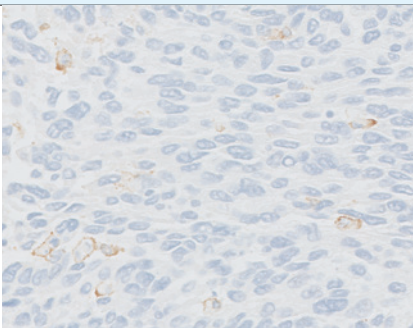
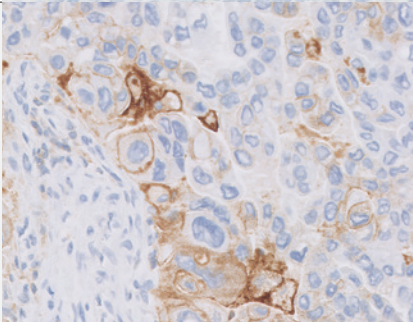
* Including tumor cells, lymphocytes, macrophages

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× magnification)
<p>< 10</p>	<p>CPS is less than 10</p>	
<p>≥ 10</p>	<p>CPS is greater than or equal to 10</p>	

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 Patients Not Eligible for Cisplatin-containing Chemotherapy With Locally Advanced or Metastatic Urothelial Carcinoma

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 with locally advanced or metastatic urothelial carcinoma 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 70–71.

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)

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

The clinical validity of PD-L1 IHC 22C3 pharmDx in evaluating PD-L1 expression in previously treated patients with urothelial carcinoma is based on the KEYTRUDA KEYNOTE-045 study sponsored by Merck Sharp & Dohme Corp. Specimens from previously treated patients with urothelial carcinoma 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 72–74.

Table 5: PD-L1 prevalence in previously treated patients with urothelial carcinoma enrolled in KEYNOTE-045

PD-L1 Expression	CPS < 10	CPS ≥ 10
Prevalence (n) †	66.8% (362)	30.2% (164)

* 9 patients had unknown PD-L1 status
 † 16 patients (10 from pembrolizumab and 6 from chemotherapy) 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.

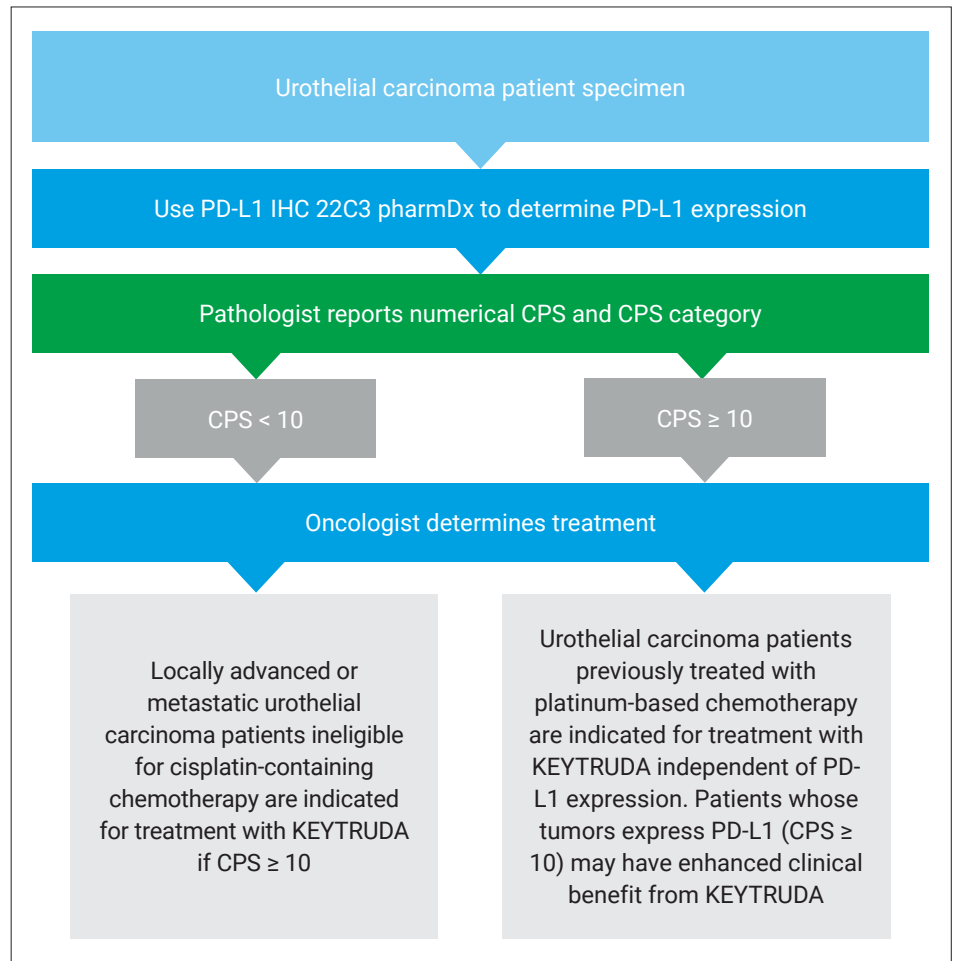


Figure 17: Testing algorithm for PD-L1 IHC 22C3 pharmDx.

Reporting Results

Suggested information to include when reporting results 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 adjacent supporting stroma with membrane and/or cytoplasmic staining (at any intensity). MICs must be directly associated with the response against the tumor

PD-L1 expression status in 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 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 24 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 urothelial carcinoma 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 20× magnification.

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

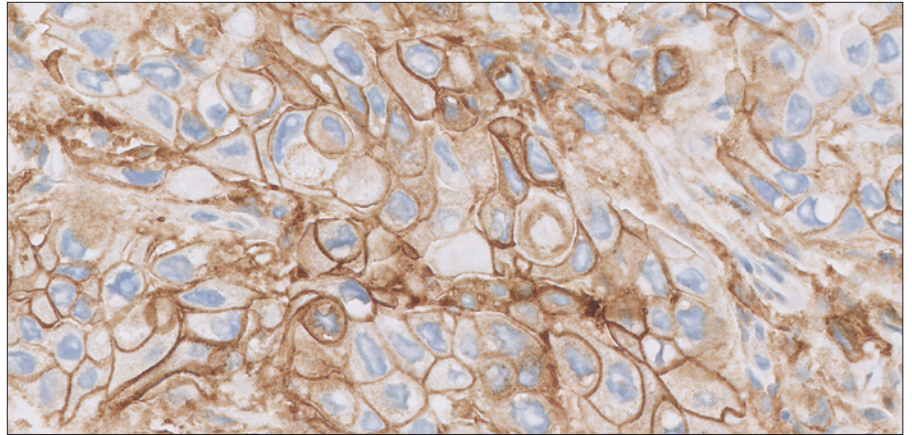


Figure 18: PD-L1 primary antibody exhibiting linear membrane staining of tumor cells (20× magnification).

Key point

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

Partial and Complete Linear Membrane Staining

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

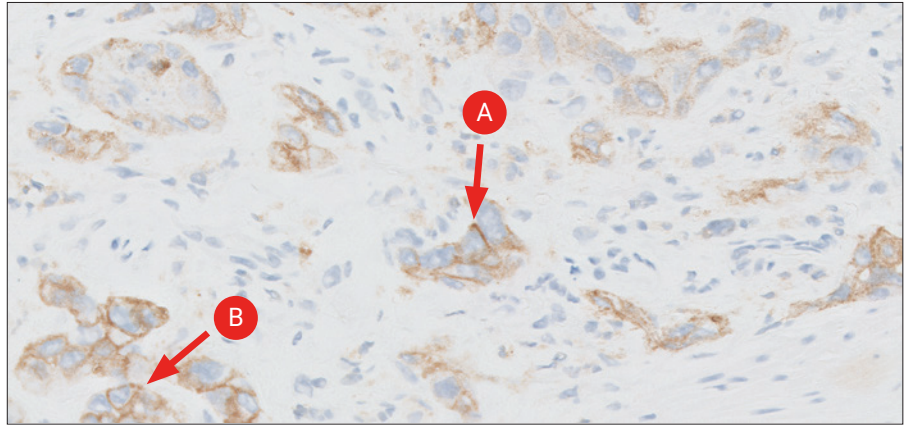


Figure 19: PD-L1 primary antibody exhibiting partial (A) and complete (B) linear membrane staining of tumor cells (20× magnification).

Key point

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

Weak Linear Membrane Staining

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

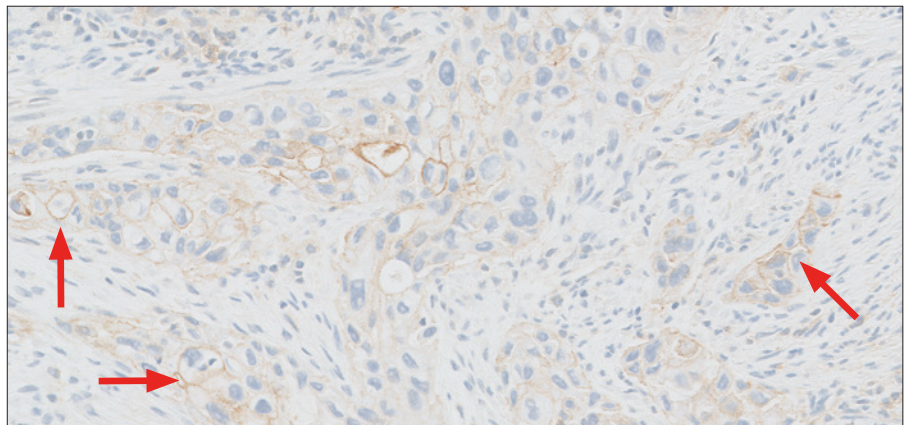


Figure 20: PD-L1 primary antibody exhibiting weak but perceptible and convincing membrane staining of tumor cells (arrows) (20× magnification).

Key point

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

Linear Membrane and Cytoplasmic Staining

Tumor cells with both convincing linear membrane staining ($\geq 1+$ intensity) and cytoplasmic staining at no higher than 20 \times magnification should be included in the CPS numerator. Tumor cells exhibiting only cytoplasmic staining are excluded from the CPS numerator, as this is considered non-specific staining.

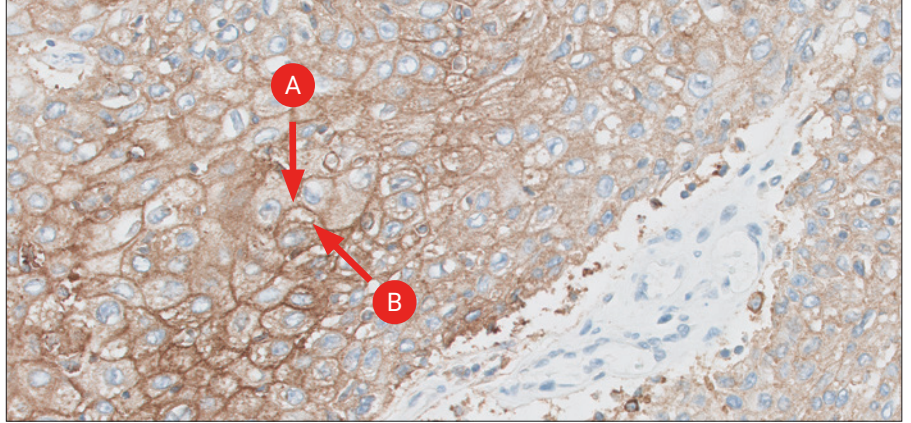


Figure 21: PD-L1 primary antibody exhibiting linear membrane (A) staining distinct from cytoplasmic (B) staining (20 \times magnification).

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 20× magnification ($\geq 1+$ intensity) are considered PD-L1 staining cells and should be included in the CPS numerator. MICs are considered tumor-associated if they are present within the tumor nests and/or adjacent supporting stroma within a 20× magnification field of view. In cases where it is difficult to tell if MICs are tumor-associated, the following is suggested as a guideline:

Move the slide so that the tumor is in the approximate center of a 20× field. Immune cells surrounding the tumor in this field should be included in scoring. Immune cells outside of this field should be excluded from scoring as long as they do not surround neighboring tumor cells. In general, include PD-L1 staining MICs that are within 0.5 mm of the tumor cells.

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.

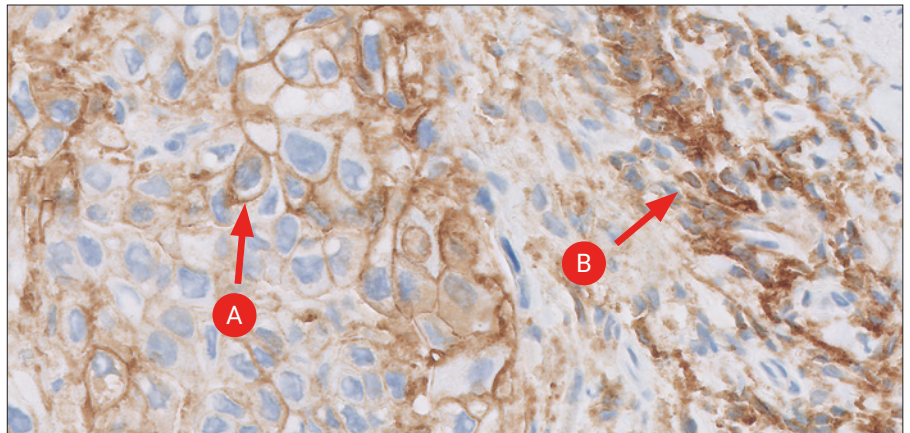


Figure 22a: PD-L1 primary antibody exhibiting staining of tumor (A) and tumor-associated mononuclear inflammatory cells (MICs) (B) (20× magnification).

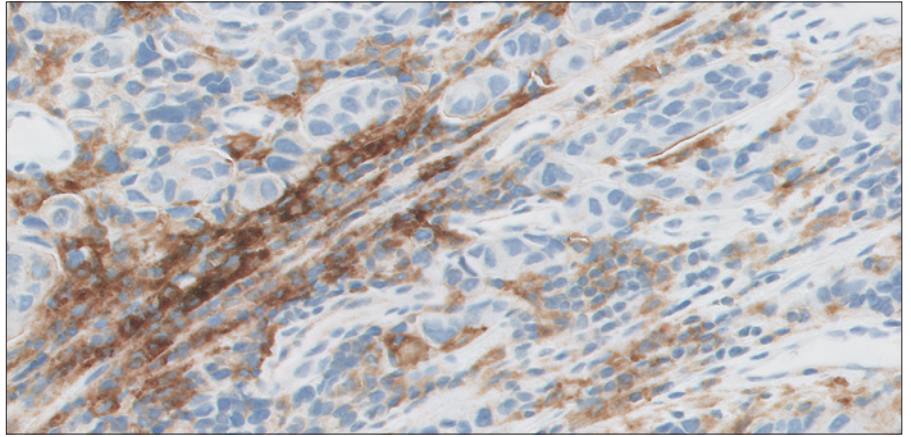


Figure 22b: PD-L1 primary antibody exhibiting staining of tumor-associated lymphocytes (20× magnification). **Note:** Tumor-associated lymphocytes are present within the tumor nests and/or adjacent supporting stroma, and are directly associated with the response against the tumor.

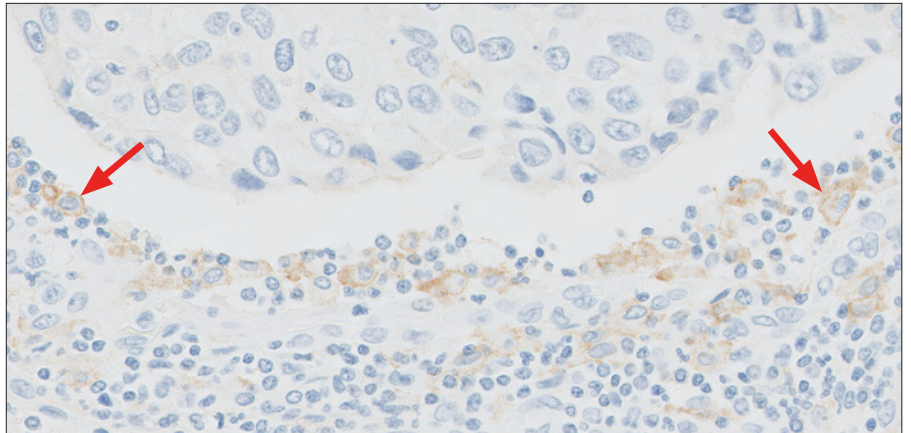


Figure 22c: PD-L1 primary antibody exhibiting moderate staining of tumor-associated macrophages (arrows) (20× magnification). **Note:** Tumor-associated macrophages are present within the tumor nests and/or adjacent supporting stroma, and are directly associated with the response against the tumor.

Key point

Tumor-associated lymphocytes and macrophages with 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 ($\geq 1+$ 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 adjacent supporting stroma, and are directly associated with the response against the tumor.

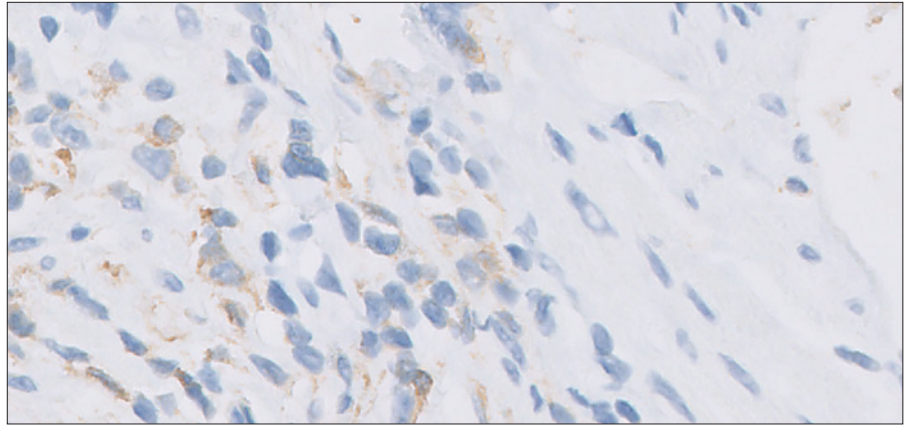


Figure 23a: PD-L1 primary antibody exhibiting weak staining of tumor-associated mononuclear inflammatory cells (MICs) (20× magnification). **Note:** Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor nests and/or adjacent supporting stroma, and are directly associated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

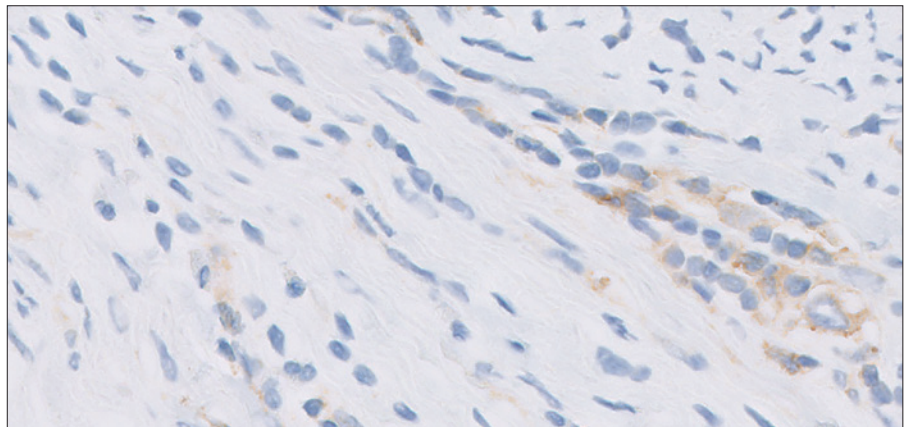


Figure 23b: PD-L1 primary antibody exhibiting weak staining of tumor-associated mononuclear inflammatory cells (MICs) (20× magnification). **Note:** Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor nests and/or adjacent supporting stroma, and are directly associated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

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

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

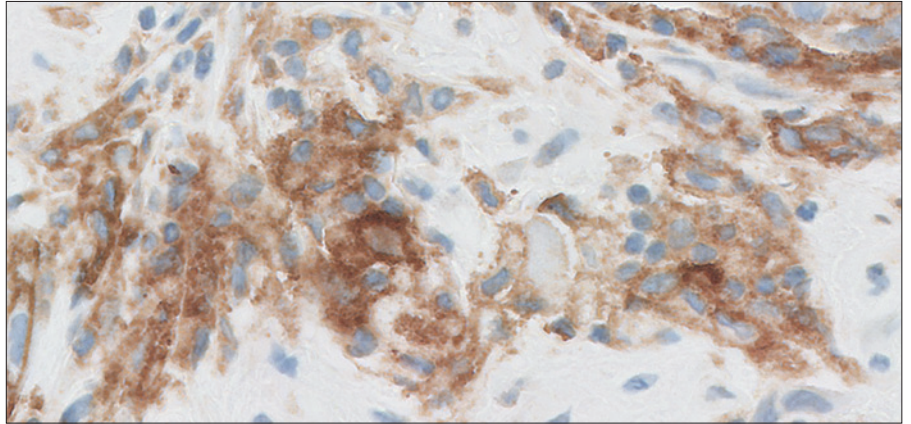


Figure 24: PD-L1 primary antibody exhibiting strong staining of tumor-associated mononuclear inflammatory cells (MICs) (20× magnification). **Note:** Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor nests and/or adjacent supporting stroma, and are directly associated 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 20x magnification, any convincing staining of tumor cells and tumor-associated lymphocytes and macrophages at any intensity should be included in the CPS numerator.

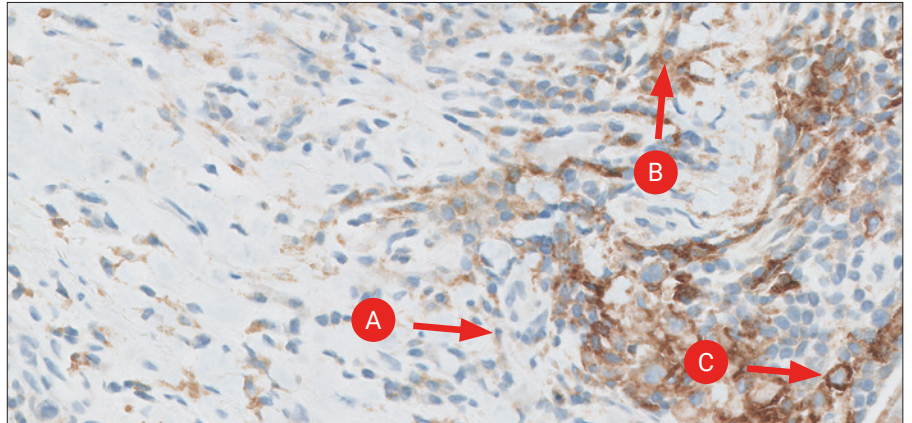


Figure 25a: PD-L1 primary antibody exhibiting heterogeneous staining intensities of mononuclear inflammatory cells (MICs) (A: 1+ intensity, B: 2+ intensity, C: 3+ intensity) (20x magnification).
Note: Tumor-associated mononuclear inflammatory cells (MICs) are present within the tumor nests and/or adjacent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

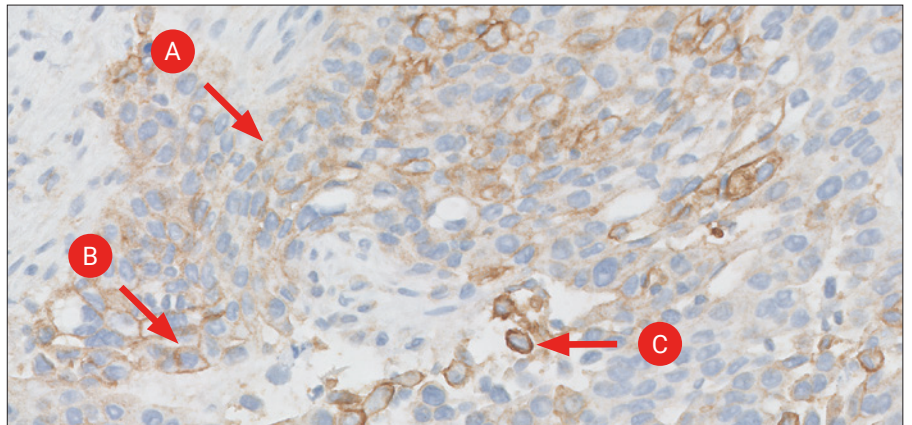


Figure 25b: PD-L1 primary antibody exhibiting heterogeneous staining intensities of tumor cells (A: 1+ intensity, B: 2+ intensity, C: 3+ intensity) (20x magnification).

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

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.

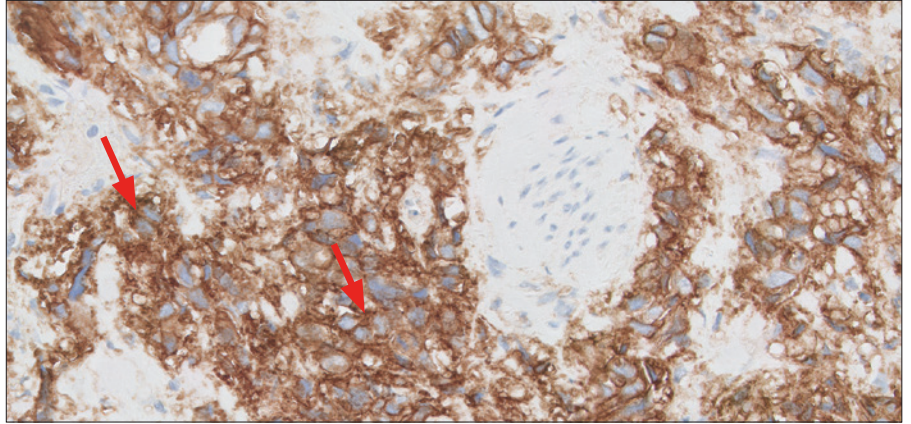


Figure 26: PD-L1 primary antibody exhibiting granular linear membrane staining pattern (arrows) (20× magnification).

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.

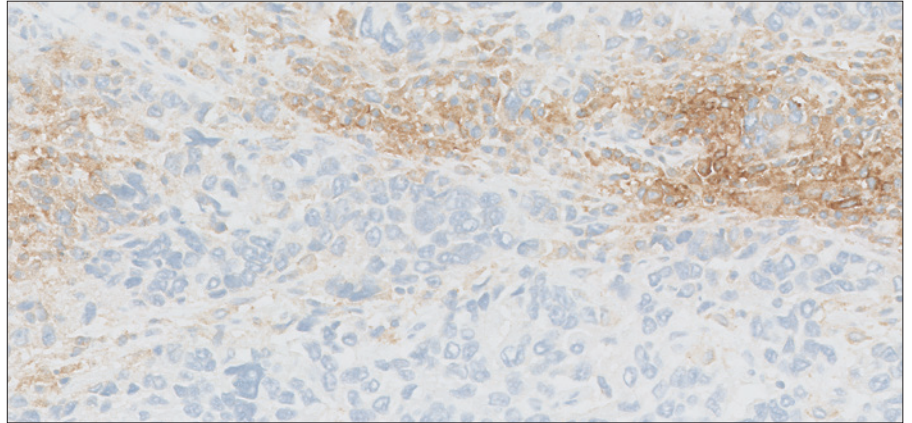


Figure 27a: Tumor and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other and exhibit PD-L1 primary antibody staining (20× magnification).

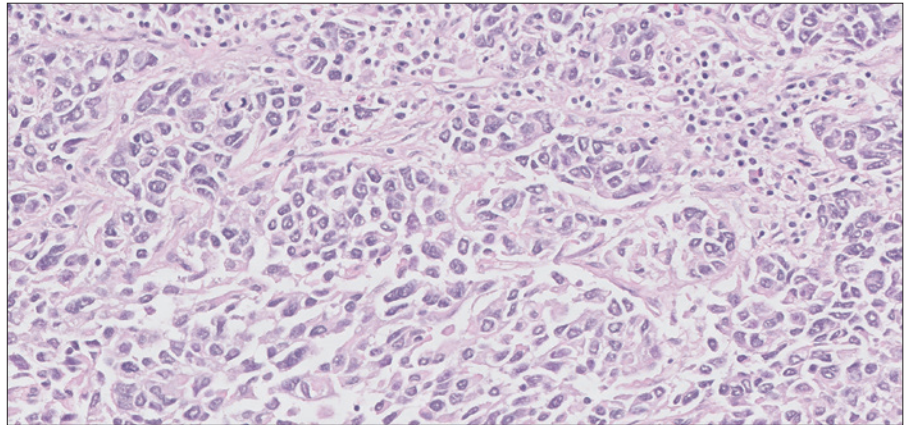


Figure 27b: Corresponding H&E to reference when tumor and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other (20× magnification).

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.

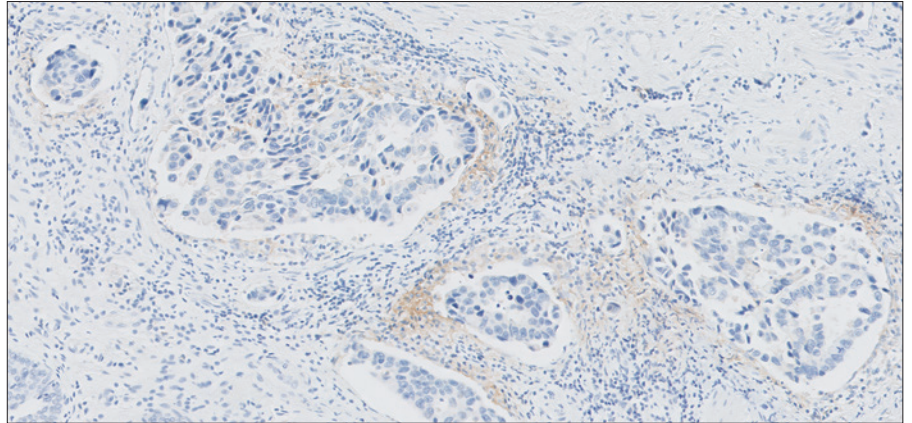


Figure 28: PD-L1 primary antibody exhibiting staining of tumor-associated mononuclear inflammatory cells (MICs) in lamina propria invasion (10× magnification).

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

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 only some exclusion elements, therefore not all exclusions are represented by images in this manual. Please refer to Tables 1 and 2 on page 24 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.

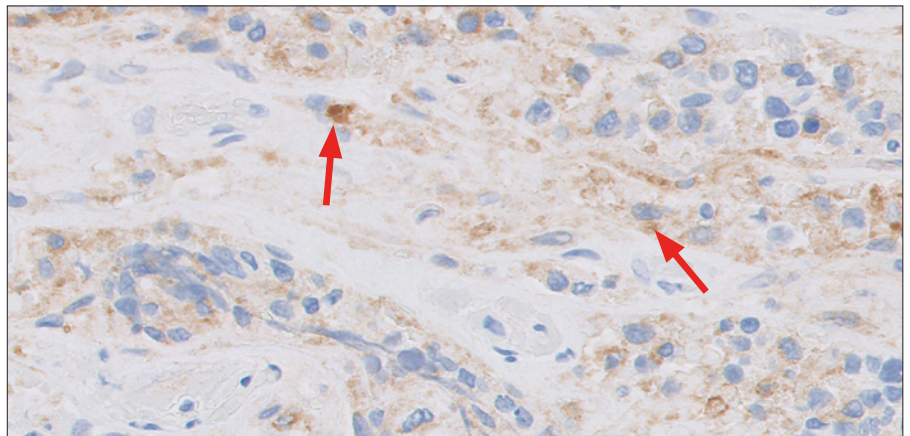


Figure 29: PD-L1 primary antibody exhibiting staining of tumor cytoplasm only (arrows) (20× magnification).

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 adjacent supportive stroma within a 20× magnification field of view; 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.

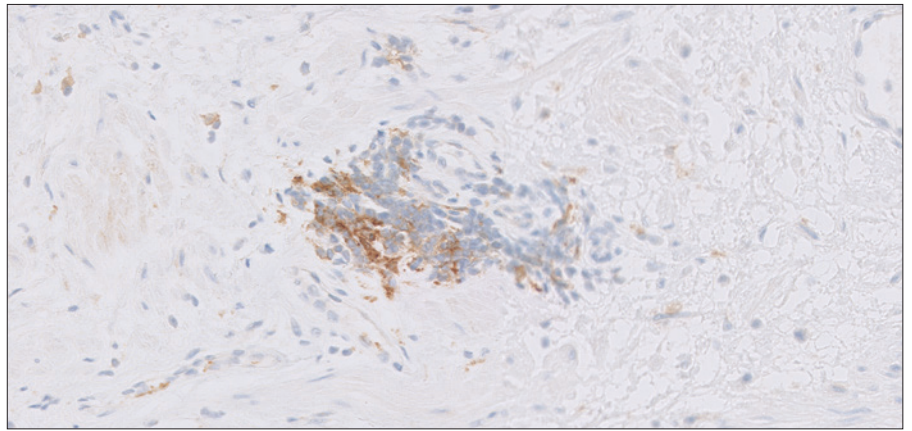


Figure 30a: Tumor cells are absent from this 20× field containing PD-L1 staining mononuclear inflammatory cells, thus none of these cells should be included in the numerator (20× magnification).

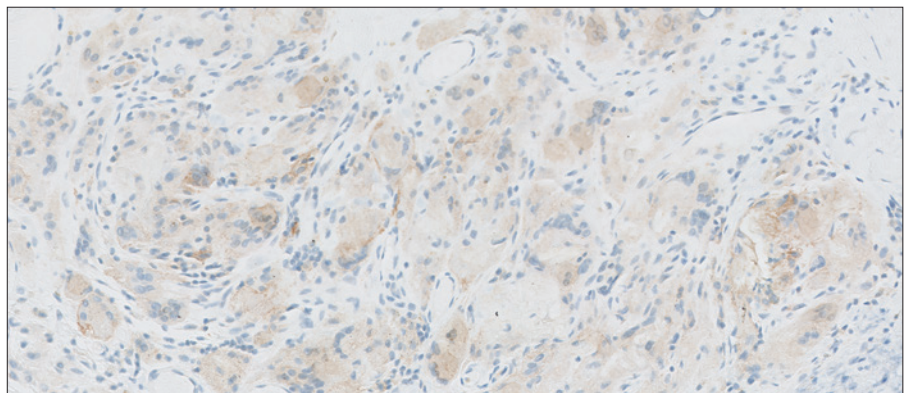


Figure 30b: PD-L1 primary antibody exhibiting staining of cells associated with BCG-induced granulomas (20× magnification).

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.

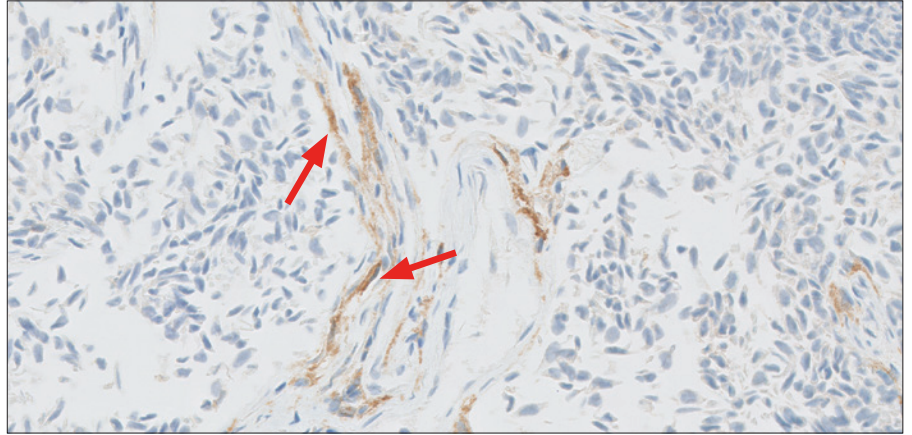


Figure 31a: PD-L1 primary antibody exhibiting staining of stromal cells (arrows) (20× magnification).

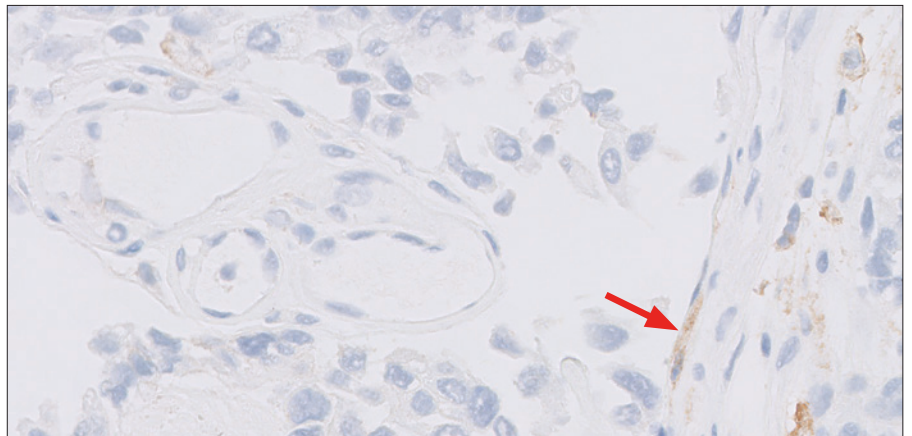


Figure 31b: PD-L1 primary antibody exhibiting staining of a fibroblast (arrow) (20× magnification).

Key point

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

Artifacts

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

Non-specific Background Staining

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

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

The non-specific background staining of the NCR-stained test specimen is useful in determining the level of background staining in the positive test specimen.

All specimens must have $\leq 1+$ non-specific background staining.

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

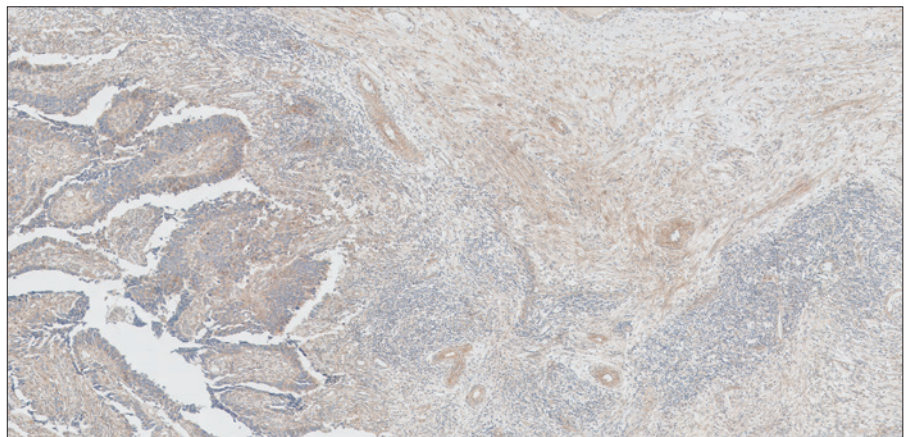


Figure 32a: Negative Control Reagent (NCR) exhibiting unacceptable non-specific background staining (5x magnification).

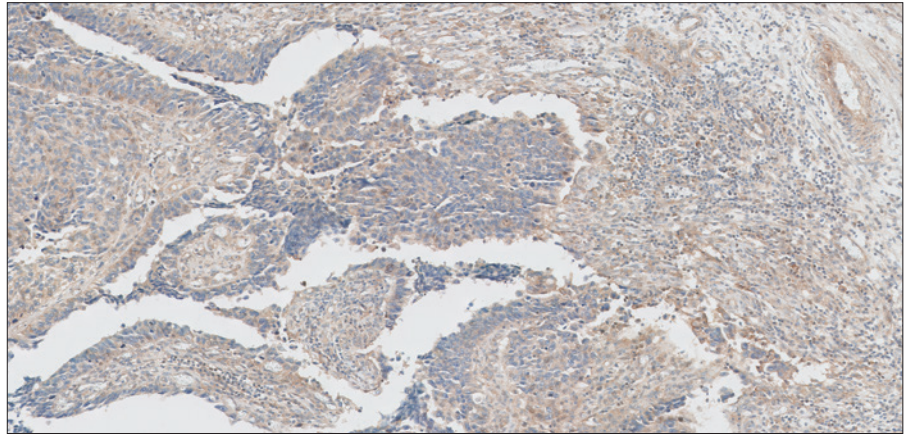


Figure 32b: PD-L1 primary antibody exhibiting blush staining; blush should be excluded from scoring (10x magnification).

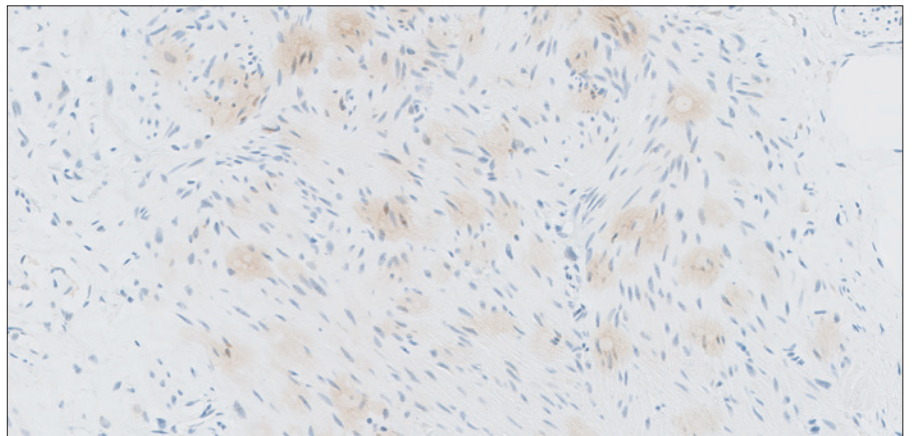


Figure 32c: Staining caused by DAB droplets should be excluded from scoring (20x magnification).

Key point

All specimens must have $\leq 1+$ non-specific background staining

Edge Artifact

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

- Thick tissue sections ($> 4 \mu\text{m}$)
- Drying of tissue prior to fixation or during staining procedure

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

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

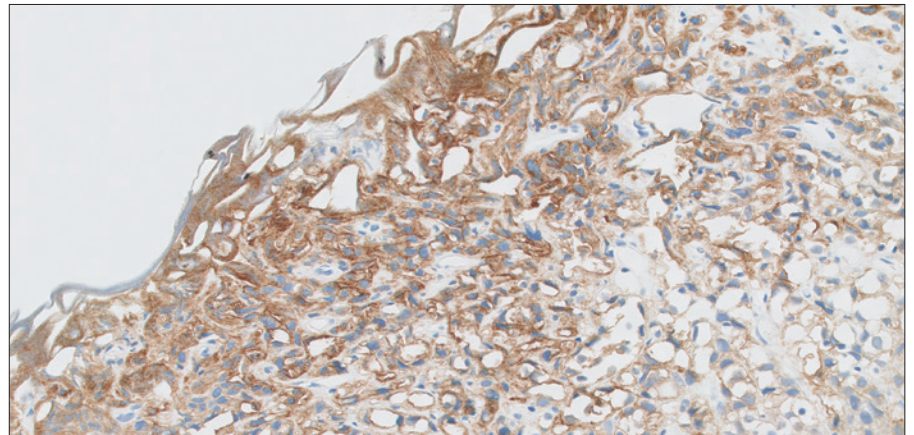


Figure 33: PD-L1 primary antibody exhibiting edge artifact staining; edge staining should be excluded from scoring (10 \times magnification).

Key point

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

Poor Fixation

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

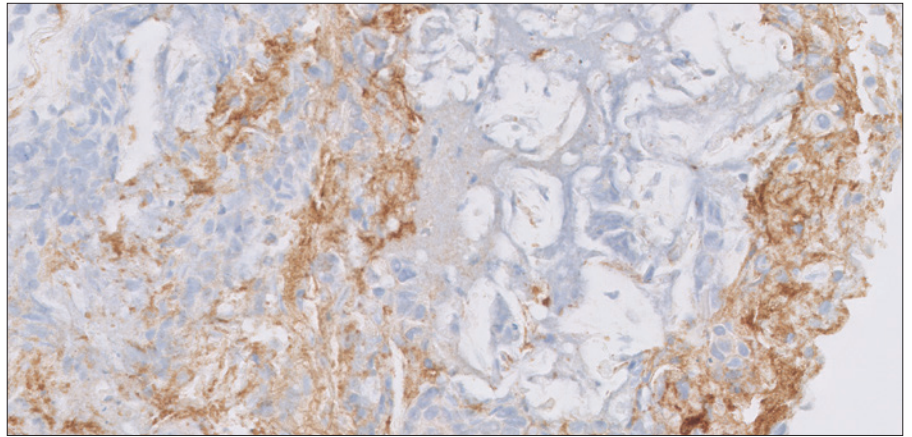


Figure 34: PD-L1 primary antibody exhibiting poor tissue fixation (20× magnification).

Key point

Proper fixation is important for accurate diagnosis

Necrosis

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

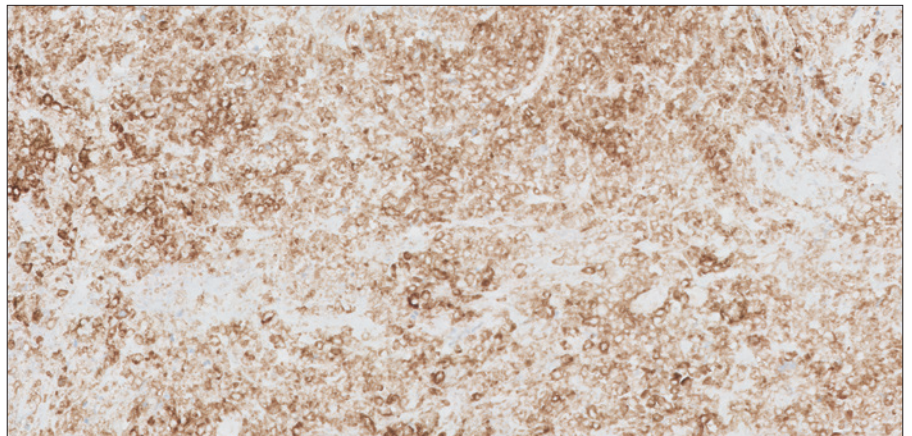


Figure 35: PD-L1 primary antibody exhibiting staining of necrosis; necrosis should not be scored (20× magnification).

Key point

Scoring of necrotic areas should be excluded from the CPS calculation

CPS Case Examples

CPS < 10 Case Examples

Case 1: CPS < 10

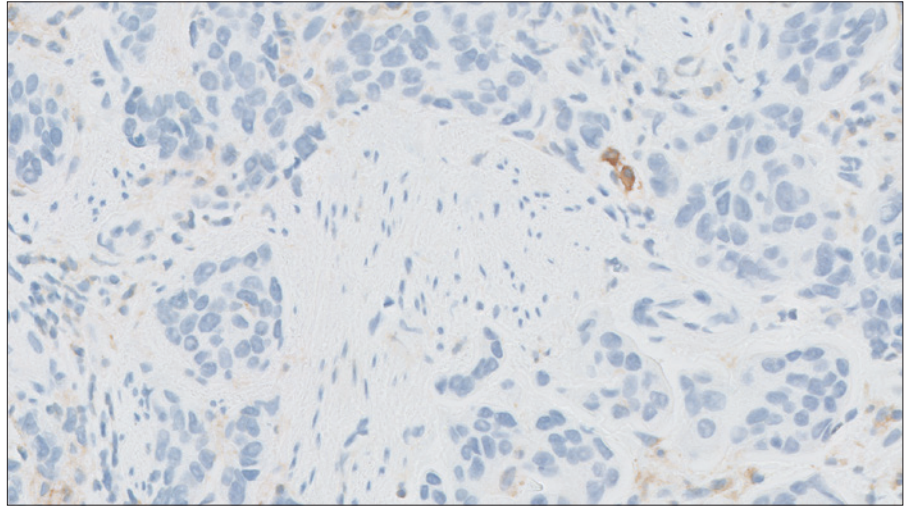


Figure 36a: 10x magnification.

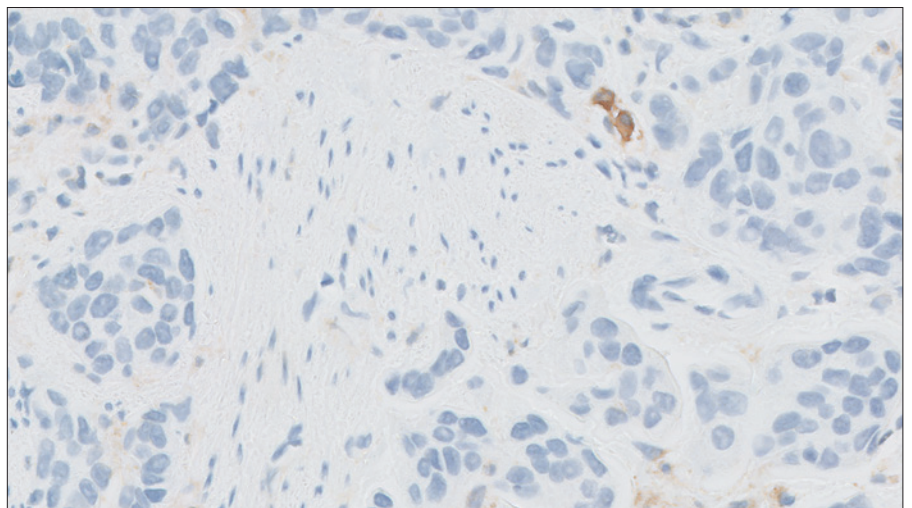


Figure 36b: 20x magnification.

PD-L1 antibody exhibiting CPS < 10 at 20x magnification.

Case 2: CPS < 10

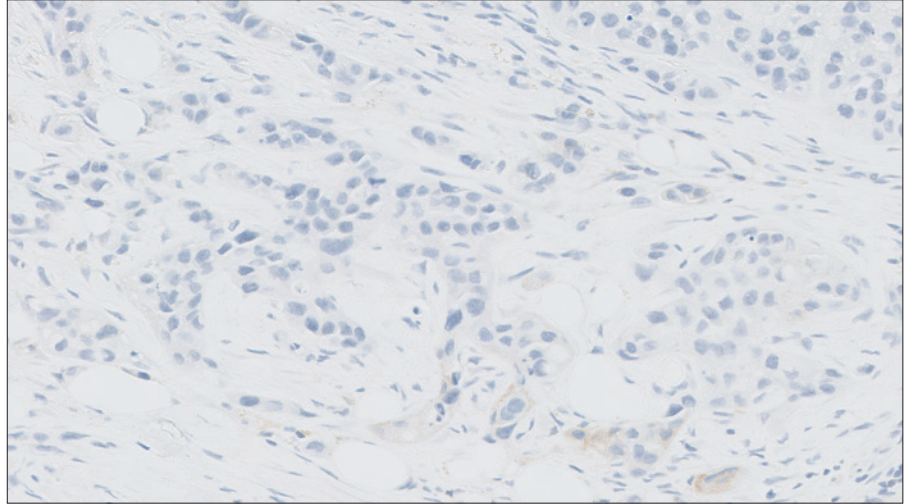


Figure 37a: 10x magnification.

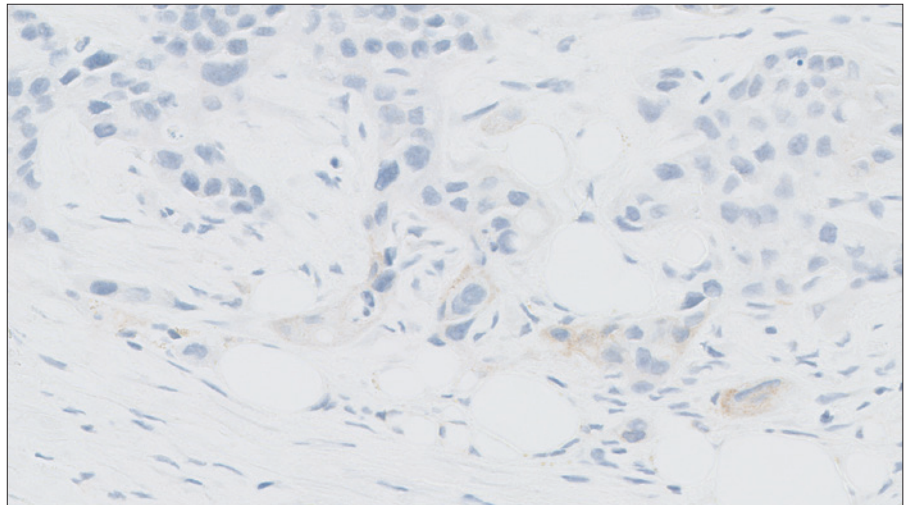


Figure 37b: 20x magnification.

PD-L1 antibody exhibiting CPS < 10 at 20x magnification.

Case 3: CPS < 10

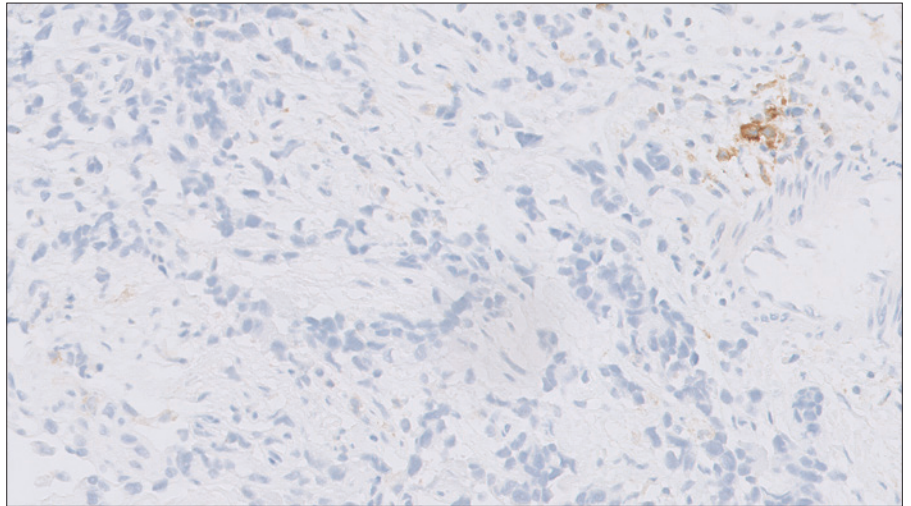


Figure 38a: 10× magnification.

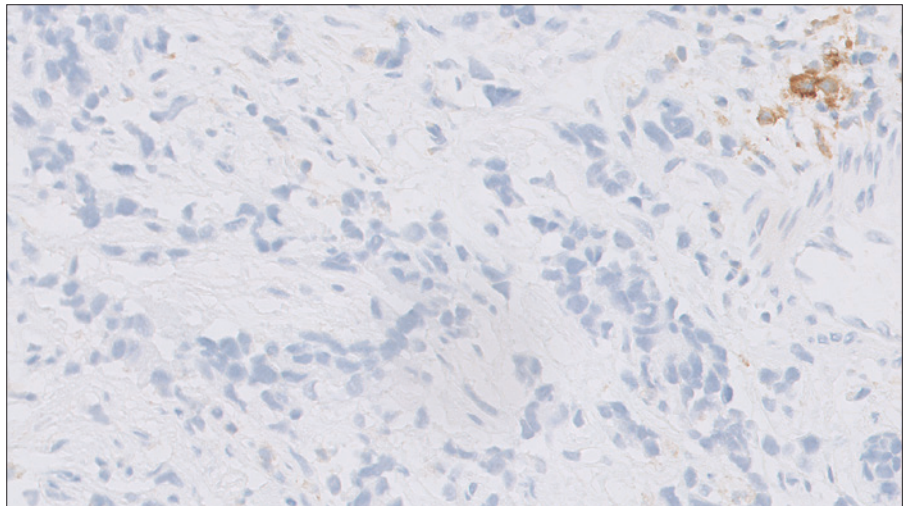


Figure 38b: 20× magnification.

PD-L1 antibody exhibiting CPS < 10 at 20× magnification.

Case 4: CPS < 10

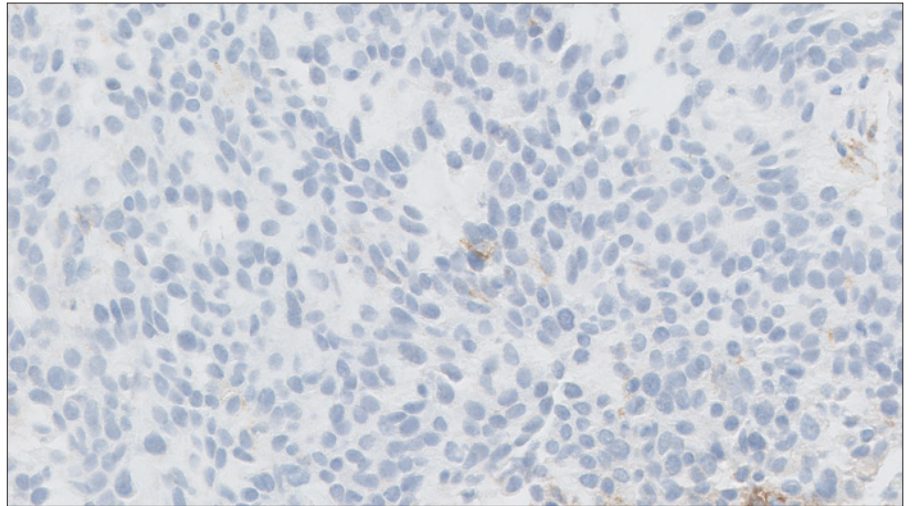


Figure 39a: 10x magnification.

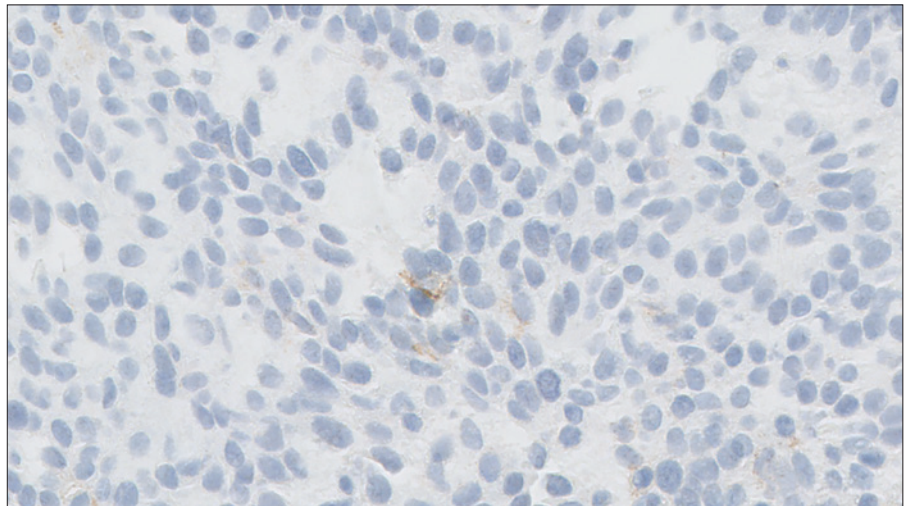


Figure 39b: 20x magnification.

PD-L1 antibody exhibiting CPS < 10 at 20x magnification.

CPS \geq 10 Case Examples

Case 5: CPS \geq 10

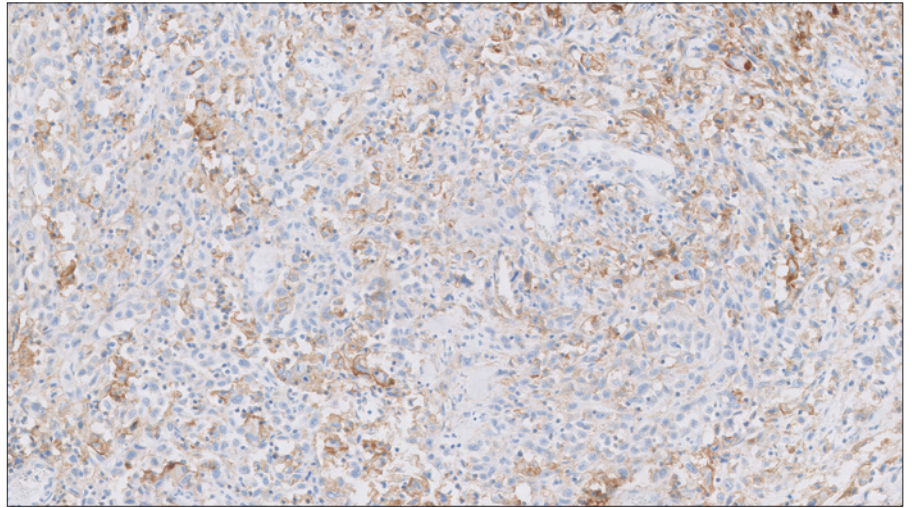


Figure 40a: 10 \times magnification.

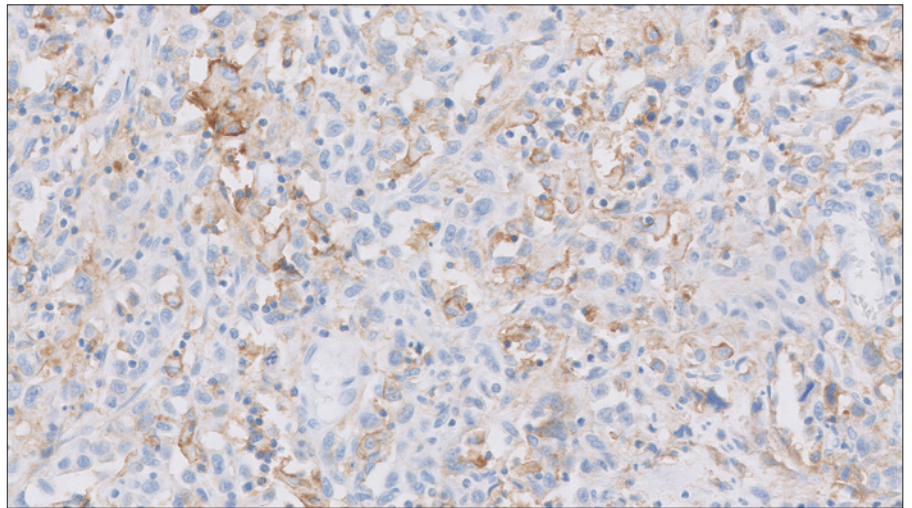


Figure 40b: 20 \times magnification.

PD-L1 antibody exhibiting CPS \geq 10 at 20 \times magnification.

Case 6: CPS \geq 10

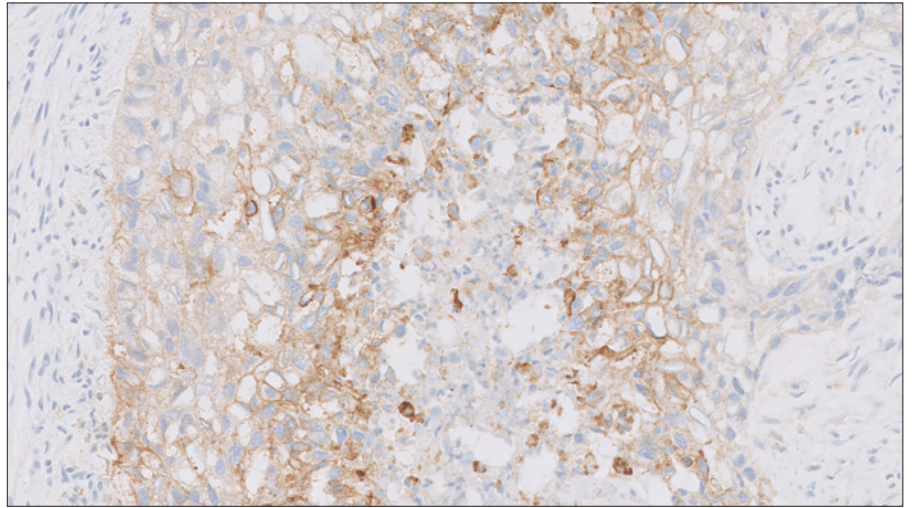


Figure 41a: 10 \times magnification.

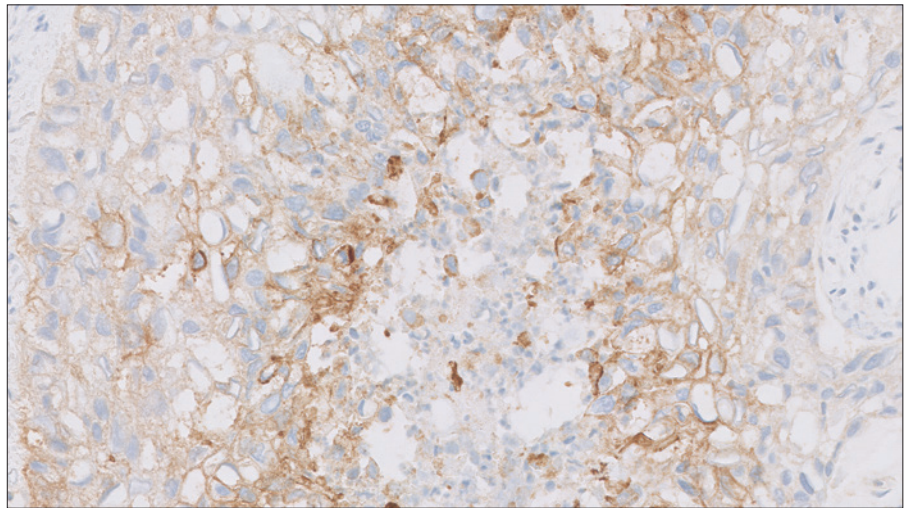


Figure 41b: 20 \times magnification.

PD-L1 antibody exhibiting CPS \geq 10 at 20 \times magnification.

Case 7: CPS \geq 10

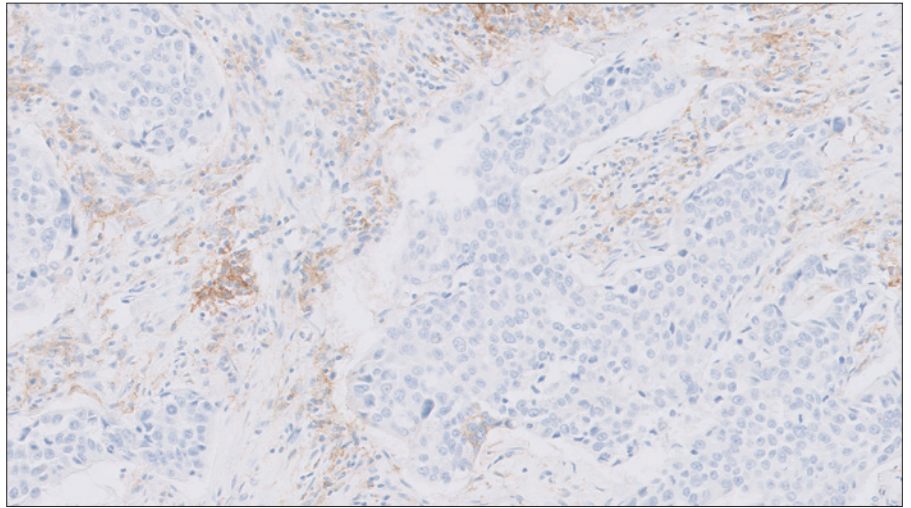


Figure 42a: 10x magnification.

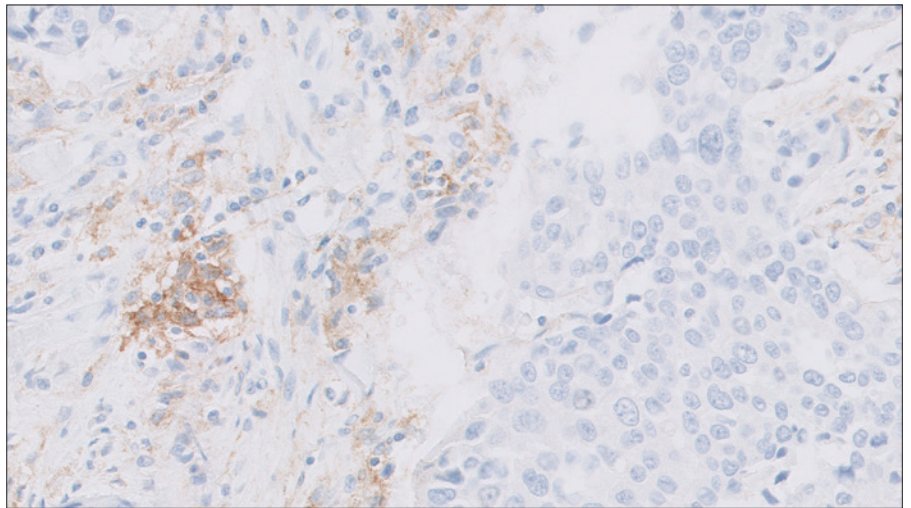


Figure 42b: 20x magnification.

PD-L1 antibody exhibiting CPS \geq 10 at 20x magnification.

Case 8: CPS \geq 10

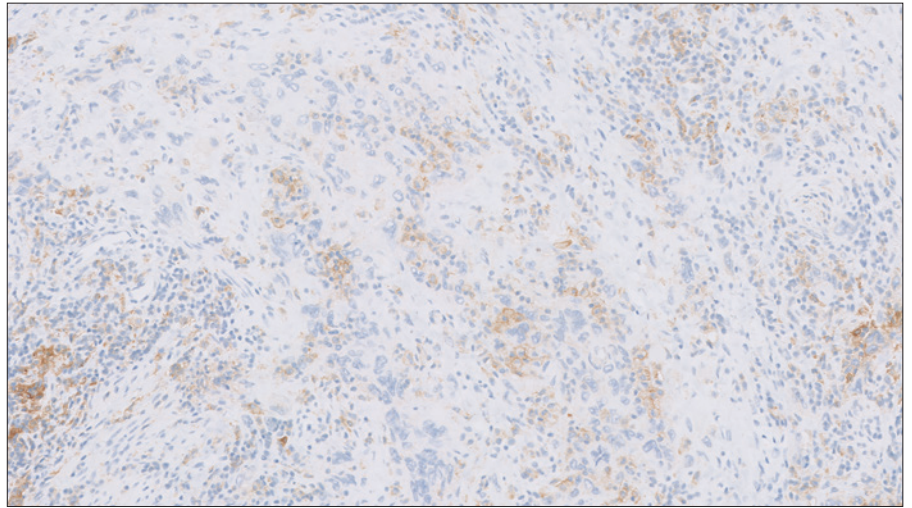


Figure 43a: 10 \times magnification.

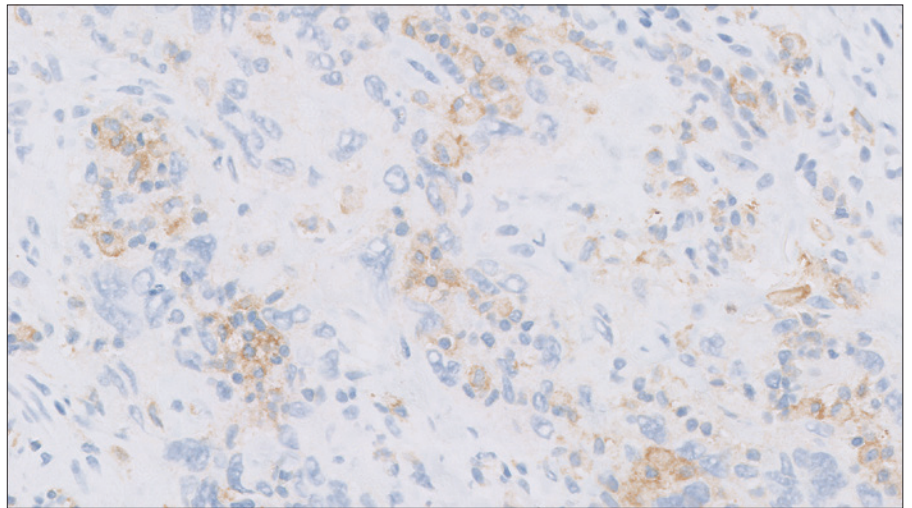


Figure 43b: 20 \times magnification.

PD-L1 antibody exhibiting CPS \geq 10 at 20 \times magnification.

Case 9: CPS \geq 10

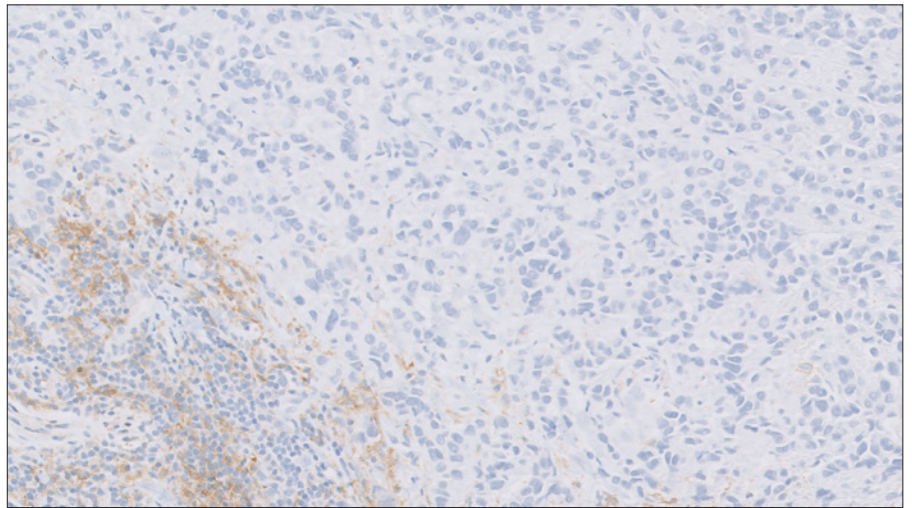


Figure 44a: 10x magnification.

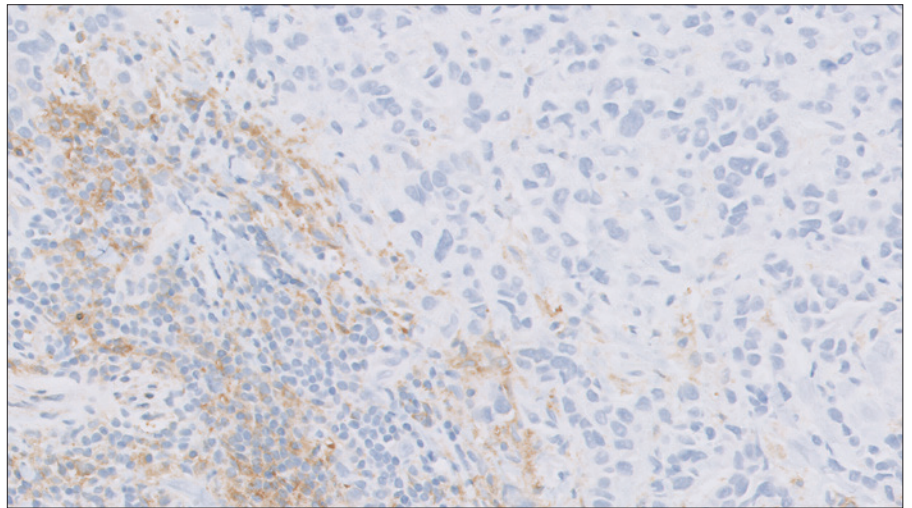


Figure 44b: 20x magnification.

PD-L1 antibody exhibiting CPS \geq 10 at 20x magnification.

Case 10: CPS \geq 10

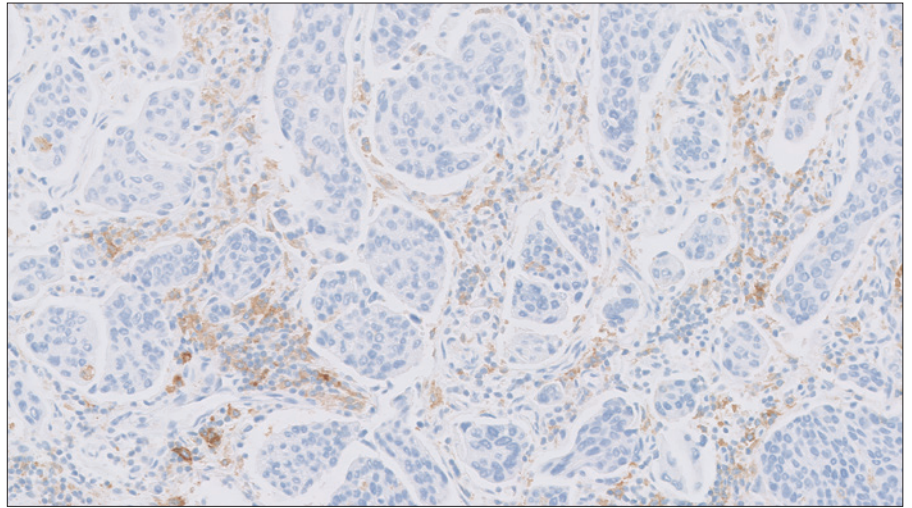


Figure 45a: 10 \times magnification.

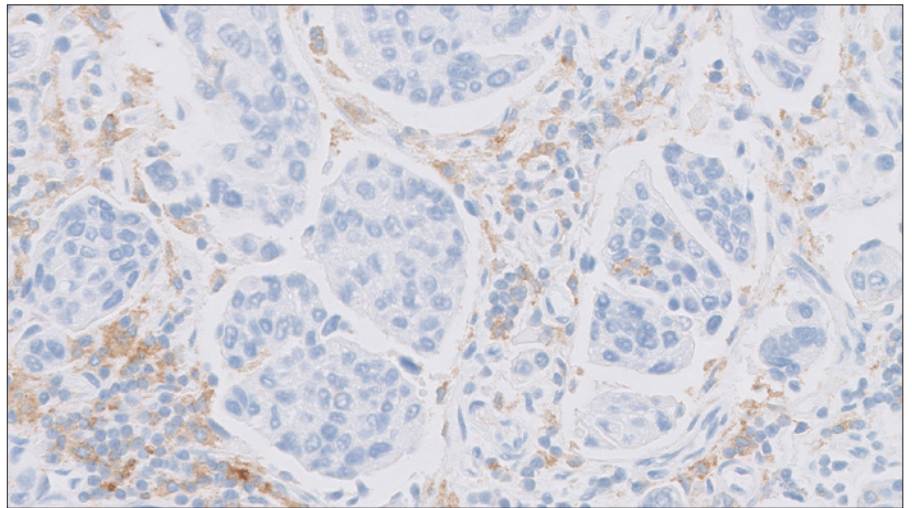


Figure 45b: 20 \times magnification.

PD-L1 antibody exhibiting CPS \geq 10 at 20 \times 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)**

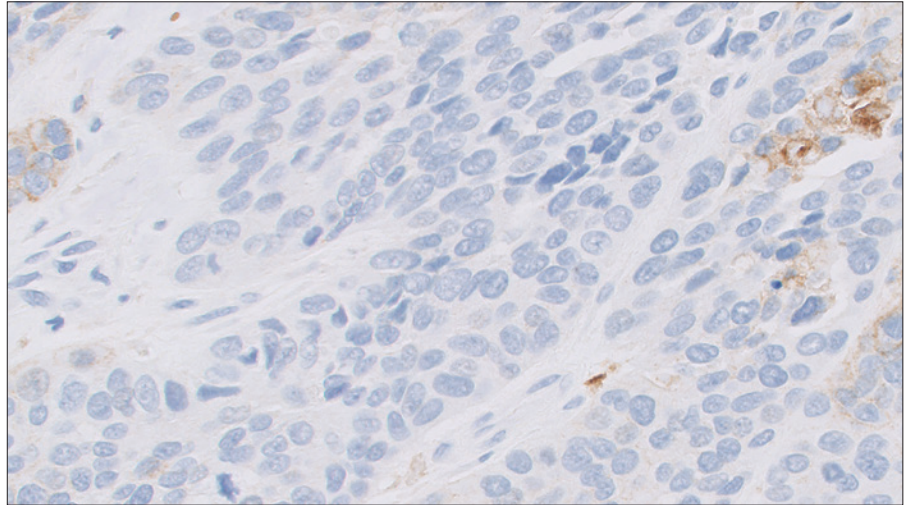


Figure 46: CPS 8 at 20× magnification.

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

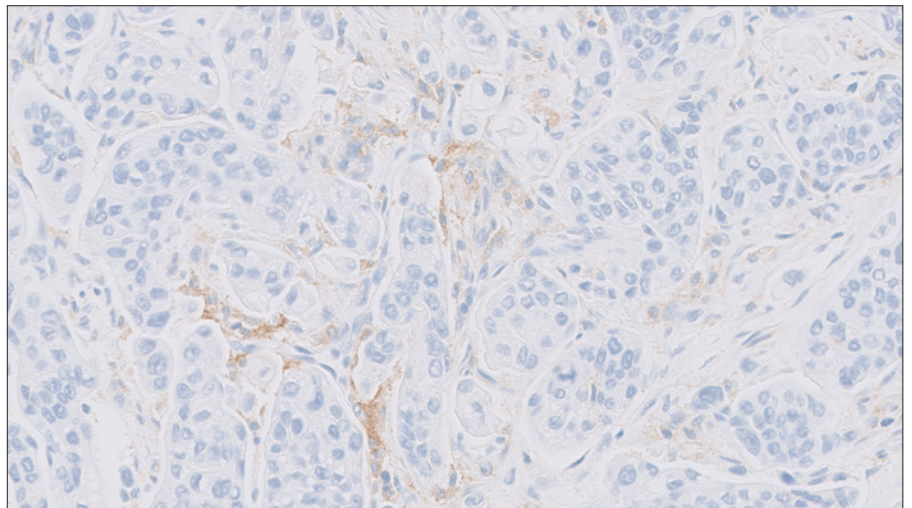


Figure 47: CPS 8 at 20× magnification.

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

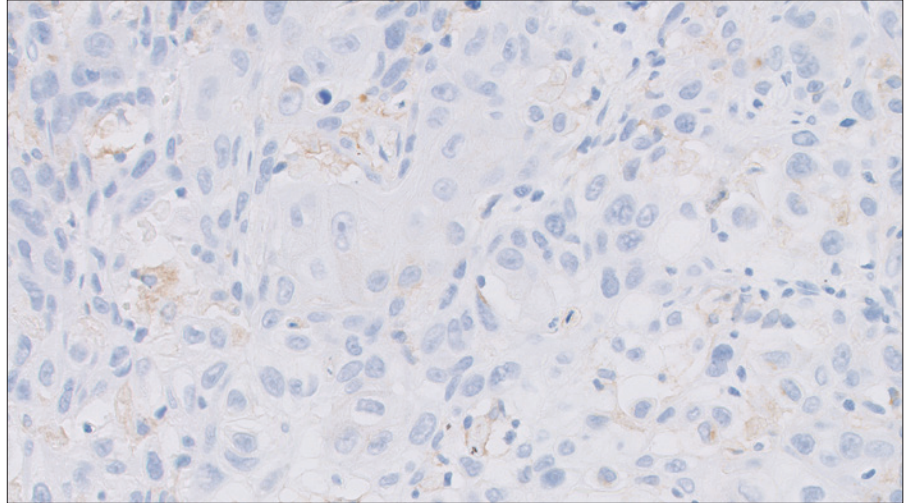


Figure 48: CPS 5 at 20× magnification.

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

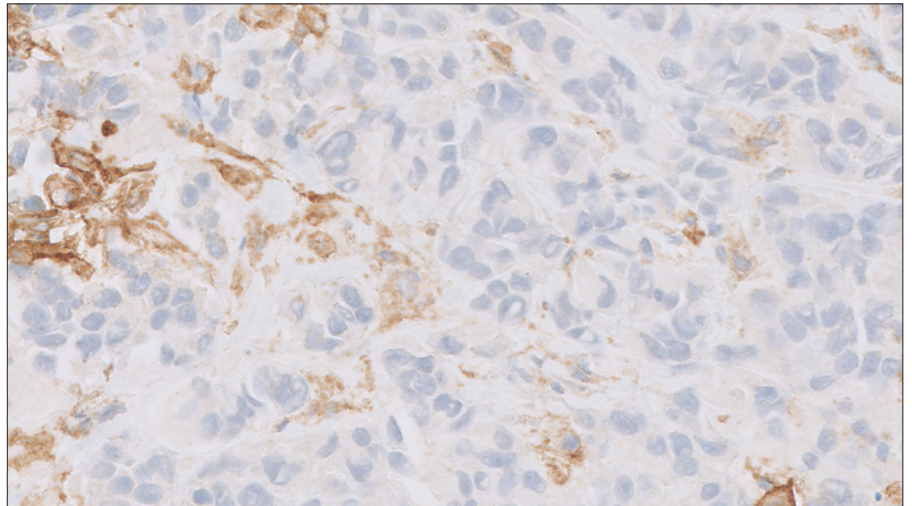


Figure 49: CPS 7 at 20× magnification.

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

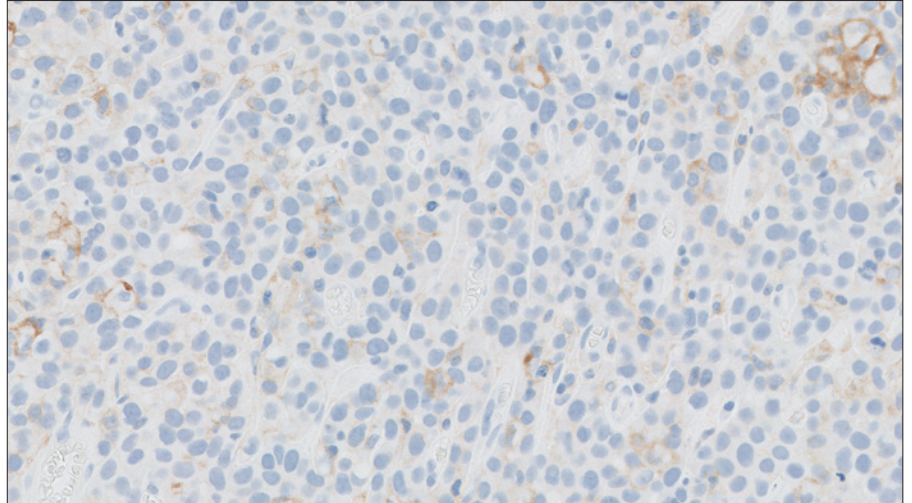


Figure 50: CPS 7 at 20× magnification.

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

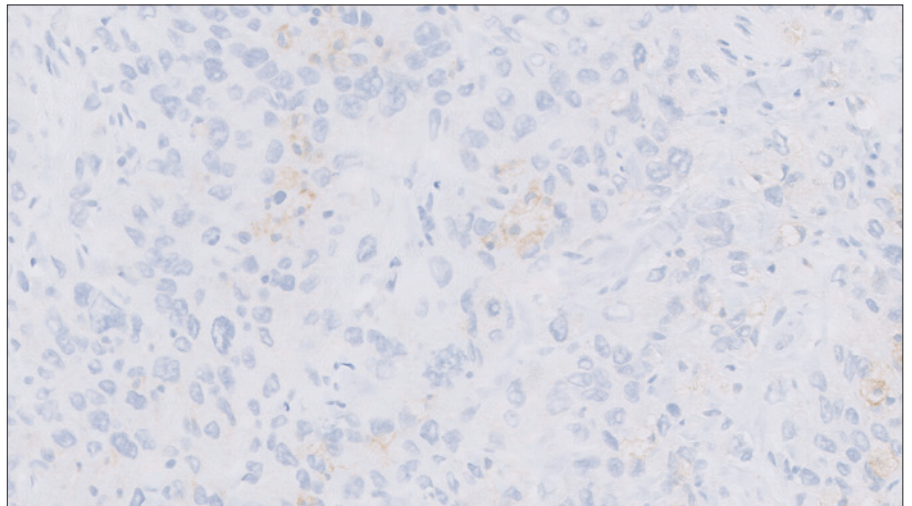


Figure 51: CPS 7 at 20× magnification.

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

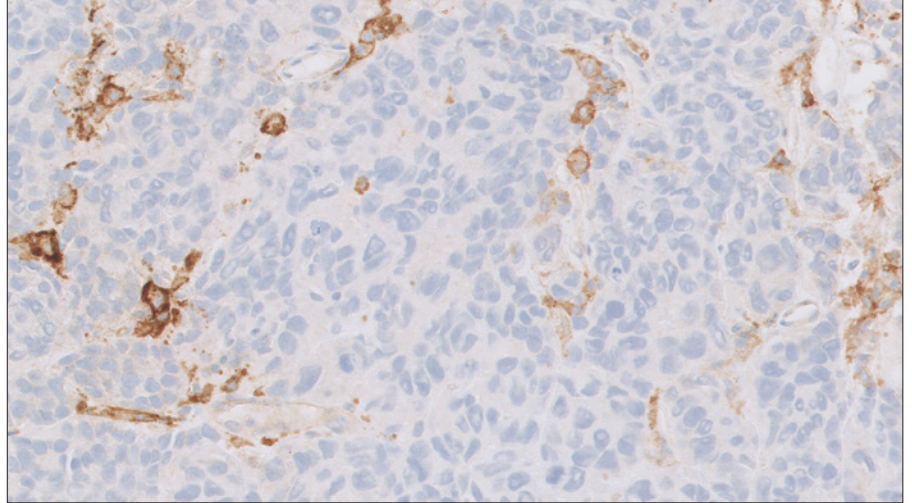


Figure 52: CPS 6 at 20× magnification.

**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)**

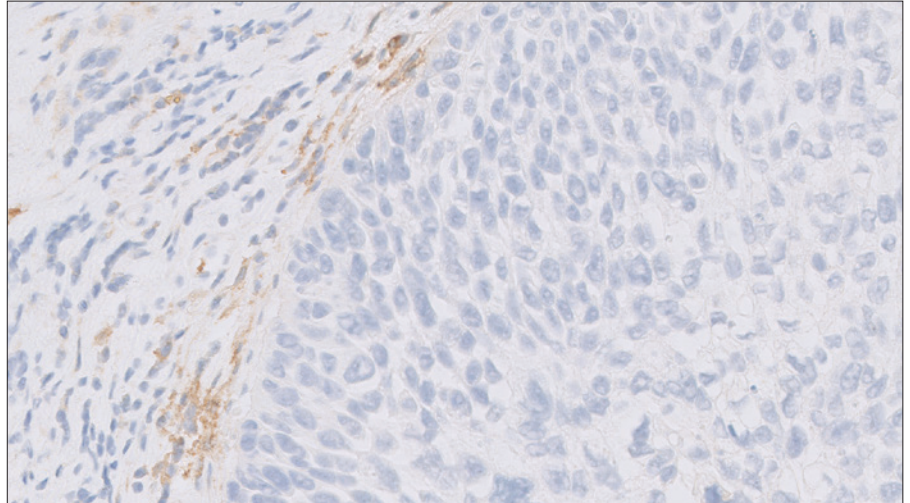


Figure 53: CPS 10 at 20× magnification.

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

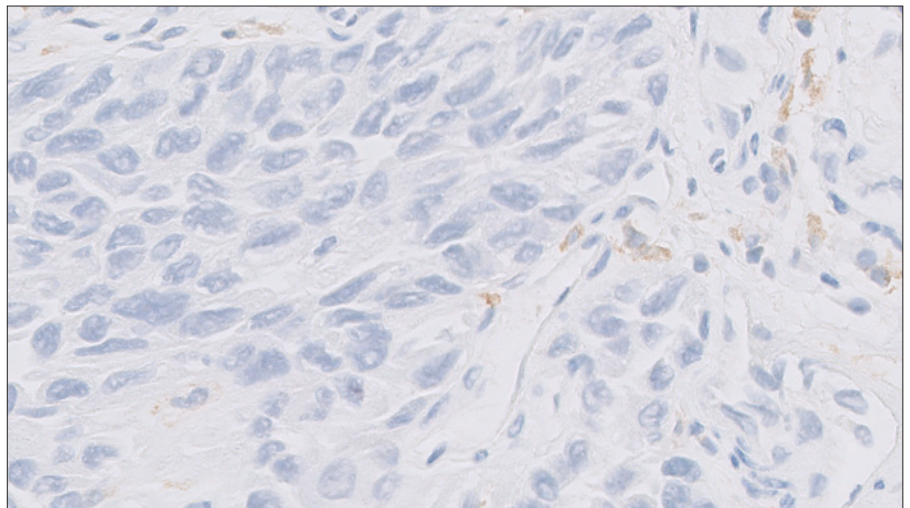


Figure 54: CPS 10 at 20× magnification.

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

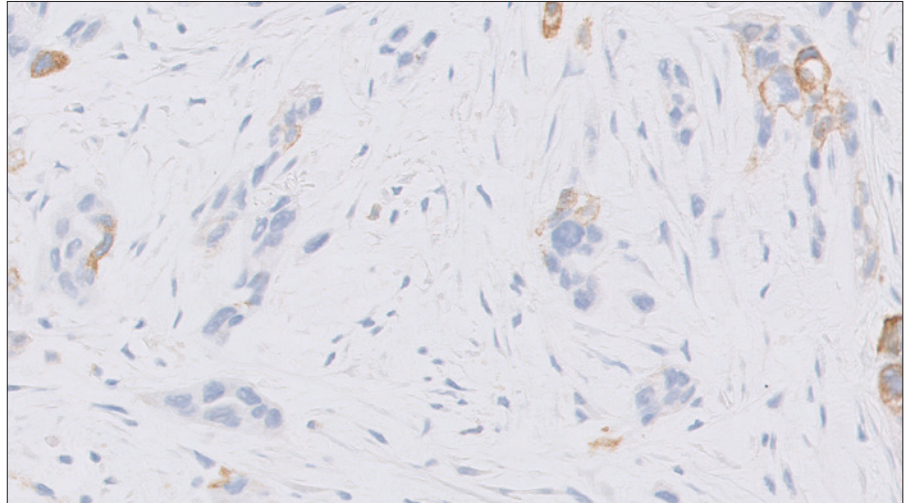


Figure 55: CPS 13 at 20x magnification.

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

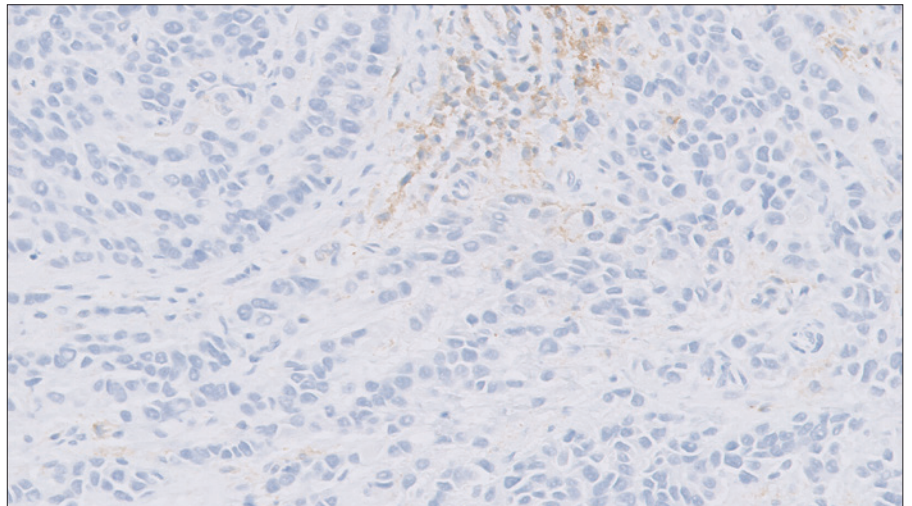


Figure 56: CPS 10 at 20x magnification.

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

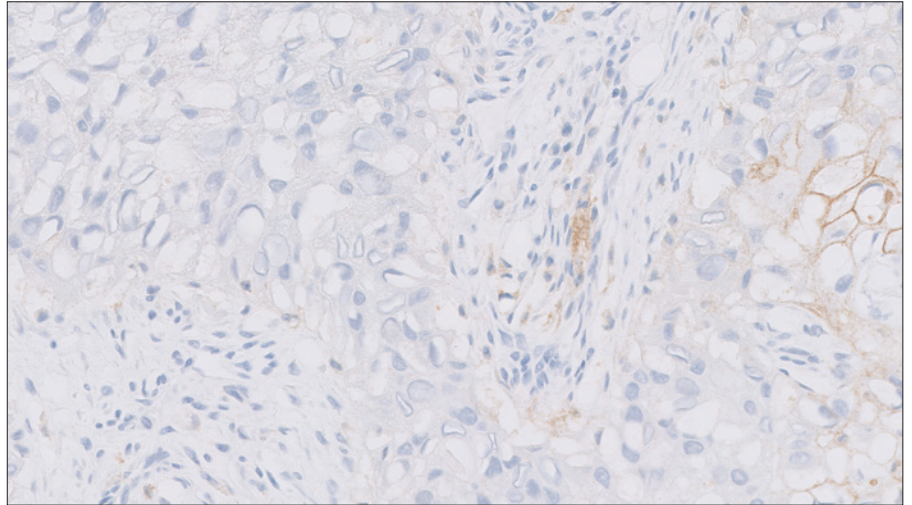


Figure 57: CPS 15 at 20× magnification.

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

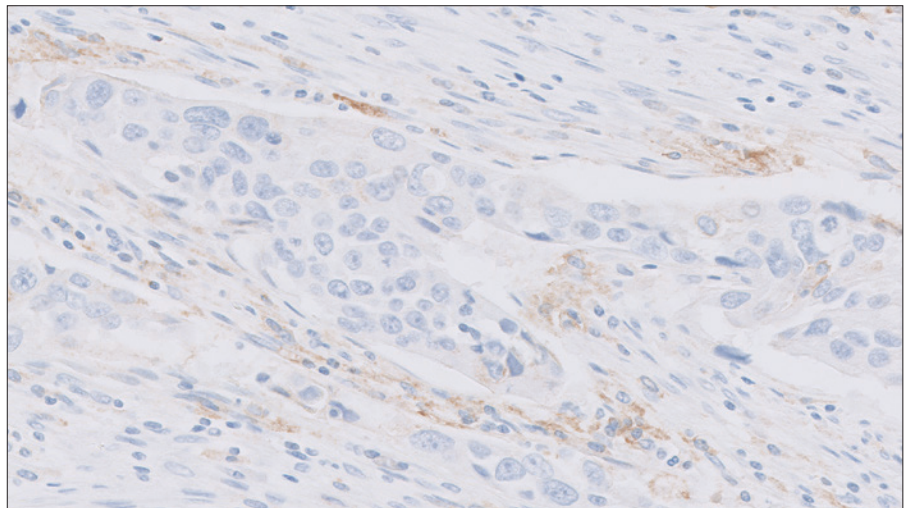


Figure 58: CPS 20 at 20× magnification.

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 pellet on the Dako-provided Control Cell Line Slide	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 charged slides (such as Fisherbrand Superfrost Plus)
	Inadequate preparation of specimens	Cut sections should be placed in a 58 ± 2 °C oven for 1 hour prior to staining
Excessively strong specific staining	Inappropriate fixation method used	Ensure that only approved fixatives and fixation methods are used
	Inappropriate wash buffer used	Use only 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

KEYNOTE-052: Open Label Trial in Urothelial Carcinoma Patients Ineligible for Cisplatin-containing Chemotherapy

The safety and efficacy of pembrolizumab were investigated in KEYNOTE-052, a multicenter, open-label study for the treatment of locally advanced or metastatic urothelial carcinoma in patients who were not eligible for cisplatin-containing chemotherapy. Patients received pembrolizumab at a dose of 200 mg every 3 weeks until unacceptable toxicity or disease progression. Treatment could continue beyond progression if the patient was clinically stable and was considered to be deriving clinical benefit by the investigator. Patients without disease progression could be treated for up to 24 months. The study excluded patients with autoimmune disease or a medical condition that required immunosuppression. Assessment of tumor status was performed at 9 weeks after the first dose, then every 6 weeks through the first year, followed by every 12 weeks thereafter.

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

Among the 370 patients, 30% (n = 110) had tumors that expressed PD-L1 with CPS \geq 10. Baseline characteristics of these patients were: median age 73 years, 68% male, and 87% 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 \geq 10. Baseline characteristics of these patients were: median age 72 years, 68% male, and 86% White. Seventy-nine percent had M1 disease, and 21% 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.

The primary efficacy outcome measure was ORR as assessed by BICR using RECIST 1.1. Secondary efficacy outcome measures included duration of response, and OS. Table 6 summarizes the efficacy results for patients with CPS ≥ 10 in the overall study and in the validation set subgroup.

Table 6 summarizes the key efficacy measures (ORR and OS) for the study population.

Table 6: ORR and OS by PD-L1 Expression

Endpoint	Subjects with CPS ≥ 10 in Overall Study (N=110)	Subjects with CPS ≥ 10 in Validation Set (N=80)
Objective Response Rate*		
ORR %, (95% CI)	47% (38, 57)	51% (40, 63)
Complete response rate	19%	21%
Partial response	28%	30%
Response Duration		
Median in months (range)	Not reached (1.4+ –26.5+)	Not reached (1.4+ –22.8+)
OS		
Median in months (95% CI)	19 (12.2,)	Not Reached (11.6,)
12-month OS rate	61%	61%

* BICR using RECIST 1.1

In patients with PD-L1 CPS < 10 in the overall study (N=251), the ORR was 21% (95% CI: 16, 26). The median OS in months and 12-month OS rate was 10 (8, 12) and 42% respectively.

In patients with PD-L1 CPS < 10 in the validation set (N=185), the ORR was 22% (95% CI: 16, 29). The median OS in months and 12-month OS rate was 11 (8, 13) and 44% respectively.

KEYNOTE-045: Controlled Trial of Urothelial Carcinoma Patients Previously Treated With Chemotherapy

The safety and efficacy of pembrolizumab were evaluated in KEYNOTE-045, a multicenter, randomized (1:1), controlled study for the treatment of locally advanced or metastatic urothelial carcinoma in patients with disease progression on or after platinum-containing chemotherapy. Patients must have received first-line platinum-containing regimen for locally advanced/metastatic disease or as neoadjuvant/adjuvant treatment, with recurrence/progression \leq 12 months following completion of therapy. Patients were randomized (1:1) to receive either KEYTRUDA 200 mg every 3 weeks (n=270) or investigator's choice of any of the following chemotherapy regimens all given intravenously every 3 weeks (n=272): paclitaxel 175 mg/m² (n=84), docetaxel 75 mg/m² (n=84), or vinflunine 320 mg/m² (n=87). Patients were treated with pembrolizumab until unacceptable toxicity or disease progression. Treatment could continue beyond progression if the patient was clinically stable and was considered to be deriving clinical benefit by the investigator. Patients without disease progression could be treated for up to 24 months.

Among the 542 randomized patients in KEYNOTE-045, baseline characteristics were: median age 66 years (range: 26 to 88), 58% age 65 or older; 74% male; 72% White and 23% Asian; 56% ECOG performance status of 1 and 1% ECOG PS of 2; and 96% M1 disease and 4% M0 disease. Eighty-seven percent of patients had visceral metastases, including 34% with liver metastases. Eighty-six percent had a primary tumor in the lower tract and 14% had a primary tumor in the upper tract. Fifteen percent of patients had disease progression following prior platinum-containing neoadjuvant or adjuvant chemotherapy. Twenty-one percent had received 2 prior systemic regimens in the metastatic setting. Seventy-six percent of patients received prior cisplatin, 23% had prior carboplatin, and 1% was treated with other platinum-based regimens. PD-L1 status was determined using PD-L1 IHC 22C3 pharmDx. Thirty percent of the patients had tumors that expressed PD-L1 with a Combined Positive Score (CPS) of \geq 10.

Table 7 summarizes the key efficacy measures for the intention-to-treat (ITT) population. The Kaplan-Meier curve is shown in Figure 59. The study demonstrated statistically significant improvements in OS and ORR for patients randomized to pembrolizumab as compared to chemotherapy. There was no statistically significant difference between pembrolizumab and chemotherapy with respect to PFS.

Further analysis was performed in KEYNOTE-045 in patients who had PD-L1 Combined Positive Score (CPS) \geq 10 and in patients who had CPS < 10 in both pembrolizumab- and chemotherapy-treated arms (see Table 8).

Table 7: Efficacy Results in KEYNOTE-045

Endpoint	Pembrolizumab 200 mg every 3 weeks n=270	Chemotherapy n=272
OS		
Number (%) of patients with event	155 (57%)	179 (66%)
Hazard ratio* (95% CI)	0.73 (0.59, 0.91)	
p-Value [†]	0.002	
Median in months (95% CI)	10.3 (8.0, 11.8)	7.4 (6.1, 8.3)
PFS[‡]		
Number (%) of patients with event	218 (81%)	219 (81%)
Hazard ratio* (95% CI)	0.98 (0.81, 1.19)	
p-Value [†]	0.416	
Median in months (95% CI)	2.1 (2.0, 2.2)	3.3 (2.3, 3.5)
Objective Response Rate[‡]		
ORR % (95% CI)	21% (16, 27)	11% (8, 16)
Complete response rate	7%	3%
Partial response	14%	8%
p-Value [§]	0.001	
Response Duration ^{††}		
Median in months (range)	Not reached (1.6+, 15.6+)	4.3 (1.4+, 15.4+)
Number (% ^{‡‡}) of patients with duration ≥ 6 months	41 (78%)	7 (40%)
Number (% ^{‡‡}) of patients with duration ≥ 12 months	14 (68%)	3 (35%)

* Hazard ratio (pembrolizumab compared to chemotherapy) based on the stratified Cox proportional hazard model

[†] Based on stratified Log rank test

[‡] Assessed by BICR using RECIST 1.1

[§] Based on method by Miettinen and Nurminen

^{††} Based on patients with a best overall response as confirmed complete or partial response

^{‡‡} Based on Kaplan-Meier estimation

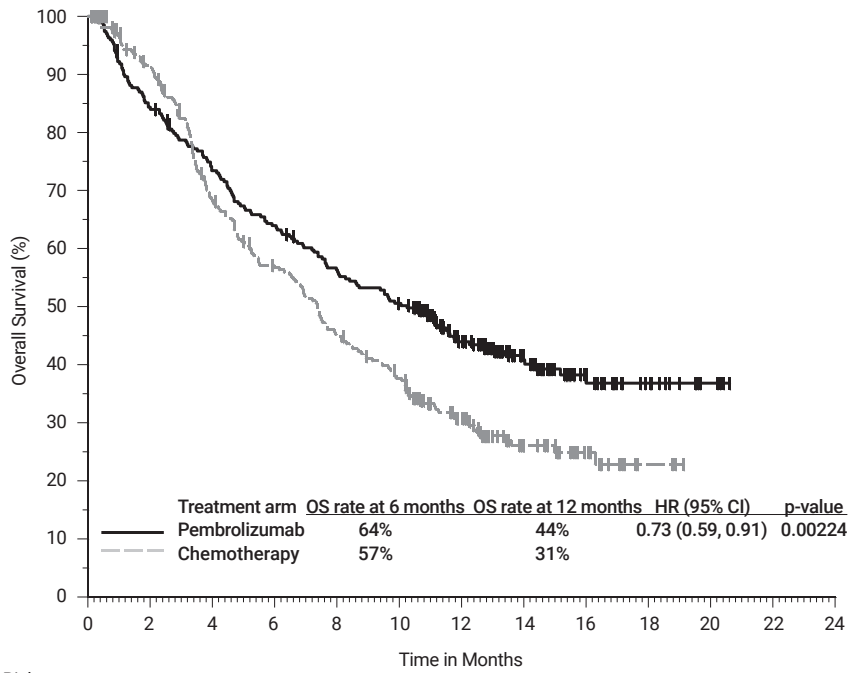
Table 8: Overall survival in KEYNOTE-045 by PD-L1 expression

PD-L1 Expression	Pembrolizumab	Chemotherapy	Hazard Ratio* (95% CI)
	OS by PD-L1 Expression Number of Events (Number of Patients)		
CPS < 10% [‡]	106 (186)	116 (176)	0.80 (0.61, 1.05)
CPS ≥ 10%	44 (74)	60 (90)	0.57 (0.37, 0.88)

* Hazard ratio (pembrolizumab compared to chemotherapy) based on the stratified Cox proportional hazard model

[†] 16 patients (10 from pembrolizumab and 6 from chemotherapy) had unknown PD-L1 status

[‡] CPS retains the same meaning with or without the percent (%) sign



Number at Risk														
		0	2	4	6	8	10	12	14	16	18	20	22	24
Pembrolizumab:	270	226	194	169	147	131	87	54	27	13	4	0	0	0
Chemotherapy:	272	232	171	138	109	89	55	27	14	3	0	0	0	0

Figure 59: Kaplan-Meier curve for OS by treatment arm in KEYNOTE-045 (ITT).

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Notes

For PD-L1 testing,
Choose PD-L1 IHC 22C3 pharmDx—the ONE assay clinical trial-proven with KEYTRUDA® (pembrolizumab)



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