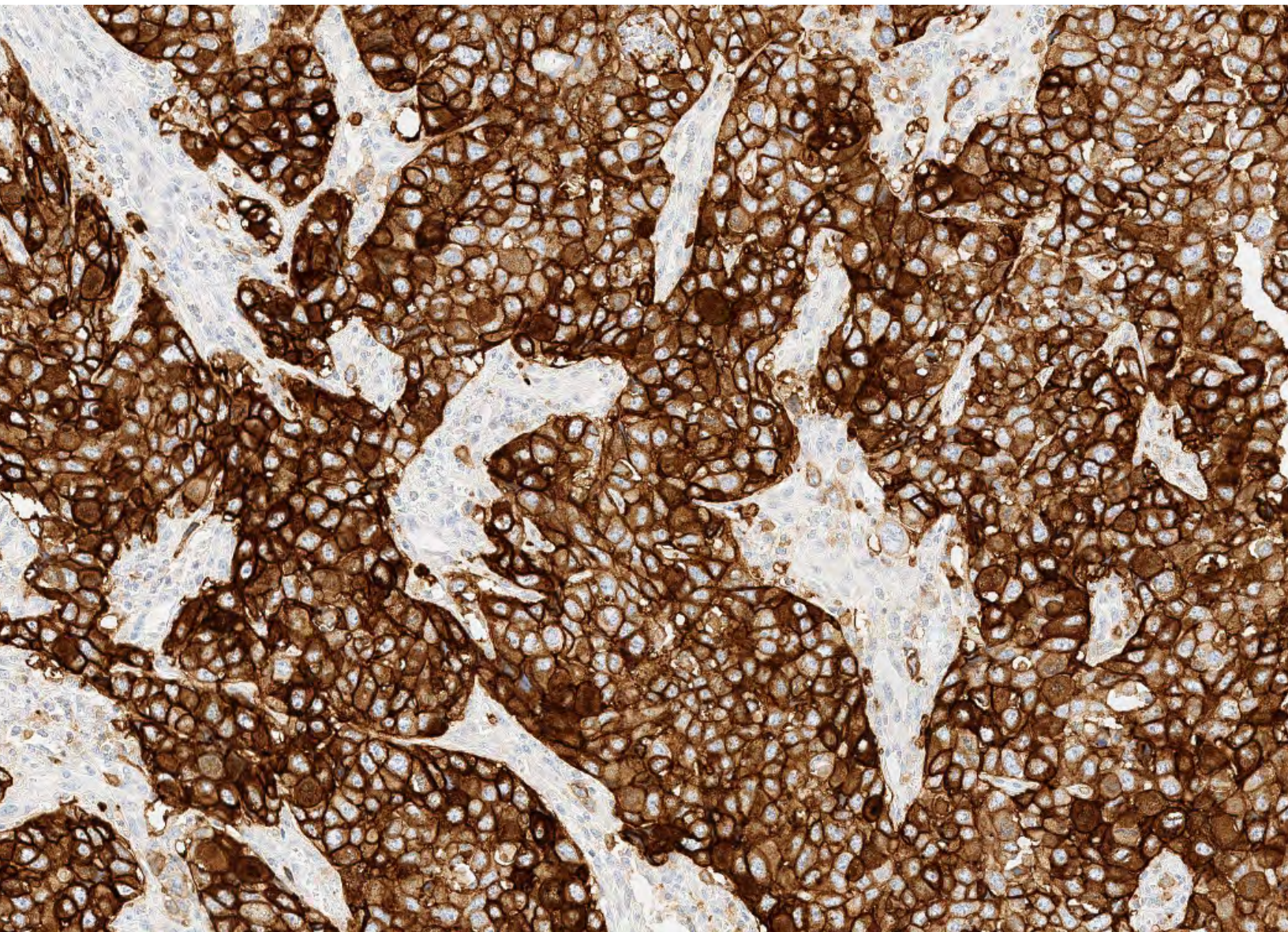


# **VENTANA PD-L1 (SP263) Assay Staining in Urothelial Carcinoma**

## *Interpretation Guide*





## Table of Contents

---

<b>Introduction</b>	<b>4</b>
Intended Use of Product	4
Purpose of Interpretation Guide	4
<b>Clinical Evaluation</b>	<b>5</b>
Evaluating Staining Patterns and Intensities	5
Tissue Requirements	10
Morphology and Background Acceptability Criteria	10
Positive Tissue Control	11
VENTANA PD-L1 (SP263) Assay Scoring Algorithm for Urothelial Carcinoma Tissue	12
Overview of PD-L1 (SP263) Scoring Algorithm for Urothelial Carcinoma	14
Evaluation of Immune Cell Staining	16
<b>PD-L1 Expression Atlas in Urothelial Carcinoma</b>	<b>25</b>
Tumor Cell Cases	25
Immune Cell Cases	31
<b>Challenging Cases</b>	<b>39</b>
Challenging cases: Weak Tumor Membrane vs Cytoplasmic Staining	40
Challenging cases: Strong Immune Cell Staining Overlapping with Tumor Cell Staining	42
Challenging cases: Cases with Multiple Tissue Fragments	44
Challenging cases: Borderline PD-L1 Status	45
Challenging cases: Obscuring Endogenous Material	48
<b>Impact of Pre-Analytical Conditions on VENTANA PD-L1 (SP263) Assay Staining</b>	<b>50</b>
Fixative Recommendations to Achieve Optimal Staining Results with the VENTANA PD-L1 (SP263) Assay	50
Impact of Tissue Thickness on Assay Staining	51
Cut Slide Stability	51
<b>References</b>	<b>51</b>

# Introduction

Urothelial carcinoma (also known as urothelial cell carcinoma, transitional cell carcinoma of the urinary tract, or urothelial bladder cancer) is the most common cancer of the urinary system worldwide. The majority of urothelial tumors arise in the bladder with the remainder originating in the renal pelvis, urethra, or ureter. Transitional cell carcinoma (TCC) is the most common histologic subtype associated with bladder cancer and accounts for greater than 90% of all urothelial carcinoma cases in the industrialized world; non-urothelial subtypes (e.g., squamous cell, adenocarcinoma, small cell carcinoma) are more frequent in other areas of the world.<sup>1</sup>

Globally, there were an estimated 429,793 new cases of bladder cancer and 165,084 deaths in 2012.<sup>2</sup> In Europe alone, for 2012, there were an estimated 151,297 new cases of bladder cancer and 52,411 deaths. In 2015, it was estimated that there would be 74,000 new cases of bladder cancer and 16,000 deaths in the United States.<sup>3</sup> Urothelial carcinoma presents as non-muscle-invasive, muscle-invasive, or metastatic disease. The overall 5-year survival rate for metastatic urothelial carcinoma (mUC) is approximately 5.4%.<sup>4</sup>

PD-L1 is a transmembrane protein that downregulates immune responses through binding to its two receptors programmed death-1 (PD-1) and B7-1 (CD80).<sup>5</sup> PD-1 is an inhibitory receptor expressed on T cells following T-cell activation, which is sustained in states of chronic stimulation such as in chronic infection or cancer.<sup>6</sup> Binding of PD-L1 with PD-1 inhibits T cell proliferation, cytokine production, and cytolytic activity, leading to the functional inactivation or exhaustion of T cells. CD80 is a molecule expressed on antigen presenting cells and activated T cells. PD-L1 binding to CD80 on T cells and antigen presenting cells can mediate downregulation of immune responses, including inhibition of T-cell activation and cytokine production.<sup>7</sup> PD-L1 expression has been observed in immune cells and tumor cells.<sup>8,9</sup> Aberrant expression of PD-L1 on tumor cells and or tumor-associated immune cells has been reported to impede anti-tumor immunity, resulting in immune evasion.<sup>6,9</sup> Therefore, interruption of the PD-L1/PD-1 pathway represents an attractive strategy to reinvigorate tumor-specific T cell immunity suppressed by the expression of PD-L1 in the tumor microenvironment. PD-L1 is expressed in a broad range of cancers including lung, melanoma, urothelial, ovarian, and colorectal cancer. Prevalence of PD-L1 expression has been reported from 12% to 100% depending on the tumor type, anti PD-L1 clone and cutoff for positivity.<sup>10</sup>

## Intended Use of Product

VENTANA PD-L1 (SP263) Assay is a qualitative immunohistochemical assay using rabbit monoclonal anti-PD-L1 clone SP263 intended for use in the assessment of the PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) urothelial carcinoma tissue stained with OptiView DAB IHC Detection Kit on a VENTANA BenchMark ULTRA instrument.

PD-L1 status is determined by the percentage of tumor cells with any membrane staining above background or by the percentage of tumor-associated immune cells with staining (IC+) at any intensity above background. The percent of tumor area occupied by any tumor-associated immune cells (Immune Cells Present, ICP) is used to determine IC+, which is the percent area of ICP exhibiting PD-L1 positive immune cell staining. PD-L1 status is considered High if any of the following are met:

- $\geq 25\%$  of tumor cells exhibit membrane staining; or,
- ICP  $> 1\%$  and IC+  $\geq 25\%$ ; or,
- ICP = 1% and IC+ = 100%.

PD-L1 High status as determined by VENTANA PD-L1 (SP263) Assay was associated with increased objective response rate (ORR) in a single arm study of IMFINZI™ (durvalumab).

This product is intended for *in vitro* diagnostic (IVD) use.

## Purpose of Interpretation Guide

This guide is intended to aid pathologists in the clinical evaluation of formalin-fixed, paraffin-embedded (FFPE) urothelial carcinoma sections stained with the Assay using the Assay Scoring Algorithms in accordance with the proposed product labeling. Specifically, this guide:

- Provides pathologists with a tool to facilitate the evaluation of formalin-fixed, paraffin-embedded (FFPE) urothelial carcinoma sections stained with the Assay using the Assay Scoring Algorithms in accordance with the proposed product labeling.
- Provides photographic images that illustrate the staining patterns and intensities that may result from staining of urothelial carcinoma tissue samples with the Assay.
- Provides guidance in the use of placenta tissue as a system-level control when stained with the Assay.
- Provides photographic images of internal controls.
- Provides a reference for relating staining patterns to specific PD-L1 scores.
- Provides example images of challenging cases to provide guidance in their evaluation.

# Clinical Evaluation

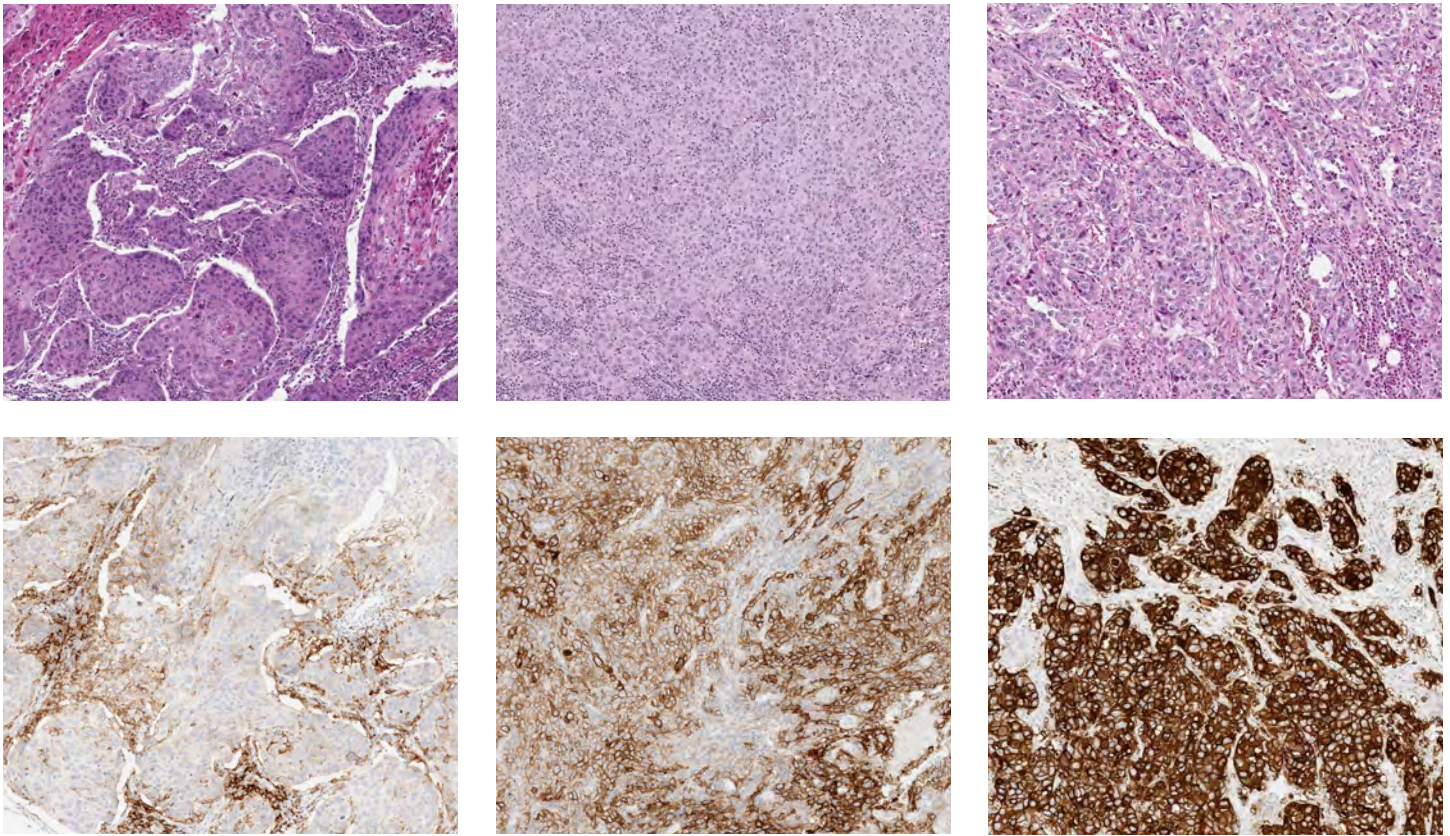
## Evaluating Staining Patterns and Intensities

Urothelial carcinoma cases stained with the Assay are assessed for both the percentage of tumor cells with membrane staining and the percentage of tumor-associated immune cells with membrane, cytoplasm, or punctate staining.

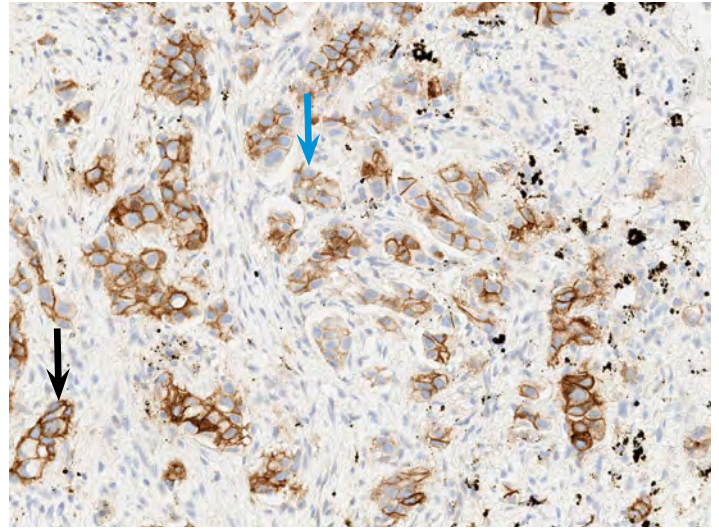
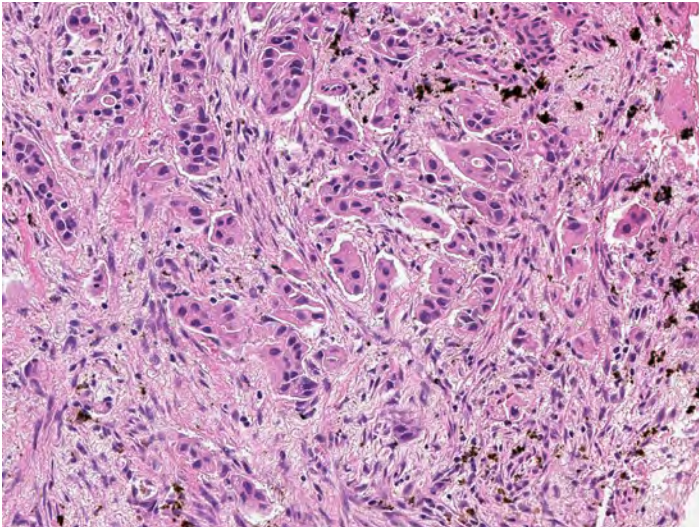
### Tumor Cell Staining:

Urothelial carcinoma neoplastic cells labeled with the Assay are evaluated for the percent of the tumor cells with membrane staining at any intensity of the diaminobenzidine (DAB) signal. The immunohistochemical staining in urothelial carcinoma

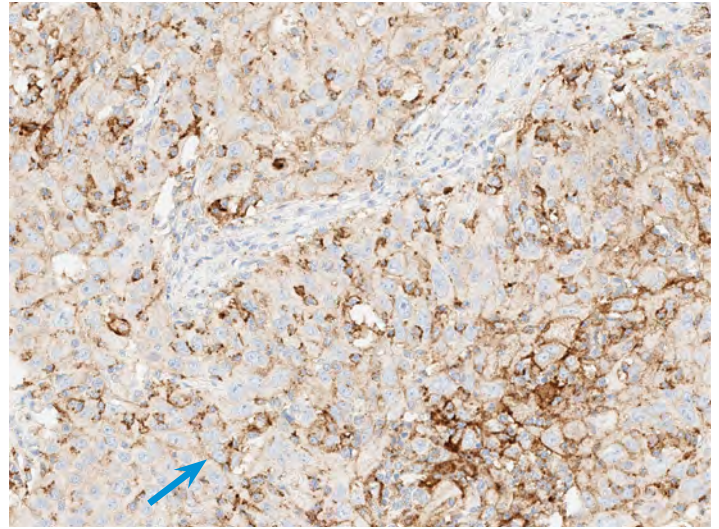
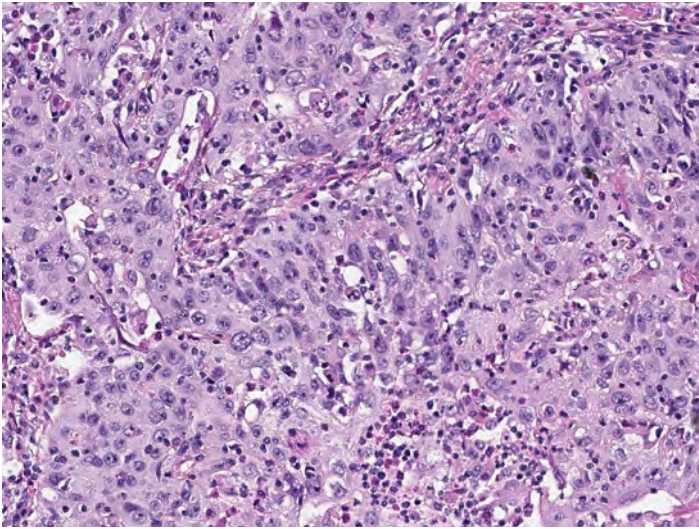
is membranous and/or cytoplasmic, and may be expressed homogeneously or heterogeneously throughout the neoplasm. Membrane staining can have a partial or complete circumferential pattern. Cytoplasmic staining is generally diffuse with some cases displaying a finely granular quality. Tumor cell cytoplasmic staining is disregarded for determining PD-L1 expression. The total percentage of membrane signal intensities is visually estimated and used to generate the PD-L1 expression level. An isotype-matched negative control antibody is used to evaluate the presence of background in test samples and establish a staining intensity baseline.



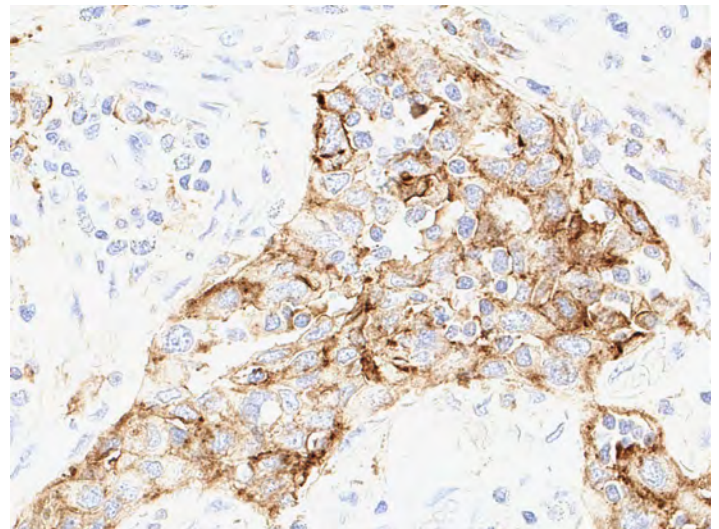
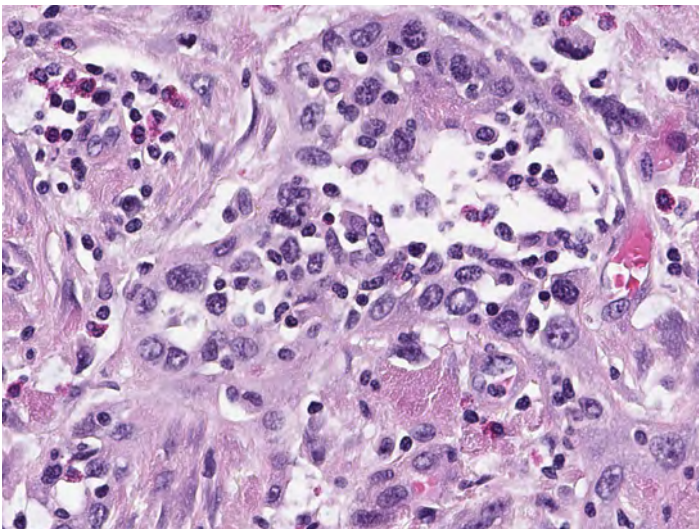
**Urothelial carcinoma, H&E and Assay:** Various cases demonstrating the range of membrane and cytoplasmic staining in tumor cells (10X)



**Urothelial carcinoma, H&E and Assay:** Tumor cells with circumferential (black arrow) and partial (blue arrow) membrane staining pattern (20X)



**Urothelial carcinoma, H&E and Assay:** Tumor cells with weak (blue arrow) membrane staining pattern (20X)



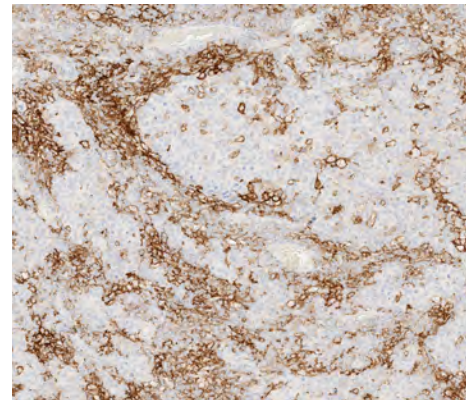
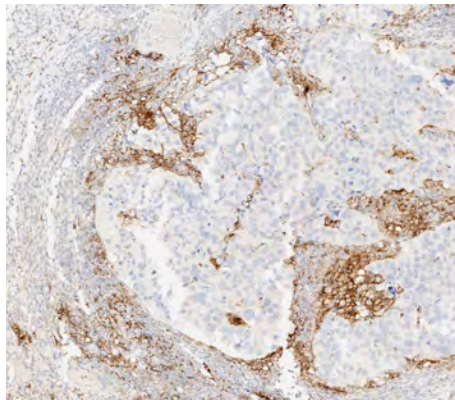
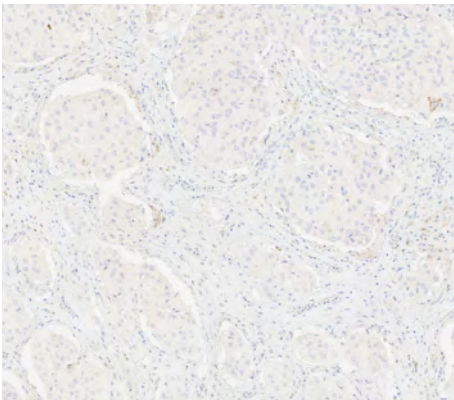
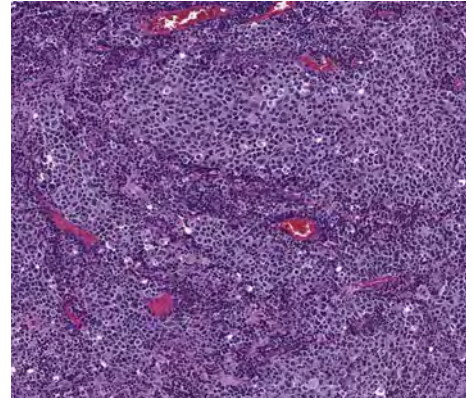
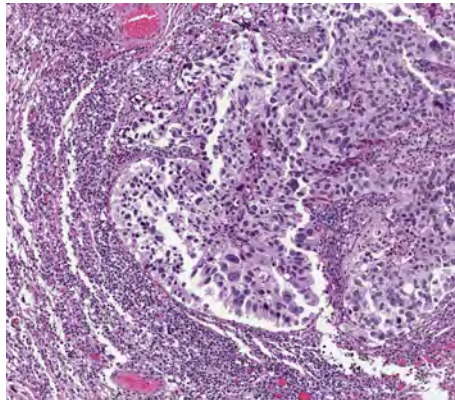
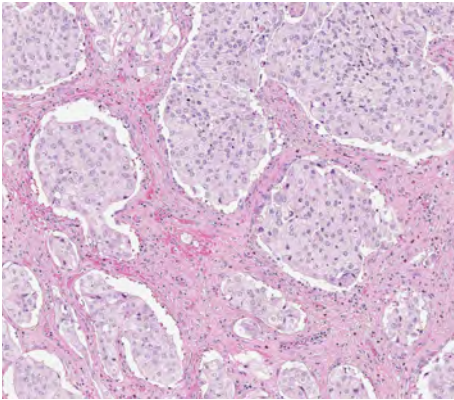
**Urothelial carcinoma, H&E and Assay:** Tumor cells show membranous and weak granular cytoplasmic staining (40X)

**Tumor-Associated Immune Cell Staining:**

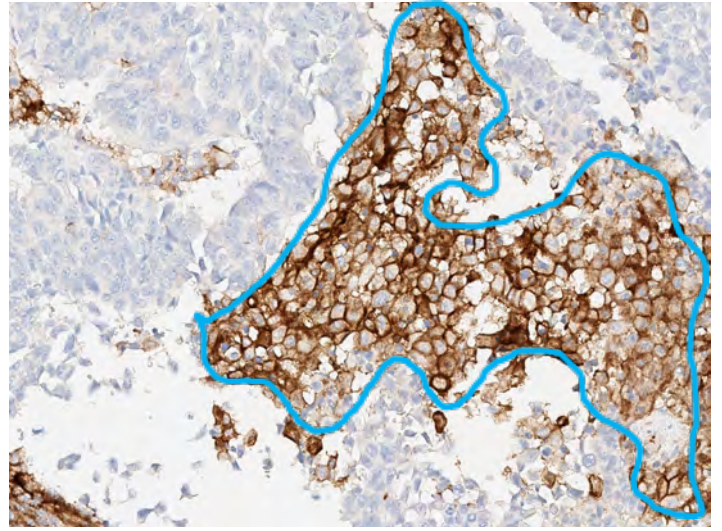
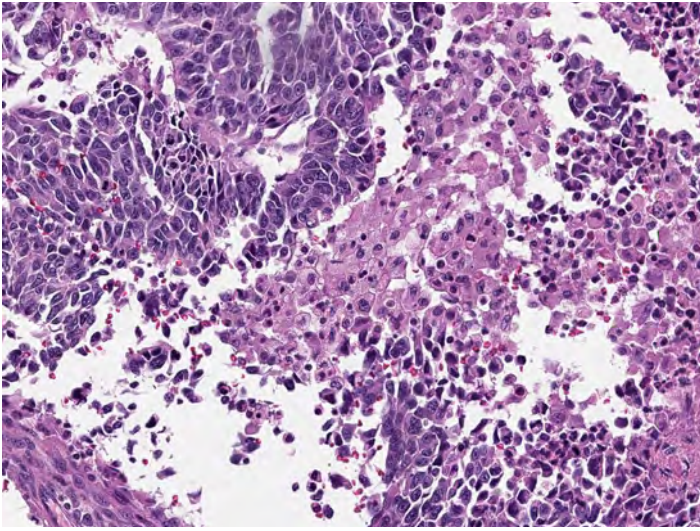
Immune cells exhibit a range of staining intensities and patterns: negative to weak diffuse cytoplasmic and/or weak to strong membranous signal. A punctate pattern of staining may be seen in association with lymphocytes. PD-L1 expression has been observed in lymphocytes, macrophages, histiocytes, plasma cells, and neutrophils.

The percentage of tumor-associated immune cells with staining in urothelial carcinoma cases is evaluated in addition to tumor cell

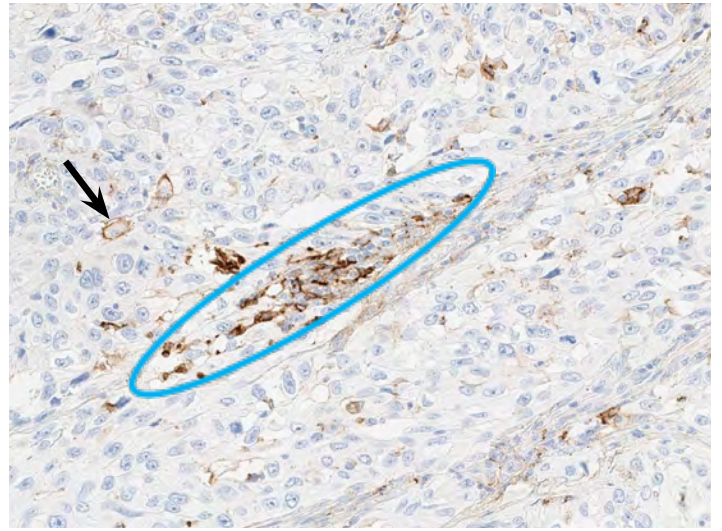
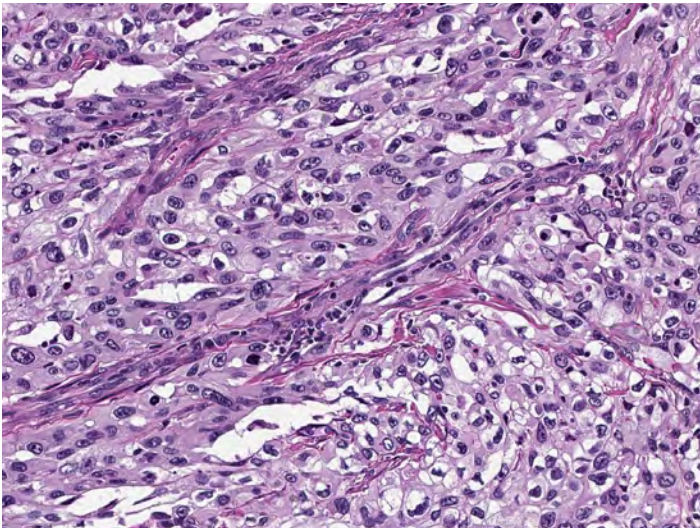
staining. Immune cell staining is assessed by initially reviewing the entire tumor area and determining the percentage of the tumor area occupied by immune cells (ICP). Next the percentage of immune cells demonstrating any pattern of PD-L1 expression within the tumor area is visually estimated (IC+).



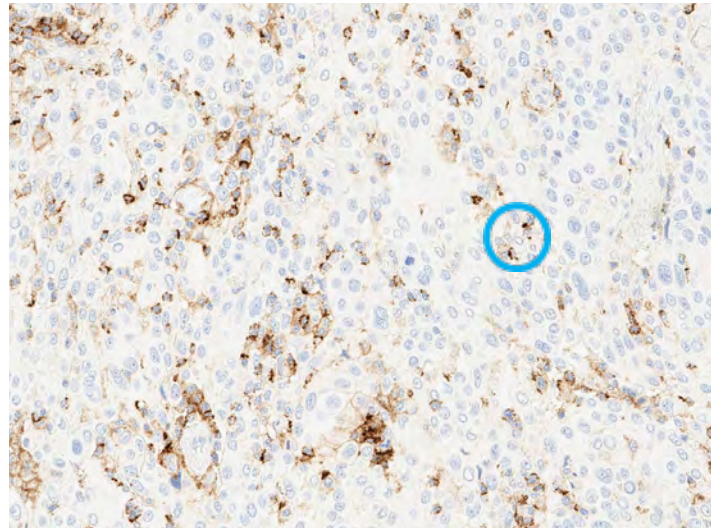
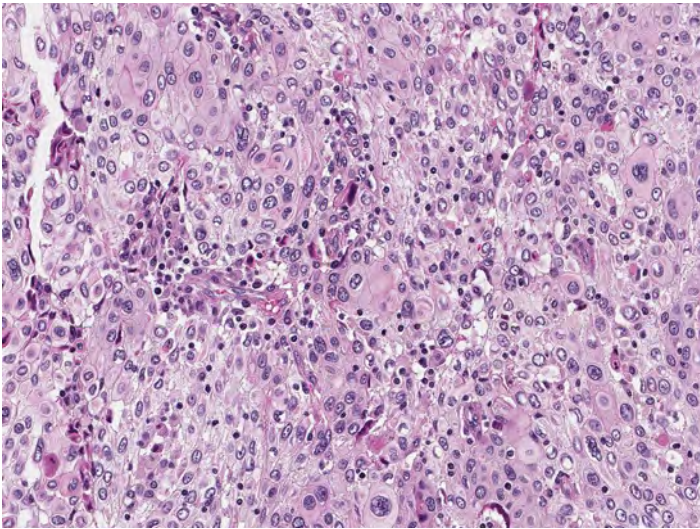
**Urothelial carcinoma, H&E and Assay:** Various cases demonstrating the range and patterns of immune cells staining (10X)



**Urothelial carcinoma, H&E and Assay:** Positive macrophage staining (blue outline) with adjacent PD-L1 negative tumor (20X)

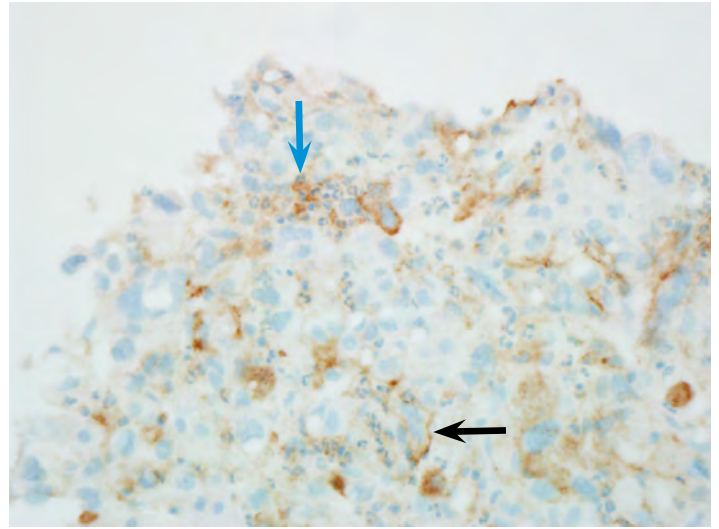
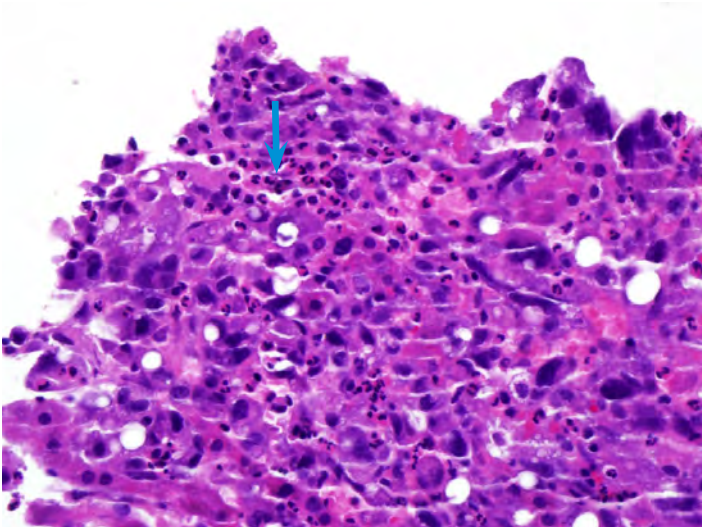


**Urothelial carcinoma, H&E and Assay:** Punctate (blue outline) and membranous (black arrow) immune cell staining with adjacent PD-L1 negative tumor (20X)



**Urothelial carcinoma, H&E and Assay:** Scattered immune cells with punctate staining infiltrating throughout tumor (single example circled in blue) (20X)





**NSCLC, H&E and Assay:** Case with rare neutrophil-only infiltrate (blue arrow) within the tumor demonstrating PD-L1 staining. Tumor cell membrane staining also present (black arrow) (40X)

## Tissue Requirements

The VENTANA PD-L1 (SP263) Assay requires one serial tissue section for hematoxylin and eosin (H&E) staining, a second serial tissue section for negative control antibody staining, and a third serial tissue section for staining with the VENTANA PD-L1 (SP263) Assay. In addition, normal human term placenta tissue can be used as a control for the PD-L1 (SP263) Assay. This tissue shows moderate to strong uniform staining of the membrane and weak to strong uniform staining of the cytoplasm of trophoblast-lineage cells. Placental stromal tissue and vasculature can be used for assessment of any background staining. If H&E evaluation indicates that the patient specimen is inadequate then a new specimen should be

obtained. Repeat staining of a specimen should be carried out on unstained slides if (1) the tissue run control slide does not exhibit acceptable staining; (2) the negative control case slide does not exhibit acceptable staining; or (3) the VENTANA PD-L1 (SP263) Assay stained case slide (the PD-L1 IHC slide) is not evaluable. If the last of these slides is not interpretable due to artifacts, edge effects, necrosis, lack of tissue, or any other reason, then the slide cannot be used for clinical evaluation. If controls are acceptable and the VENTANA PD-L1 (SP263) Assay stained slide is evaluable, the slide can be evaluated by a trained pathologist as described in the Scoring Criteria.

## Morphology and Background Acceptability Criteria

Tissue morphology and background acceptability are assessed for each patient case using the criteria described in Tables 1 and 2.

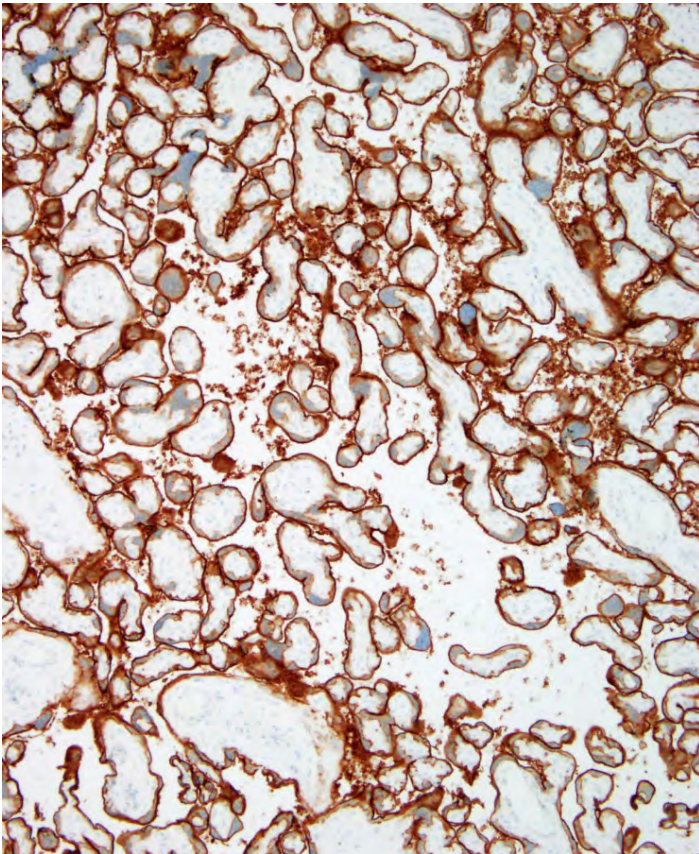
<b>Table 1: Morphology Acceptability Criteria</b>	
<b>Interpretation</b>	<b>Microscope Observation</b>
Acceptable	Cellular elements of interest are visualized allowing interpretation of the stain.
Not Acceptable	Cellular elements of interest are not visualized compromising interpretation of the stain.

<b>Table 2: Background Acceptability Criteria</b>	
<b>Interpretation</b>	<b>Microscope Observation</b>
Acceptable	Non-specific staining that is not obtrusive to interpretation of specific staining.
Not Acceptable	Non-specific staining that is obtrusive to interpretation of specific staining.

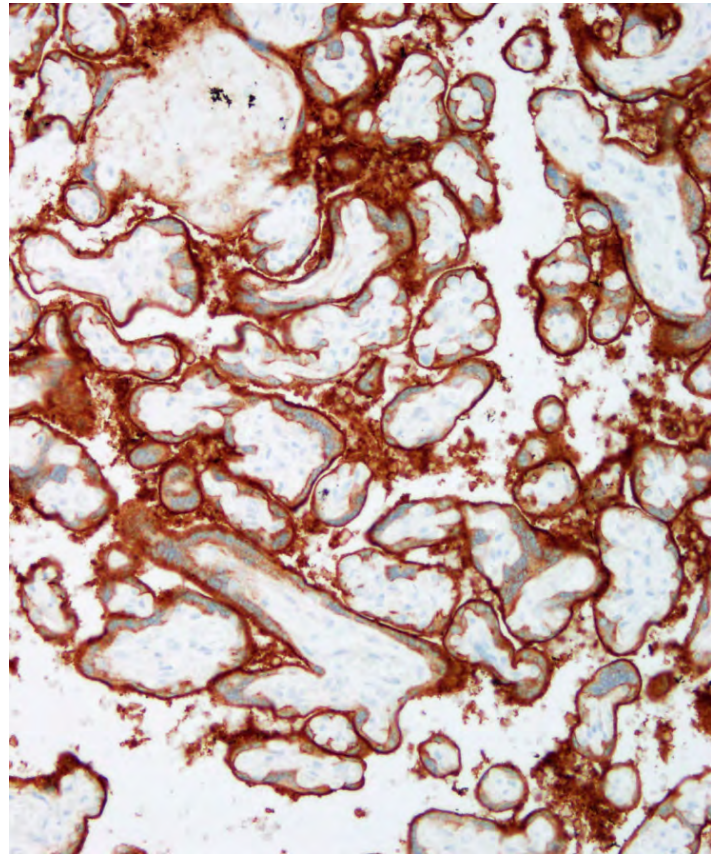
## Positive Tissue Control

A known positive control tissue fixed and processed in the same manner as the patient specimens should be run for each set of test conditions and with every VENTANA PD-L1 (SP263) Assay staining procedure performed. The control tissue (an index case) should be a fresh autopsy, biopsy, surgical specimen prepared and fixed as soon as possible in a manner identical to patient specimens. This tissue may be used to monitor all steps of specimen processing and staining. A tissue section fixed or processed differently from the test specimen can be used as a control for reagents and staining but not for fixation or tissue preparation. A positive urothelial carcinoma case with moderate staining is more suitable for quality control than one that stains strongly; it can be used to detect minor levels of reagent

degradation or out-of-specification issues that might be instrument-related. Positive membrane staining of neoplastic cells in the control tissue confirms that the VENTANA PD-L1 (SP263) antibody was applied and the instrument functioned properly. The positive tissue control should be used only to monitor performance; it should not be used to aid the clinical diagnosis of patient samples. Additionally, the VENTANA PD-L1 (SP263) Assay can utilize as a positive control human term placental tissue, which shows moderate to strong uniform staining of the membrane and weak to strong uniform staining of the cytoplasm of trophoblast-lineage cells. Placental stromal tissue and vasculature can be used for assessment of any background staining. Please refer to Table 3.



Placenta, VENTANA PD-L1 (SP263) Assay: Strong uniform membrane staining and moderate cytoplasmic staining of trophoblast-lineage cells (10X)



Placenta, VENTANA PD-L1 (SP263) Assay: Stroma and vasculature within villi show no PD-L1 staining (20X)

**Table 3: Placenta Tissue Control Evaluation Criteria for the Ventana PD-L1 (SP263) Assay**

Acceptable	Moderate to strong uniform membrane staining of trophoblast-lineage cells, and placental stroma and vasculature with no staining.
Not Acceptable	No to weak uniform membrane staining of trophoblast lineage cells and/or specific staining within placental stromal and vascular tissue.

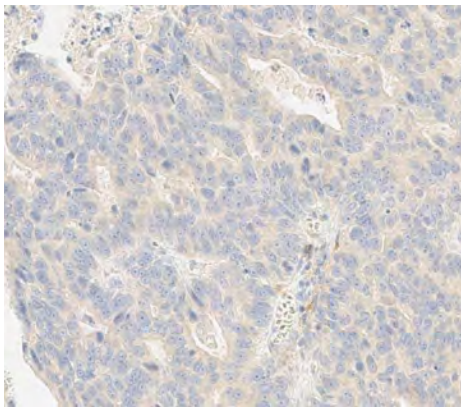
## VENTANA PD-L1 (SP263) Assay Scoring Algorithm for Urothelial Carcinoma Tissue

PD-L1 status and expression level is assigned by a trained pathologist based on their evaluation of the percentage of specific staining for both tumor and tumor-associated immune cells. PD-L1 status is based on the total percent of tumor cells with membrane staining or the total percent of tumor-associated immune cells with staining (IC+) at any intensity. Specifically, this guide highlights an expression level of greater than or equal to 25% of tumor cells with membrane staining or of tumor-associated immune cells with staining. In cases where the percent of tumor-associated immune cells in the tumor area (ICP) is 1%, IC+ is scored as either 0%, <100% or 100% due to the difficulties in estimating the percent staining in small volumes of immune cells in low measures. The small amount of PD-L1 staining observed in cases with < 100% IC+, should be considered as < 25% PD-L1 expression. Please refer to Table 4.

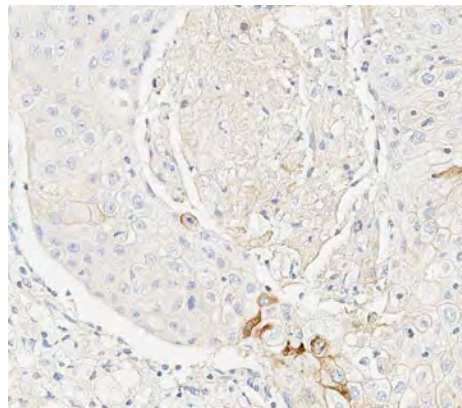
Interpretation of urothelial carcinoma cases stained with the Assay is based on the criteria noted in the table below. Images of various expression level staining patterns are provided in the subsequent sections. Please refer to Table 4.

Table 4: VENTANA PD-L1 (SP263) Assay Scoring Algorithm for Urothelial Carcinoma	
PD-L1 Interpretation	Staining Description
PD-L1 status is determined by the percentage of tumor cells with any membrane staining above background or by the percentage of tumor-associated immune cells with staining (IC+) at any intensity above background. Percent of tumor area occupied by any tumor-associated immune cells (ICP) is used to determine IC+, which is the percent area of ICP exhibiting PD-L1 positive immune cell staining.	
High	PD-L1 Status is considered high if any of the following are met: <ul style="list-style-type: none"> <li>▪ <math>\geq 25\%</math> of tumor cells exhibit membrane staining; or,</li> <li>▪ ICP <math>&gt; 1\%</math> and IC+ <math>\geq 25\%</math>; or,</li> <li>▪ ICP = 1% and IC+ = 100%.</li> </ul>
Low/negative	PD-L1 Status is considered low/negative if: <ul style="list-style-type: none"> <li>▪ none of the criteria for PD-L1 High Status are met.</li> </ul>

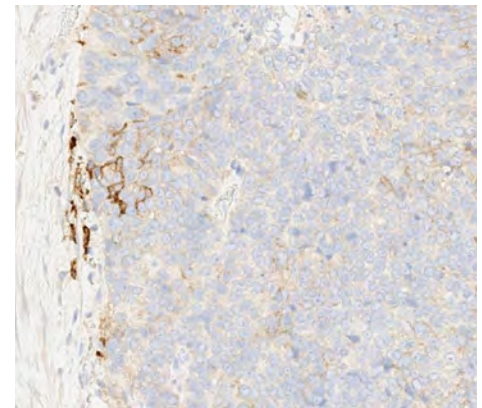
### PD-L1 LOW/NEGATIVE STATUS: TUMOR CELL EXPRESSION < 25%



0%

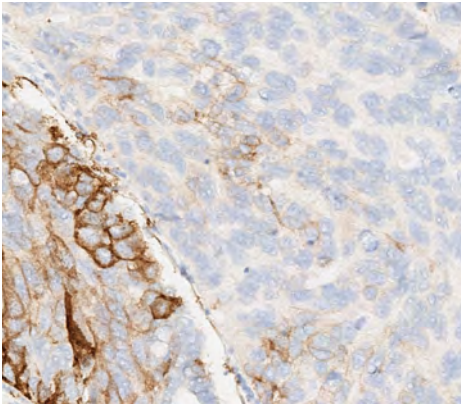


5%

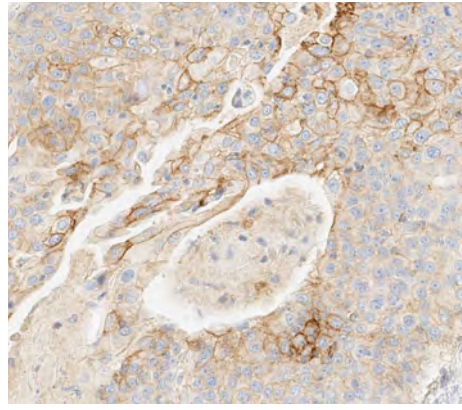


10%

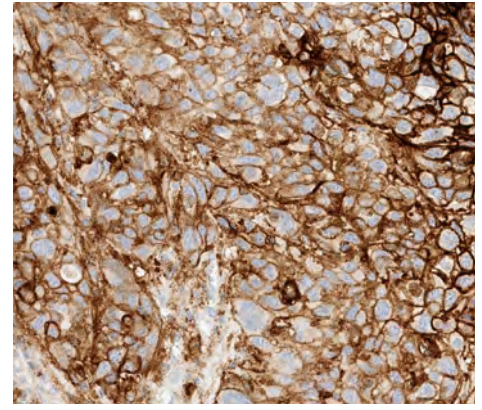
**PD-L1 HIGH STATUS: TUMOR CELL EXPRESSION  $\geq$  25%**



25%

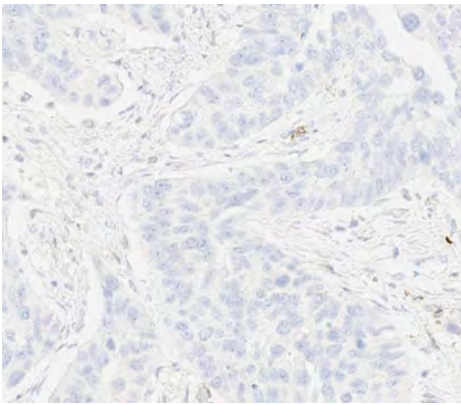


50%

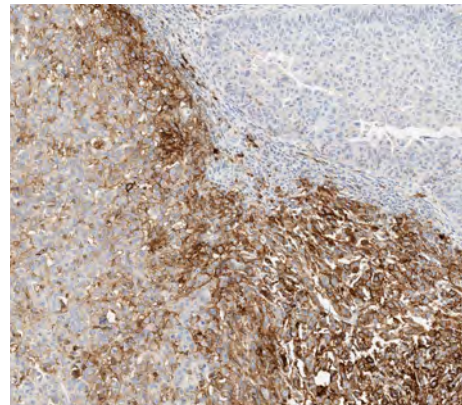


100%

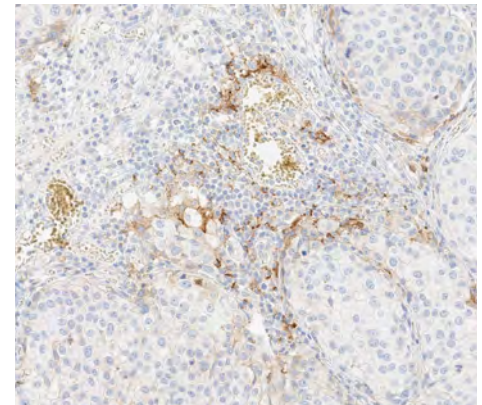
**PD-L1 LOW/NEGATIVE STATUS: TUMOR-ASSOCIATED IMMUNE CELL EXPRESSION  $<$  25%**



0% IC+

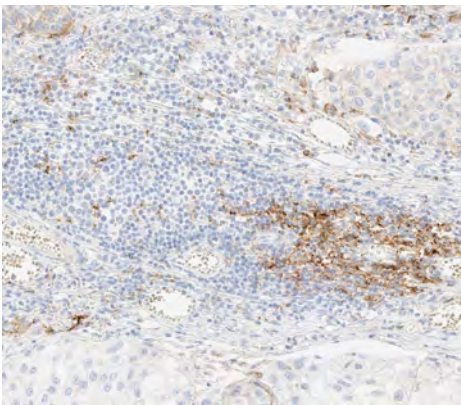


10% IC+

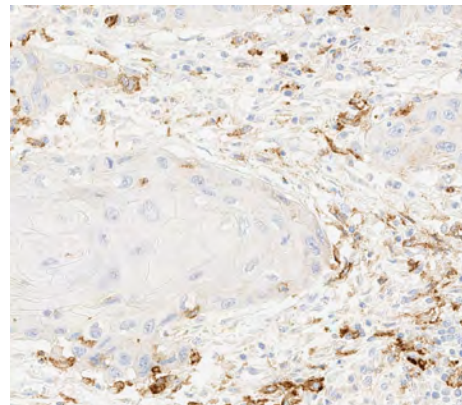


20% IC+

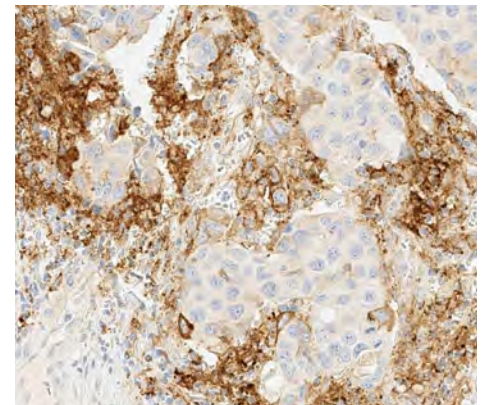
**PD-L1 HIGH STATUS: TUMOR-ASSOCIATED IMMUNE CELL EXPRESSION  $\geq$  25%**



30% IC+



60% IC+

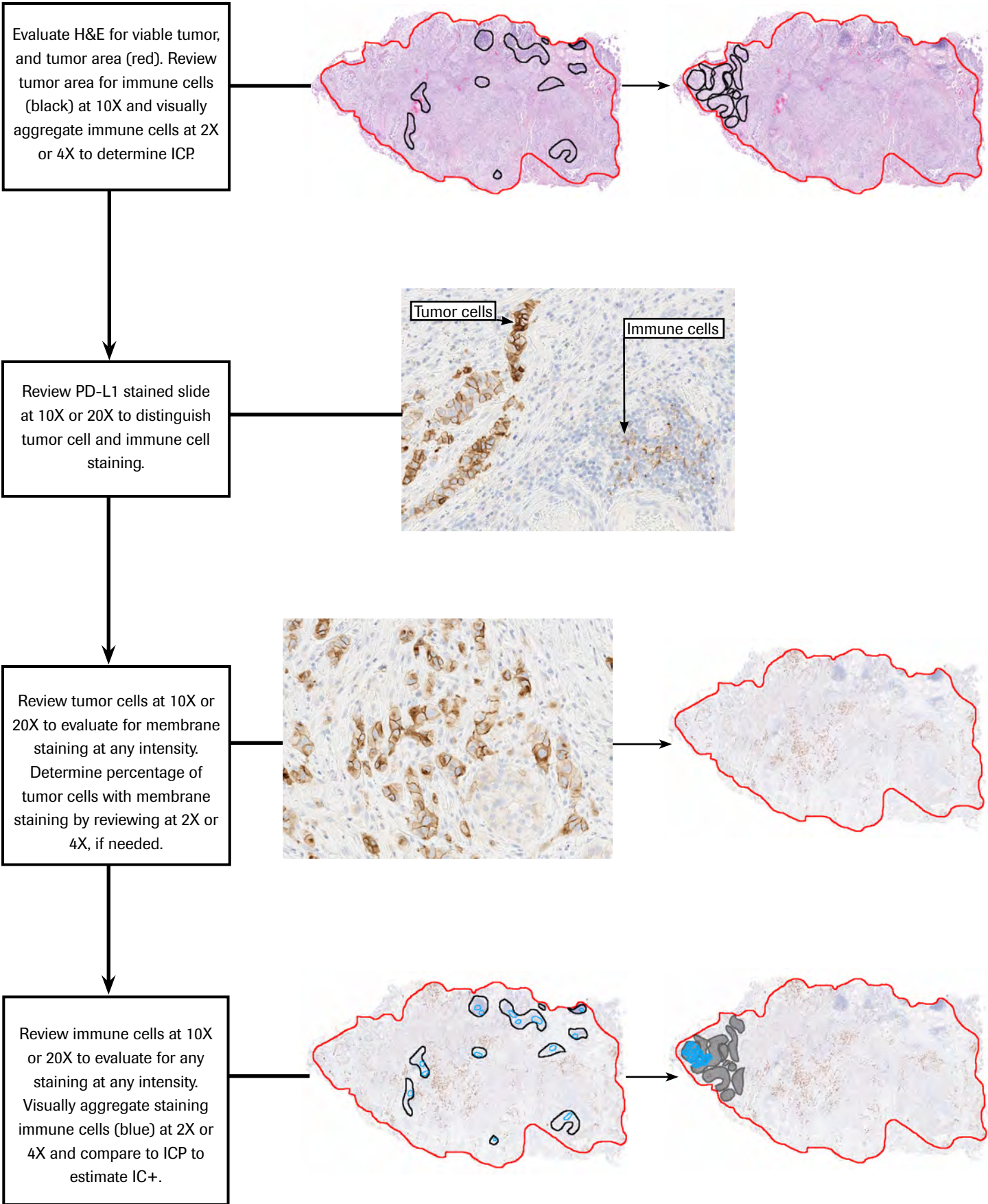


80% IC+

## Overview of PD-L1 (SP263) Scoring Algorithm for Urothelial Carcinoma

1. H&E slide is reviewed for viable tumor, tumor-associated immune cells and tumor area.
  - a. Tumor area encompasses the tumor proper, associated desmoplastic stroma and immune cells infiltrating the tumor and contained within the desmoplasia.
2. Visually, tumor-associated immune cells are densely aggregated (with no intervening stroma) within the tumor area when estimating Immune Cells Present (ICP).
  - a. Tumor associated immune cells include those present within the tumor reactive stroma, between the tumor islands and those invading the tumor proper.
3. The ICP percentage is scored as 0%, 1%, 5%, and deciles and quartiles (10, 20, 25, 30, 40, 50, 60, 70, 75, 80, 90, 100).
  - a. If a raw percentage falls between a decile or quartile, standard mathematical rounding is used to score to the nearest decile or quartile.
  - b. When differentiating between 1% and 5% ICP, a raw percentage estimate of 2% is rounded to 1% and an estimate of 3% to 4% is rounded to 5%.
4. PD-L1 stained slide is reviewed for tumor cell and immune cell staining.
5. Estimate the percentage of tumor cells with partial or complete membrane staining at any intensity.
6. The percentage of immune cells (within the ICP) expressing PD-L1 (IC+) is estimated.
  - a. Immune cells with any staining (membranous, cytoplasmic, or punctate) at any intensity are visually aggregated within the ICP to estimate IC+.
  - b. IC+ is scored as deciles and quartiles (0, 10, 20, 25, 30, 40, 50, 60, 70, 75, 80, 90, 100).
7. For cases with 1% ICP, the percentage estimate for IC+ is reported as 0%, <100%, or 100%.

# Overview of PD-L1 (SP263) Scoring Algorithm for Urothelial Carcinoma



## Evaluation of Immune Cell Staining

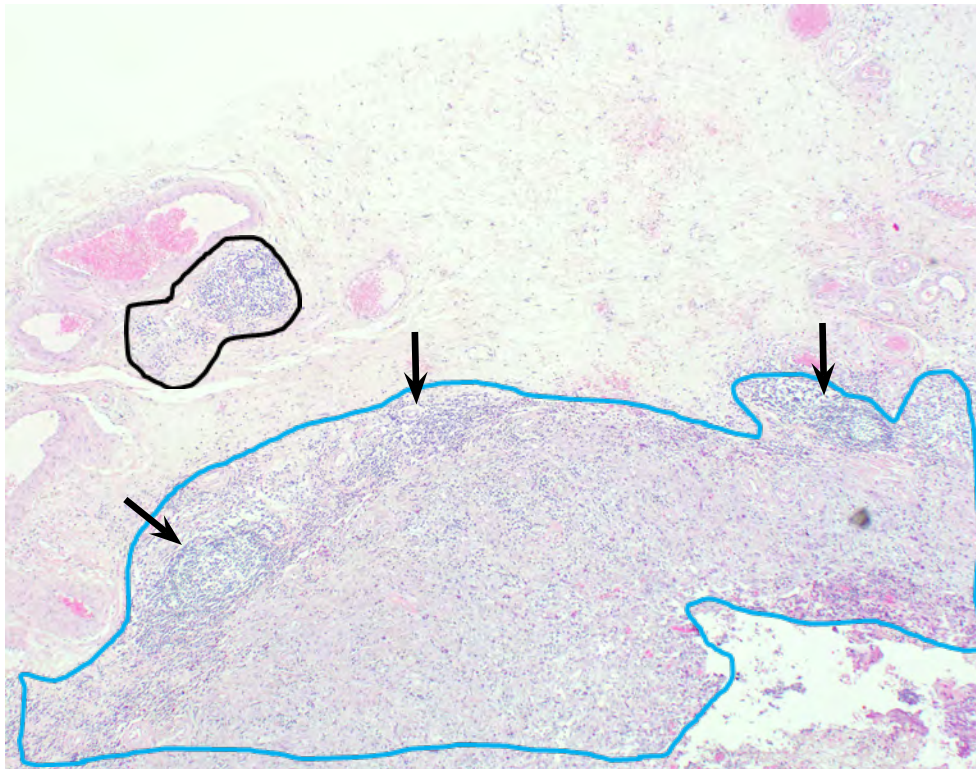
A variety of immune cells display staining with the Assay, and include lymphocytes, macrophages, histiocytes, reticular dendritic cells, plasma cells and neutrophils. The H&E-stained slide is initially examined to determine the total percentage of the tumor area (tumor cells and any desmoplastic stroma) involved by immune cells with no intervening stroma (ICP). Areas not considered part of the tumor area include non-viable tumor such as those with cautery or crush artifacts, and extensive necrosis. Normal lymphoid tissue uninvolved by the neoplasm, as seen in lymph nodes with metastatic tumor, is not considered as a part of the tumor area or immune cells involving the tumor.

The PD-L1 IHC slide is then scored for the percentage of tumor-associated immune cells staining for PD-L1 (IC+). Membranous, cytoplasmic, and punctate PD-L1 immune cell staining are all included in the estimation. In cases where positively-staining immune cells are intermixed with positively-staining tumor cells, it can be difficult to quantify the amount of staining for each component. Examples of immune cell interpretation are given in the following photo sets.

The following steps describe the estimation of tumor-associated immune cell staining percentage.

### Tumor-associated immune cell staining percentage estimation:

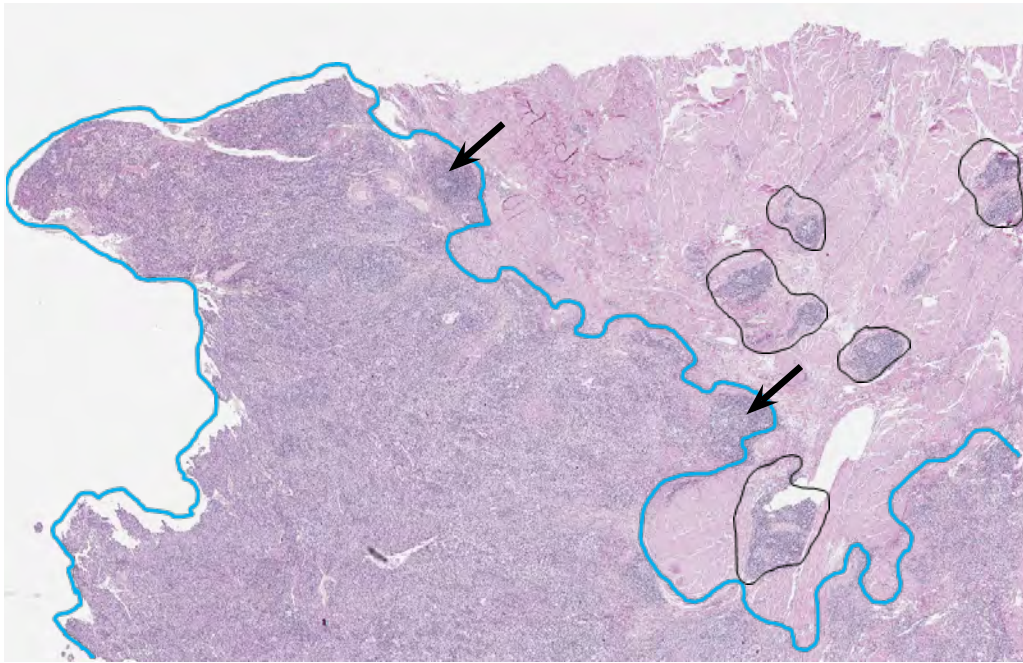
1. Review case H&E to assess viable tumor with attached desmoplastic stroma containing tumor-associated immune cells. Tumor associated immune cells include those present within the tumor reactive stroma, between the tumor islands and those invading the tumor proper. Tumor within lymphatics is not included within the tumor area.



**Immune Cell scoring 1:** Tumor-associated immune cells (black arrows) within the tumor area (blue outline) are present at the leading edge of the tumor and are included in ICP estimation. A separate aggregate of lymphocytes is not within the tumor area (black outline) (4X)

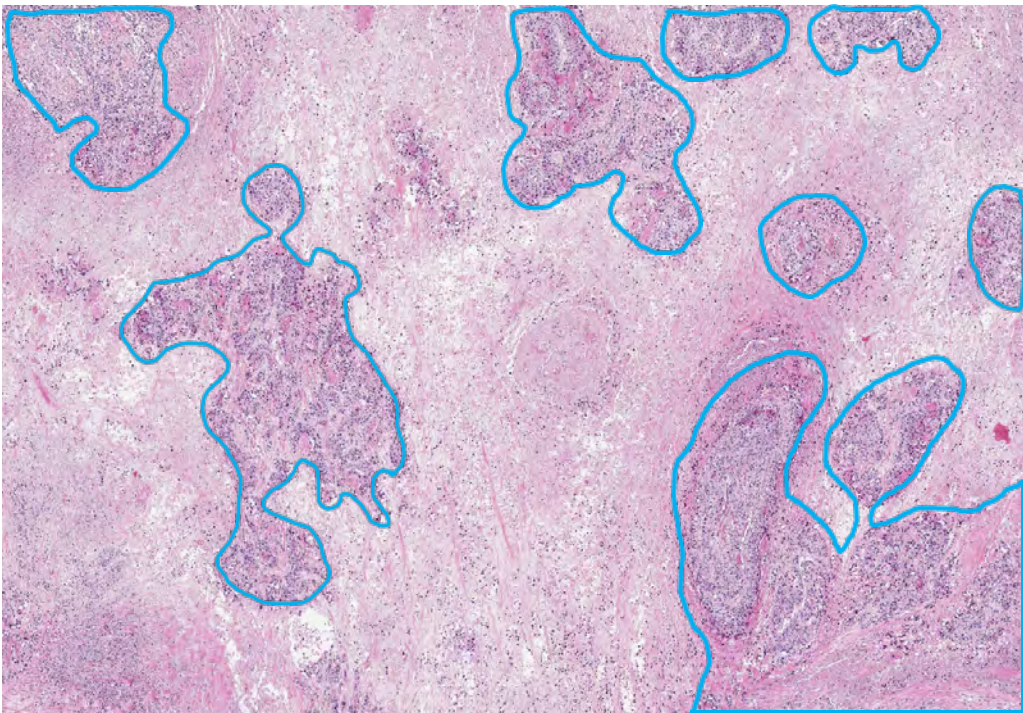


2. Lower magnification review is recommended to perform the percentage estimate of immune cells within and adjacent to the tumor area (ICP). Immune cells located within blood vessels and lymphatics are disregarded.



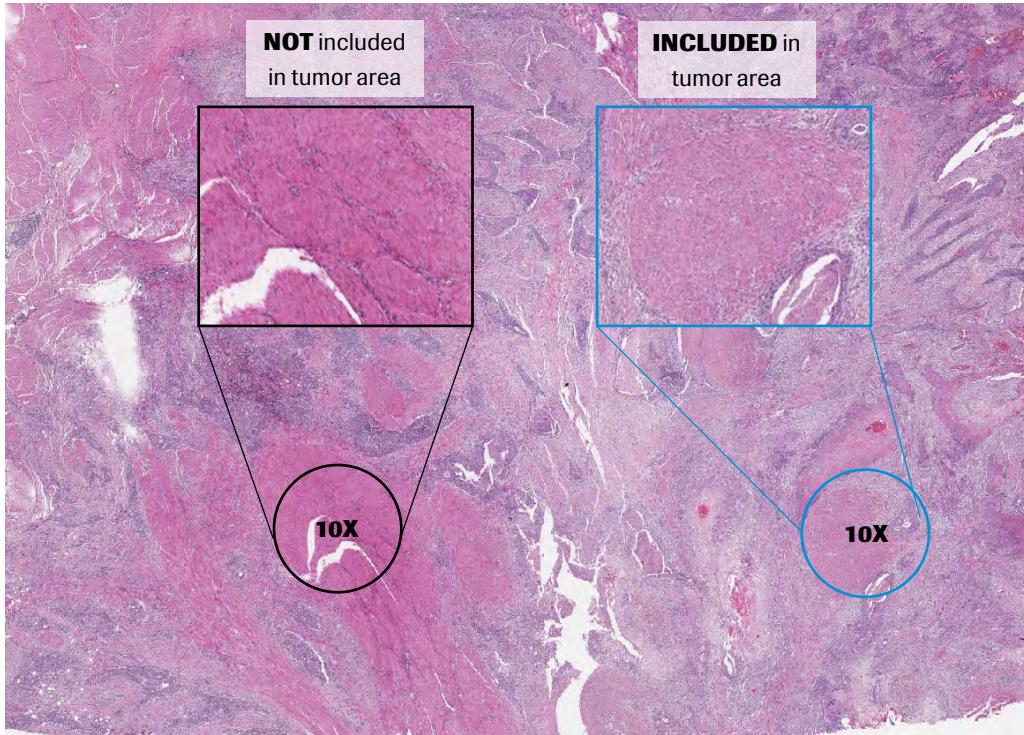
**Immune Cell scoring 2:** Tumor area containing tumor cells, desmoplastic stroma and tumor-associated immune cells is outlined in blue, which includes immune cell aggregates identified by black arrows. Immune cells outside of the tumor area are also present (black outlines) (1X)

3. Exclude non-neoplastic areas not involved by tumor, areas with necrotic tumor, crush and cautery artifacts.



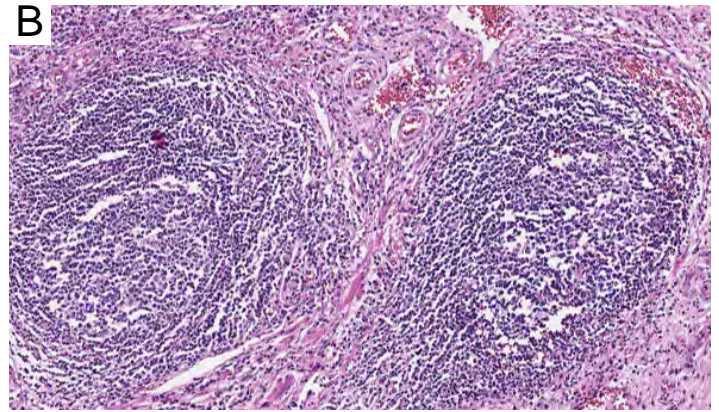
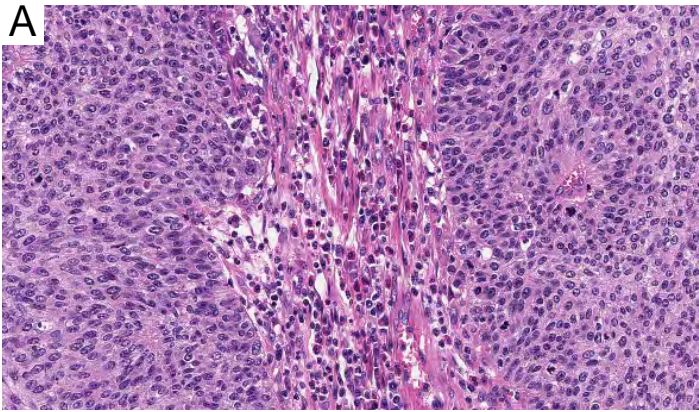
**Immune Cell scoring 3:** Tumor area consists of only viable areas of tumor and desmoplasia (areas outlined in blue). Any necrosis, cautery artifact, crush artifact or large areas of non-neoplastic tissue are disregarded (3X)

4. If tumor islands are separated by muscle or stroma, they are included as part of the tumor area if the tumor borders on both sides within a 10X field.



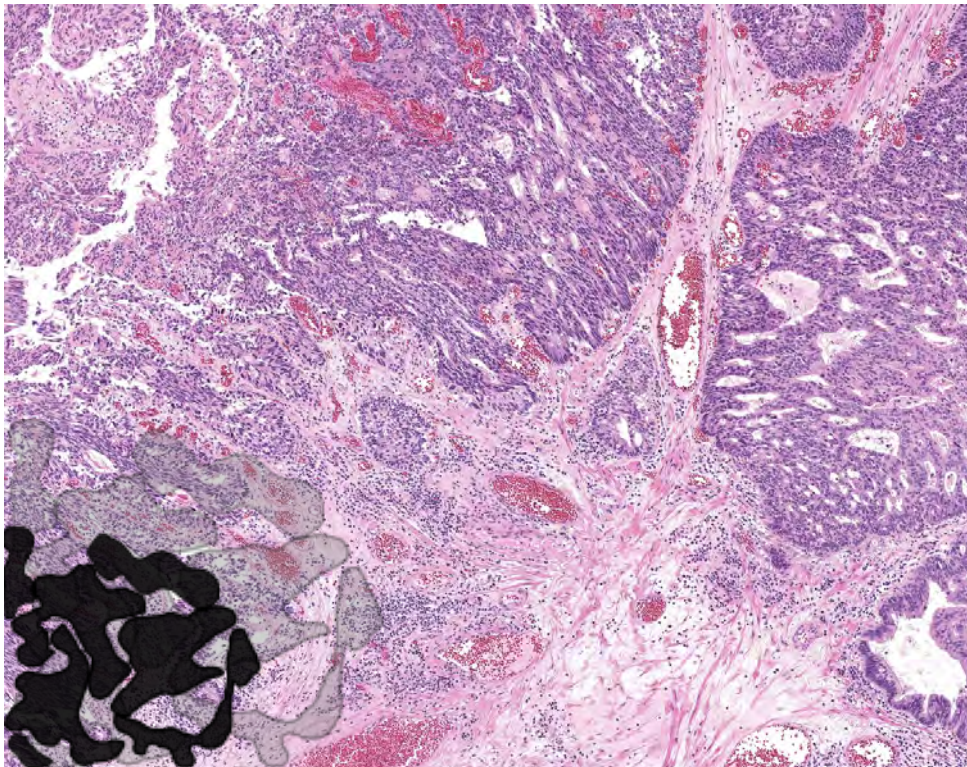
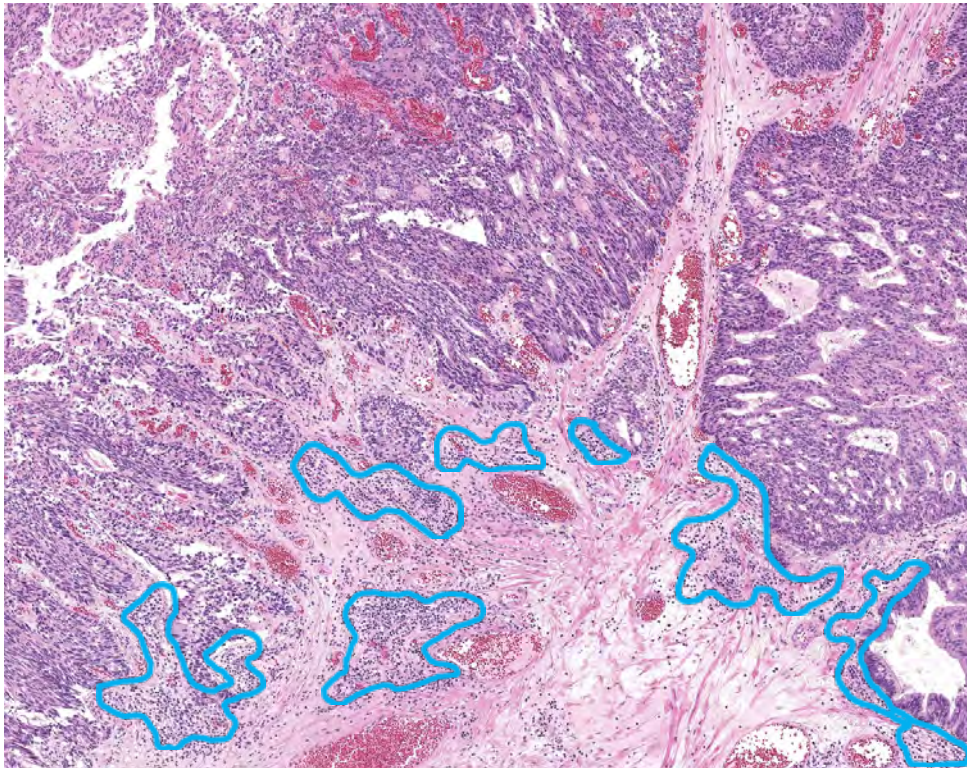
**Immune Cell scoring 4:** The circled areas show large areas of fibromuscular stroma uninvolved by tumor. When viewed at higher power (10X), the stroma with tumor on both sides within a single field of view (blue) is included in the overall tumor area. When the large bundle of fibromuscular stroma is not bordered by tumor within a single 10X field (black), it is not included in the overall tumor area. (1X)

5. For noninvasive urothelial carcinoma (papillary carcinoma), include the immune cells within the fibrovascular cores as well as within the immediately adjacent base of the frond/stalk.

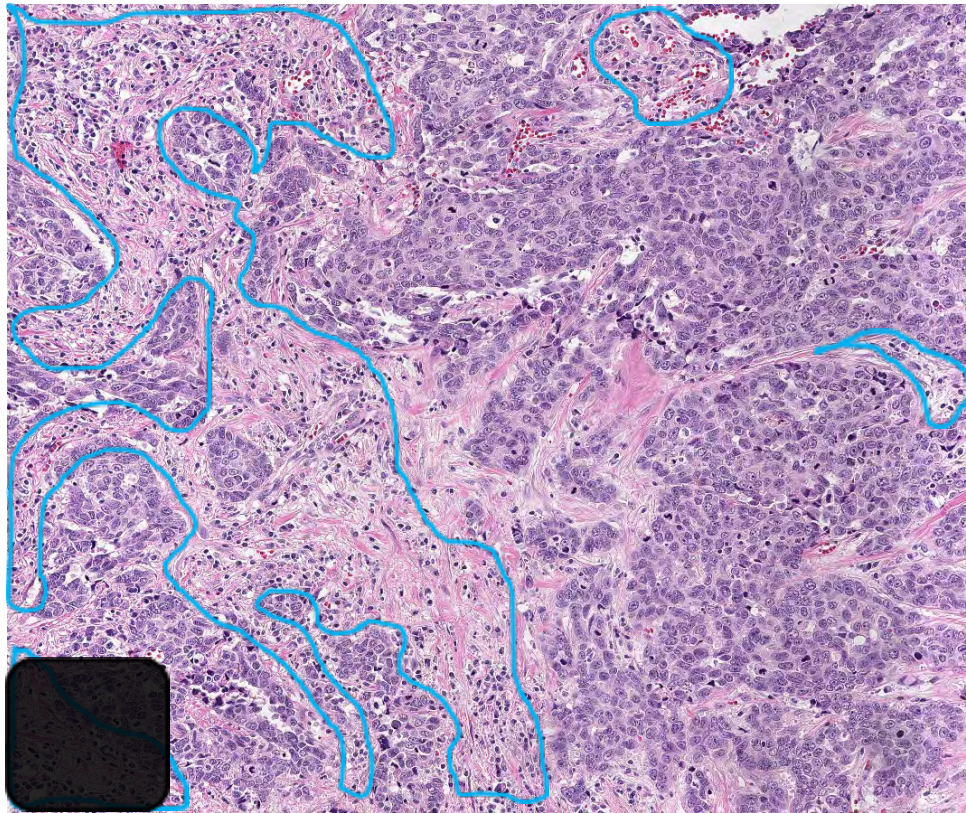


**Immune Cell scoring 5:** Double sided arrow (black) shows base of papillary tumor with the tumor area for this noninvasive urothelial carcinoma outlined (blue). Immune cells from the intratumoral fibrovascular core (**A**) (10X) and adjacent base of the stalk (**B**) (5X) are included in estimation of ICP (0.8X)

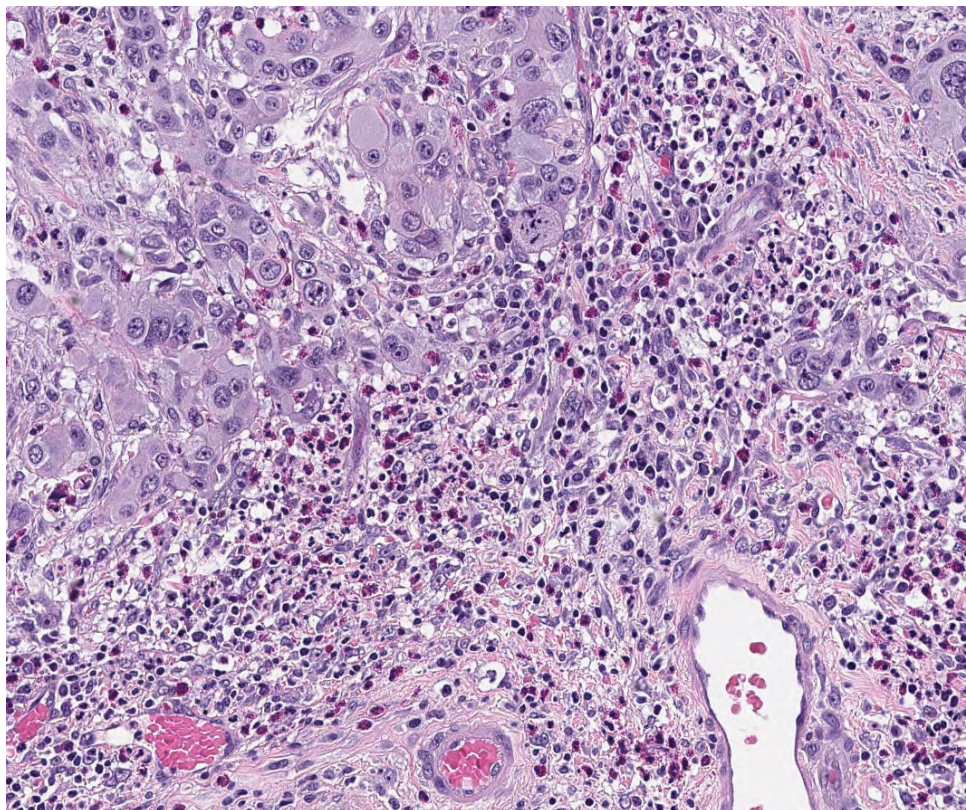
6. Visually estimate the area occupied by the tumor-associated immune cells relative to the total tumor area (ICP = percent of tumor area occupied by immune cells).



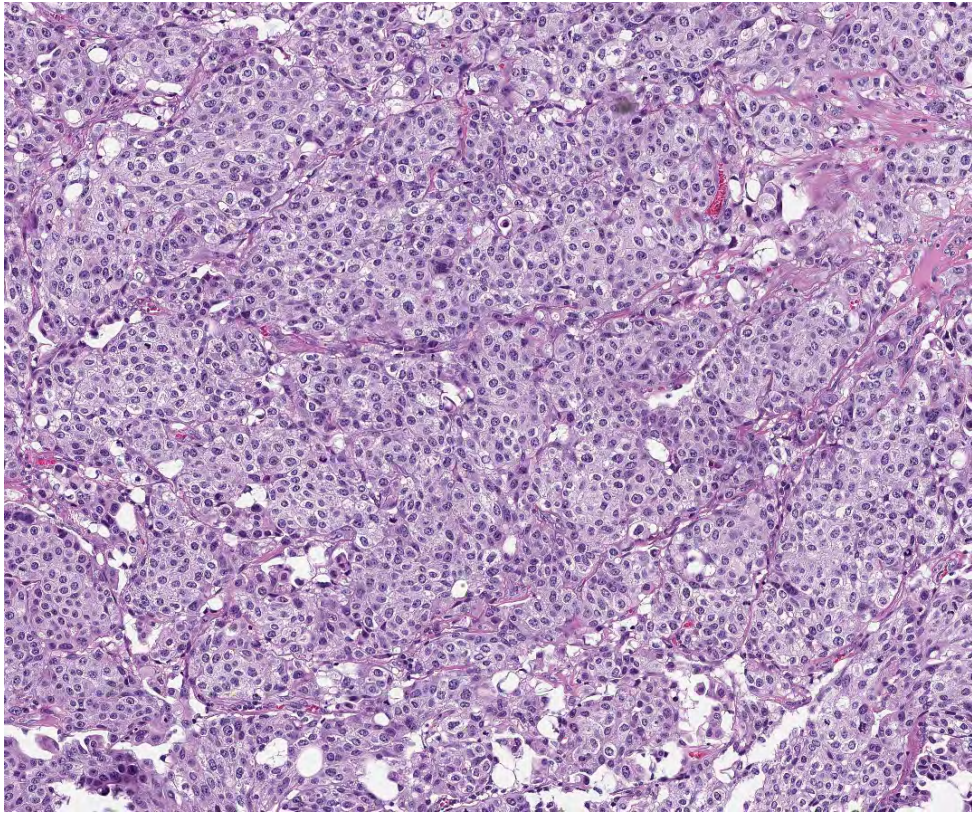
**Immune Cell scoring 6:** The regions occupied by immune cells within the tumor area in the upper image (blue outlines) are identified in the lower image (gray outlines) and then densely aggregated (illustrated by the smaller black areas) in order to estimate the percentage of the tumor area with immune cells, or ICP – in this example 10% (black areas together relative to the tumor area) (5X)



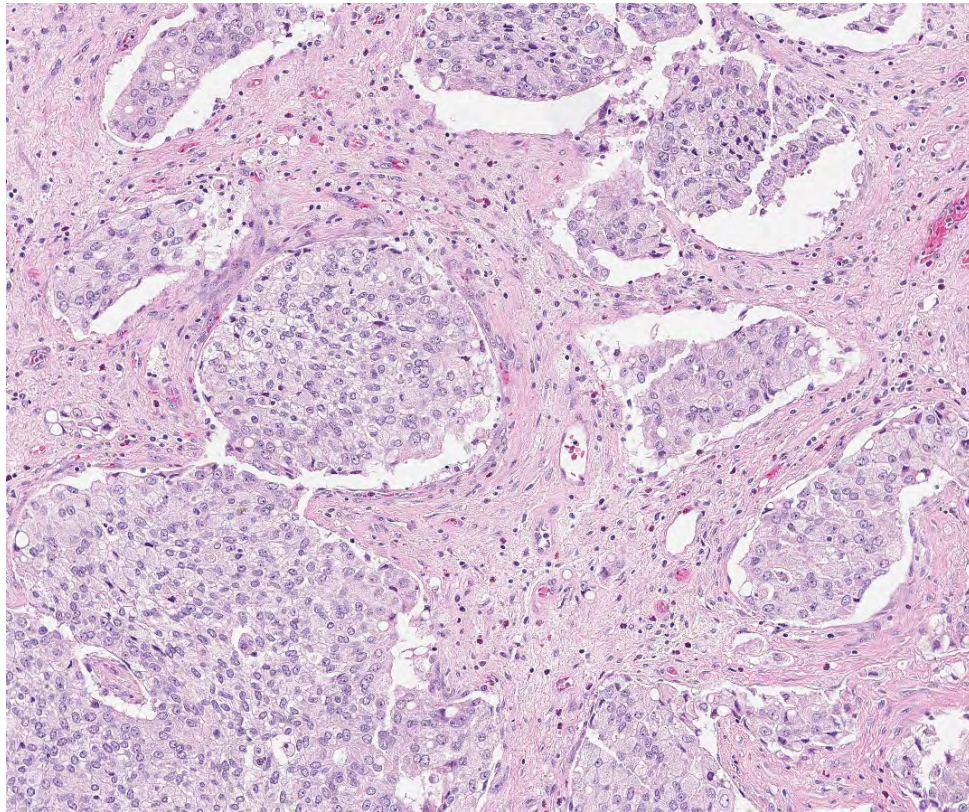
**Immune Cell scoring 7:** The stromal regions occupied by widely dispersed immune cells (blue outlines) are identified. When the immune cells are densely aggregated (represented by the black area) in order to estimate the percentage of the tumor area with immune cells, the ICP in this example is 5% (10X)



**Immune Cell scoring 8:** The immune cell infiltrates with neutrophils and eosinophils, are included in the ICP estimation. The ICP in this example is 25% (10X)

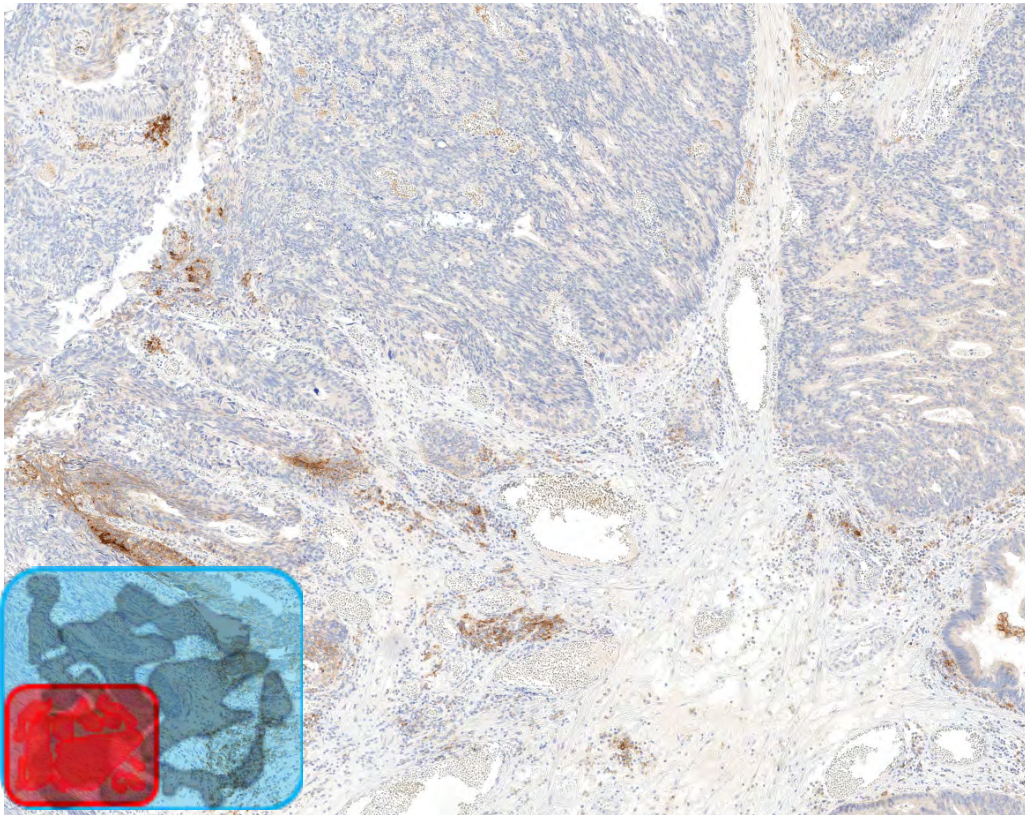
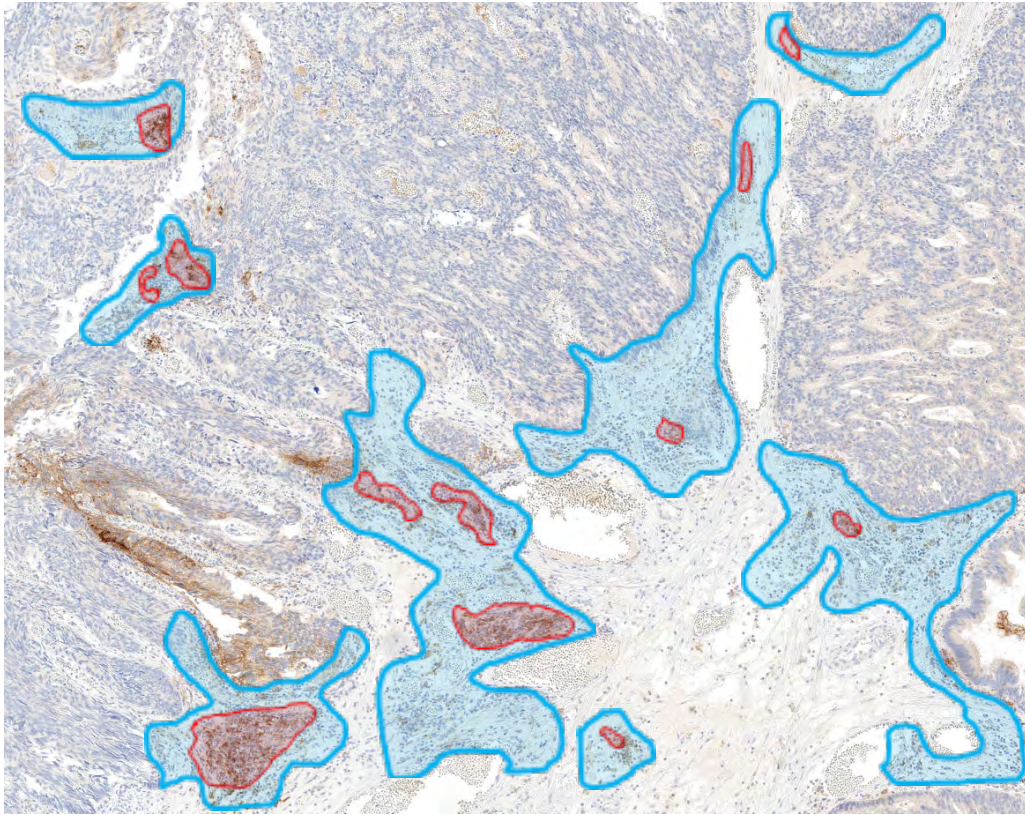


**Immune Cell scoring 9:** In this field of view, the ICP for the small amount of immune cells in the upper right corner is 1% (10X)



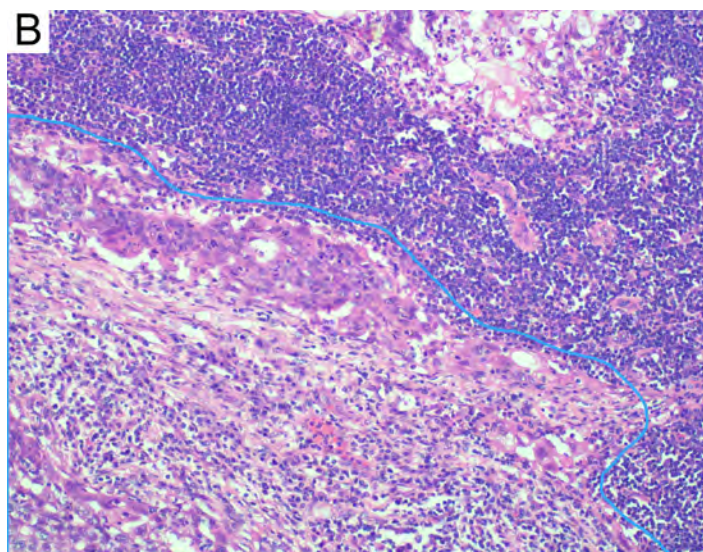
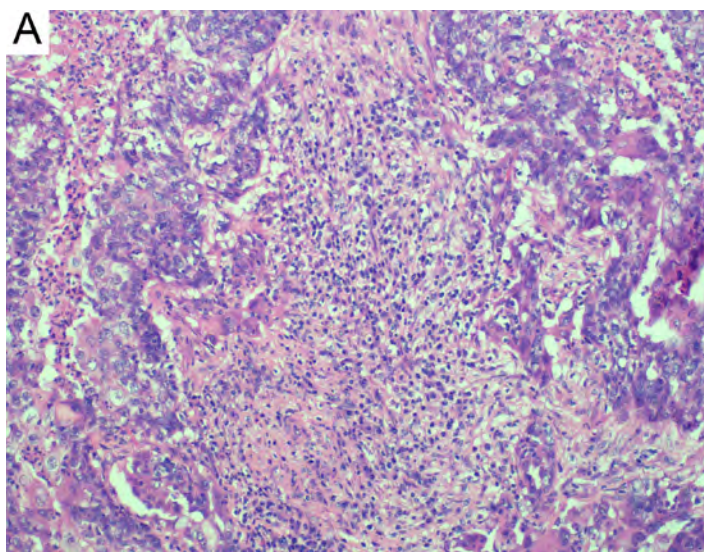
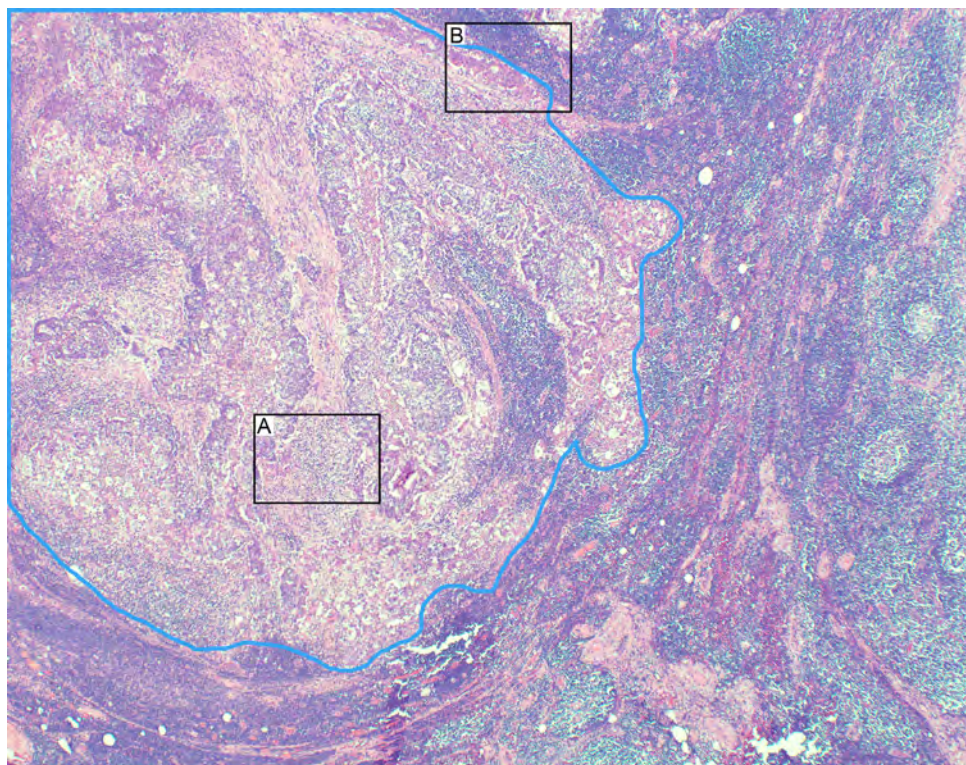
**Immune Cell scoring 10:** The ICP for this field of view when densely aggregated is 5% (10X)

7. Review PD-L1-stained slide and estimate the percentage of tumor-associated immune cells which demonstrate PD-L1 expression (IC+) including diffuse cytoplasmic, linear membrane and punctate immune cell staining.



**Immune Cell scoring 11:** The immune cells comprising ICP are outlined in blue (upper image) and can be seen densely aggregated in the lower image. The immune cells with PD-L1 staining (IC+) are highlighted in red (upper image) and when aggregated constitute 25% of the ICP (lower image) (5X). Please refer to the associated H&E image contained within this guide, "Immune Cell scoring 6."

8. When evaluating lymph node metastases the reactive stroma generated by the tumor is included as part of the tumor area when determining ICP. In cases where the tumor does not generate a stromal response, the tumor area is limited to the tumor nests and adjacent immune cells in direct contact with the tumor only. Any immune cells part of the uninvolved lymphoid tissue are disregarded for ICP.



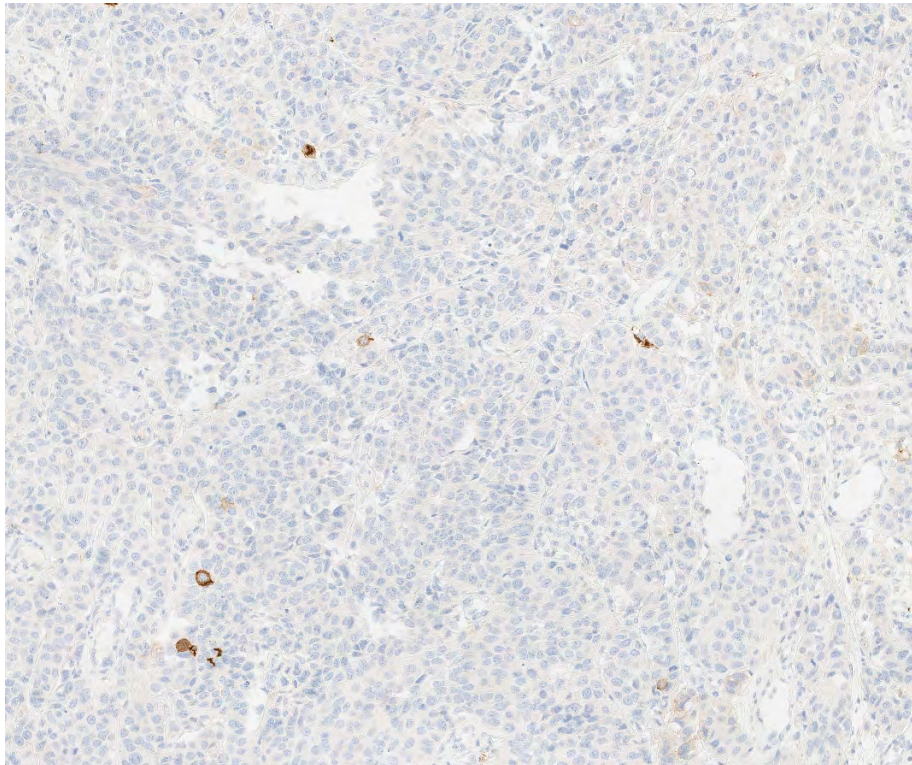
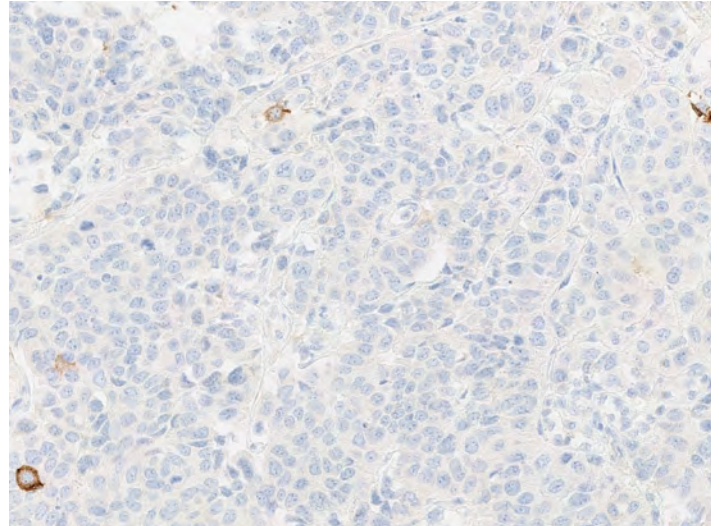
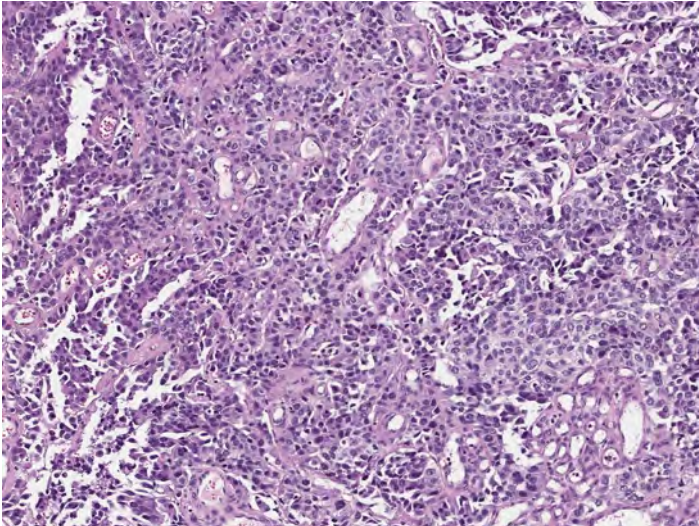
**Immune Cell scoring 12:** In this metastatic tumor to a lymph node, the tumor area containing tumor cells, desmoplastic stroma and immune cells is outlined in blue (4X). Higher power shows immune cells that are included as part of ICP within the reactive stroma (inset A (20X)). Along the periphery of the metastatic nodule only immune cells immediately adjacent to the tumor cells outlined in blue are included as part of ICP (inset B (20X)).

9. Only in cases where the ICP equals 1%, the IC+ is only scored as 0%, <100% or 100% due to the difficult nature of quantifying expression in such small amounts of cells.
10. If the PD-L1-stained slide shows more immune cell staining than the initial ICP estimation from the H&E slide, refer back to the H&E slide and revise the ICP percentage, if necessary.

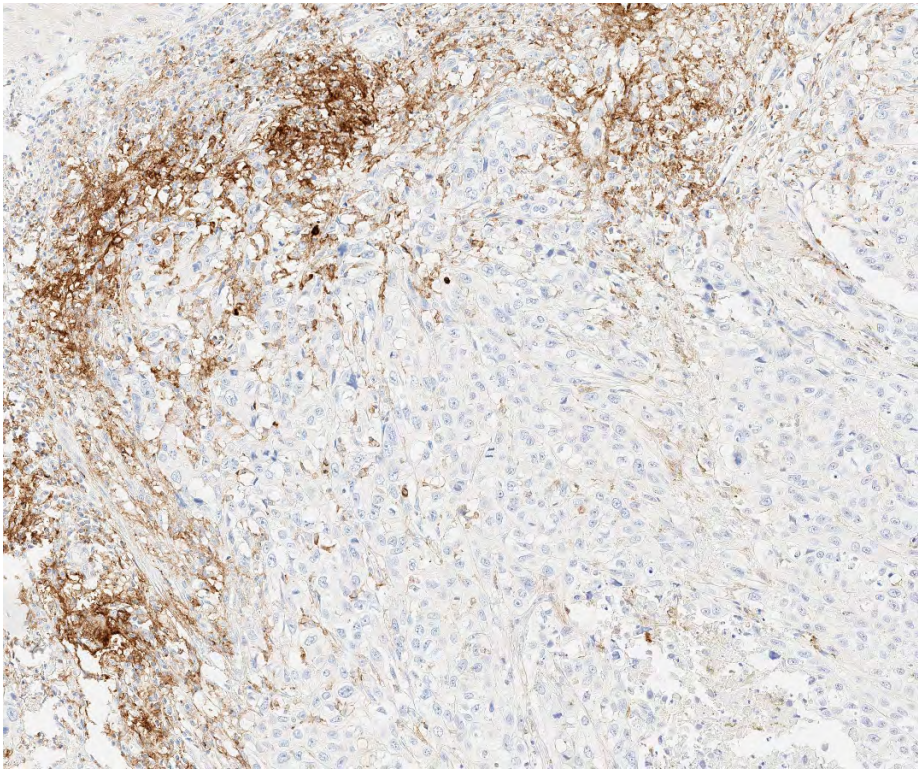
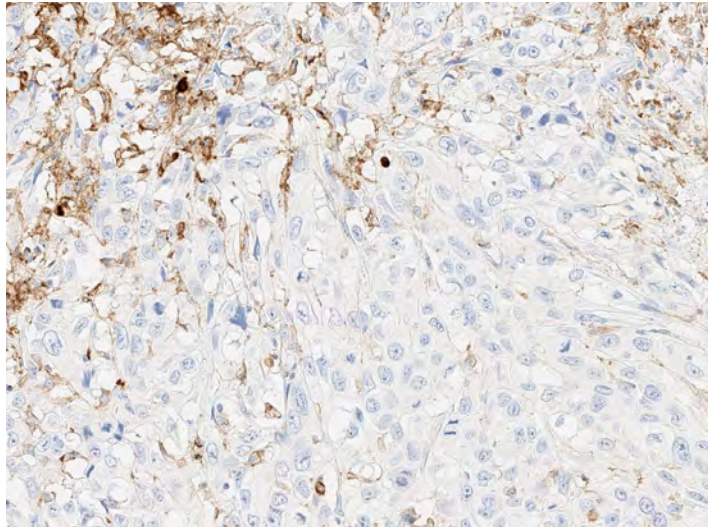
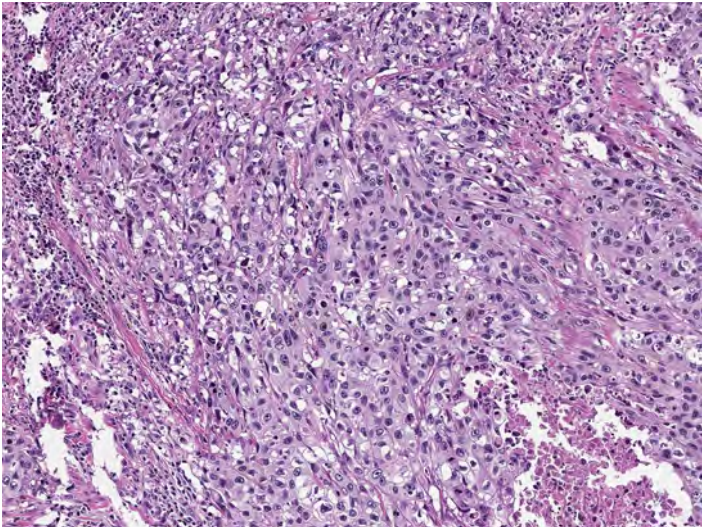


# PD-L1 Expression Atlas in Urothelial Carcinoma

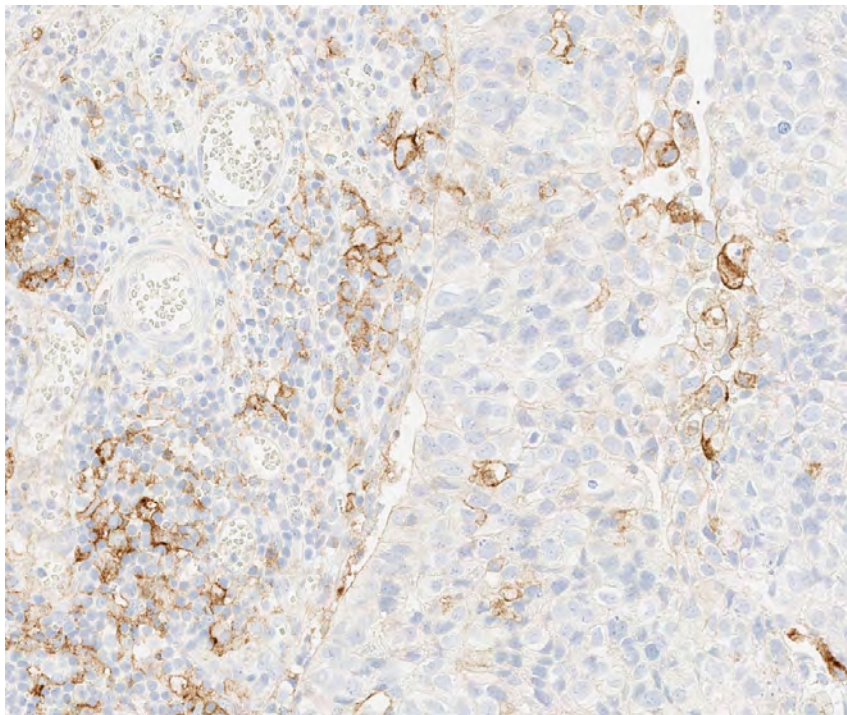
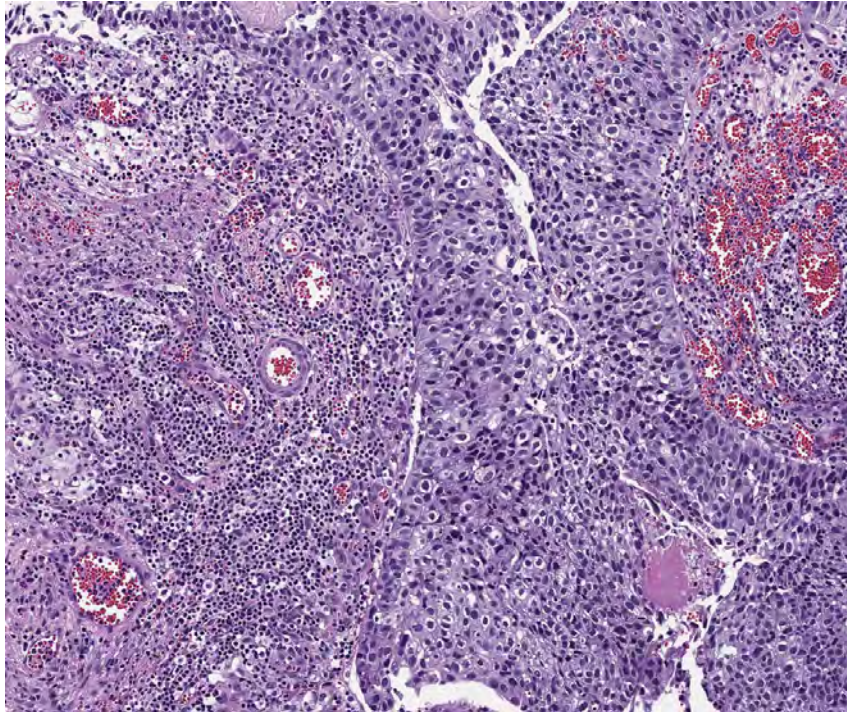
## Tumor Cell Cases



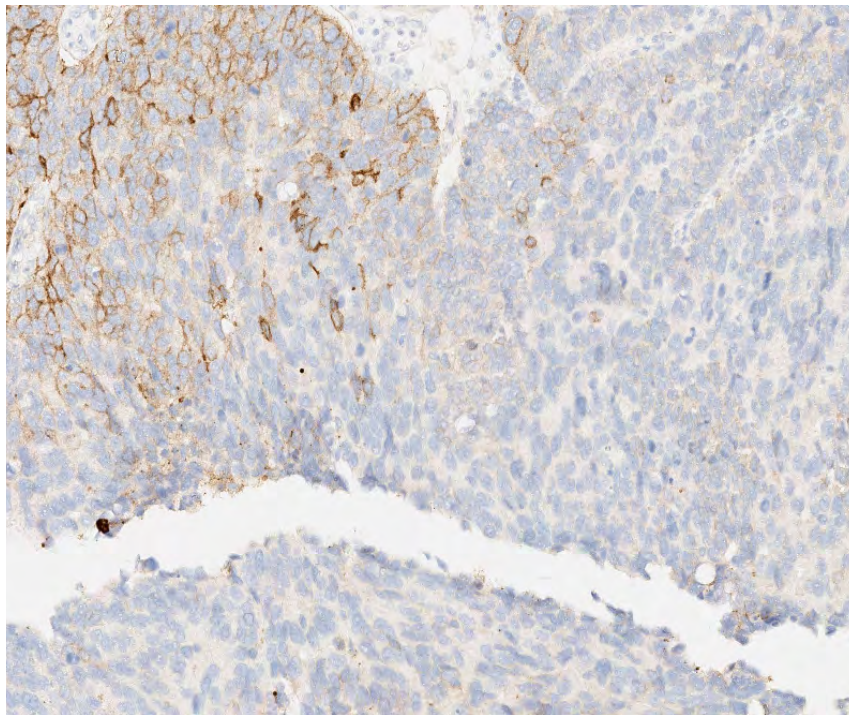
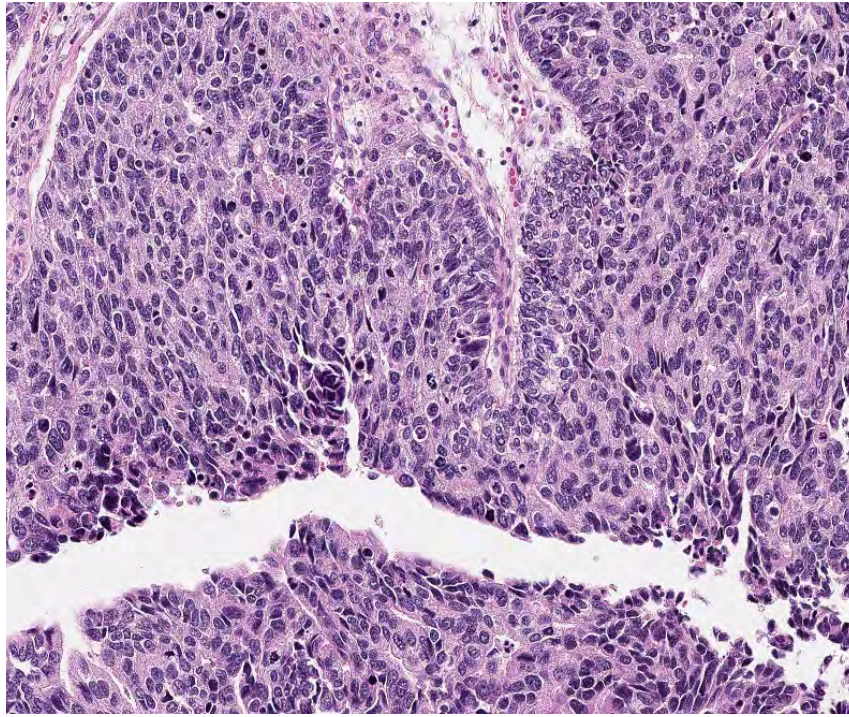
**Tumor Cell Case 1:** Tumor cells with 0% staining, Immune cells with 0% staining. (IC+) (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)



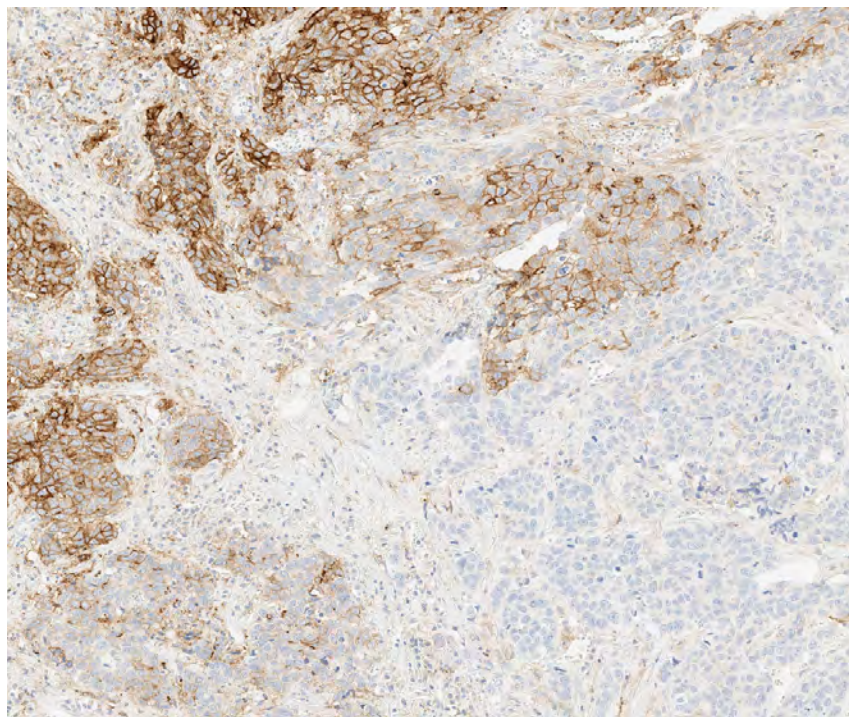
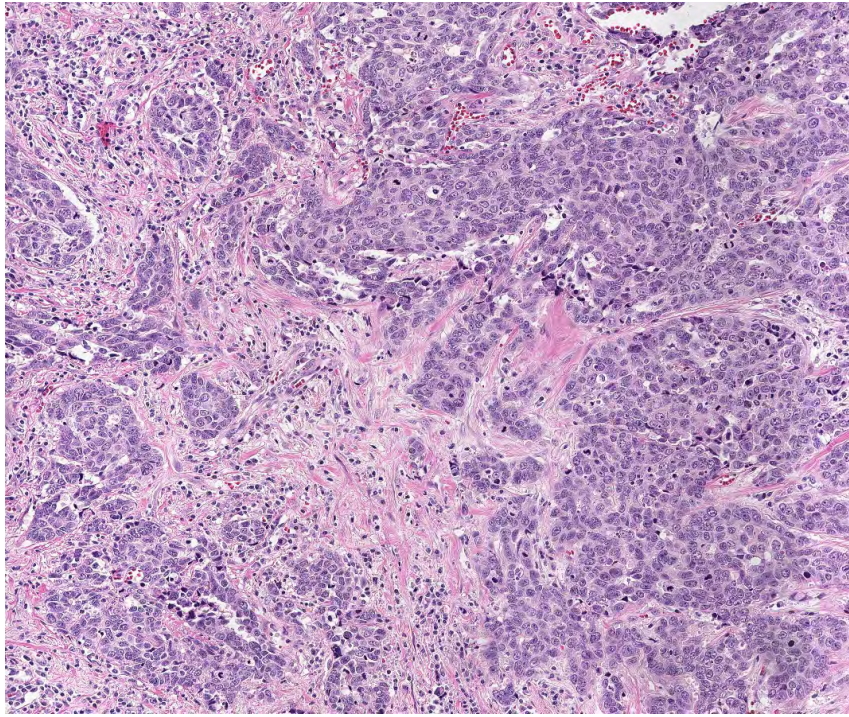
**Tumor Cell Case 2:** Tumor cells with 0% staining, Immune cells with 80% staining. (IC+) (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)



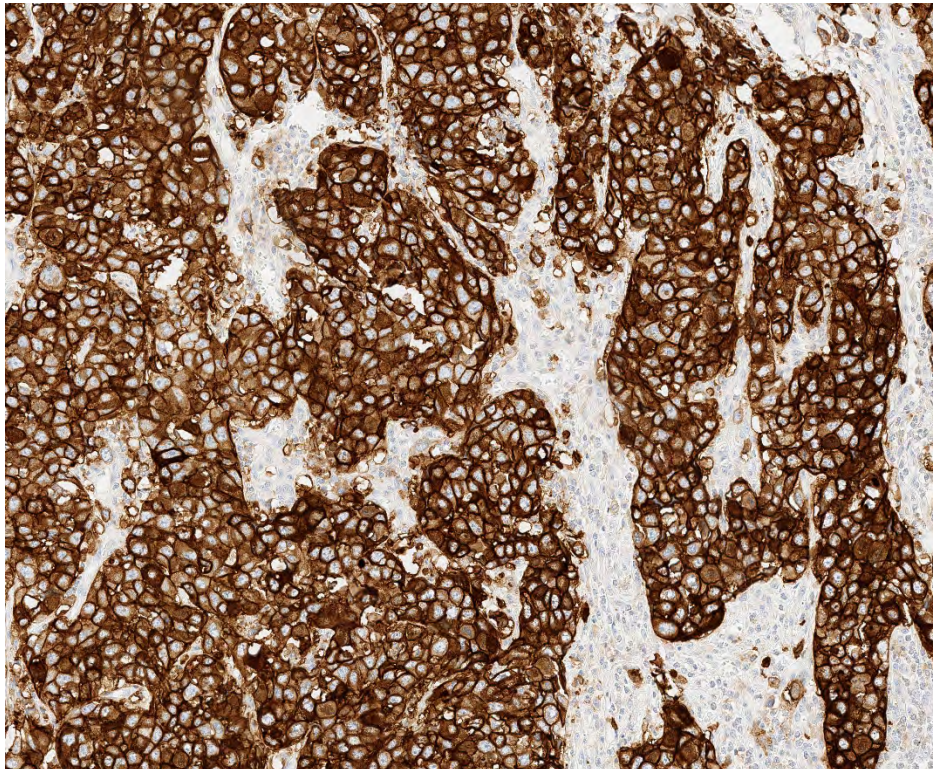
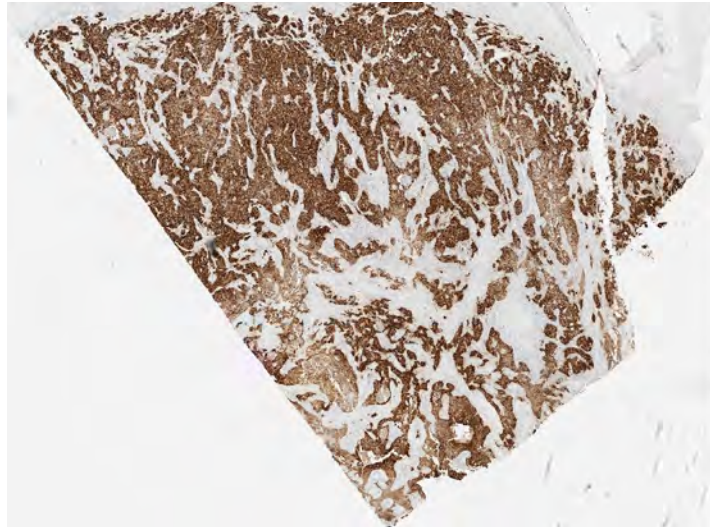
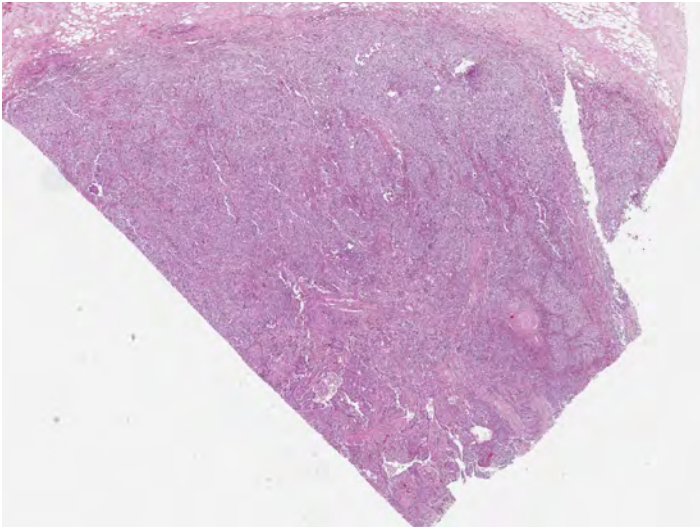
**Tumor Cell Case 3:** Tumor cells with 10% staining, Immune cells with 40% staining (IC+) (10X)



**Tumor Cell Case 4:** Tumor cells with 25% staining, Immune cells with 10% staining (IC+) (10X)

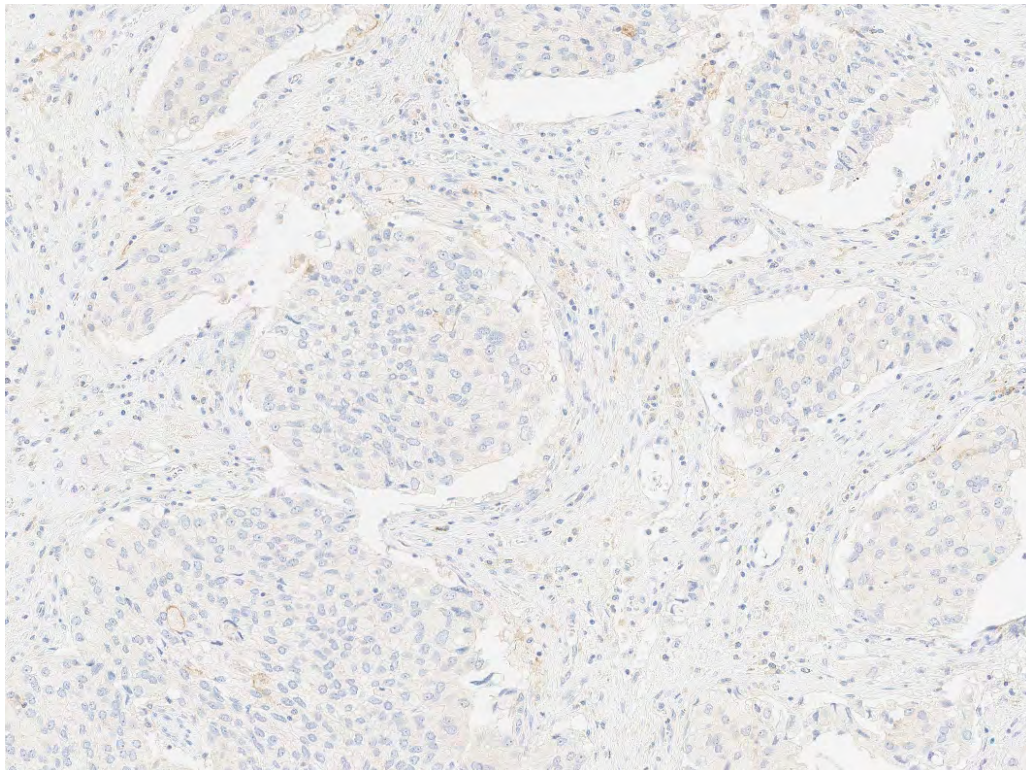
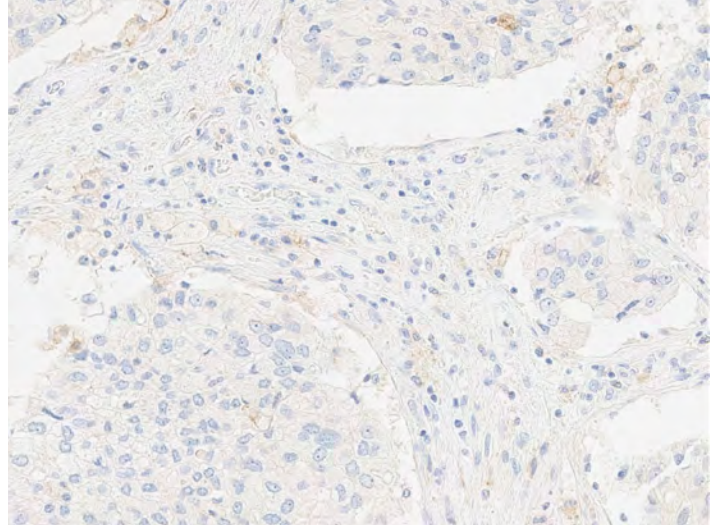
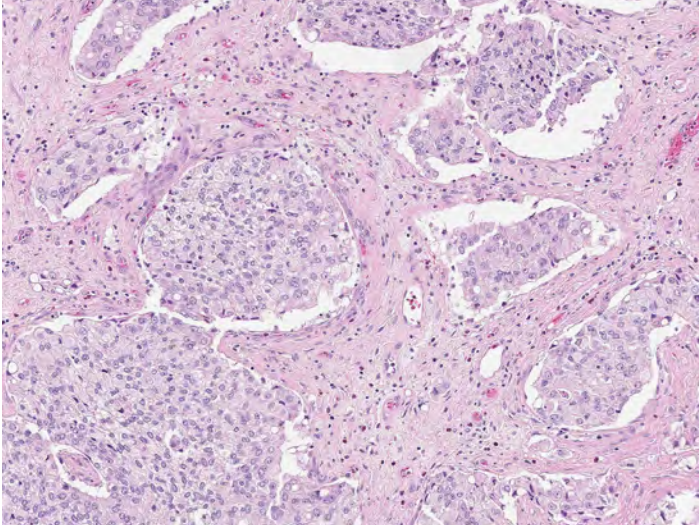


**Tumor Cell Case 5:** Tumor cells with 50% staining, Immune cells with 20% staining (IC+) (10X)

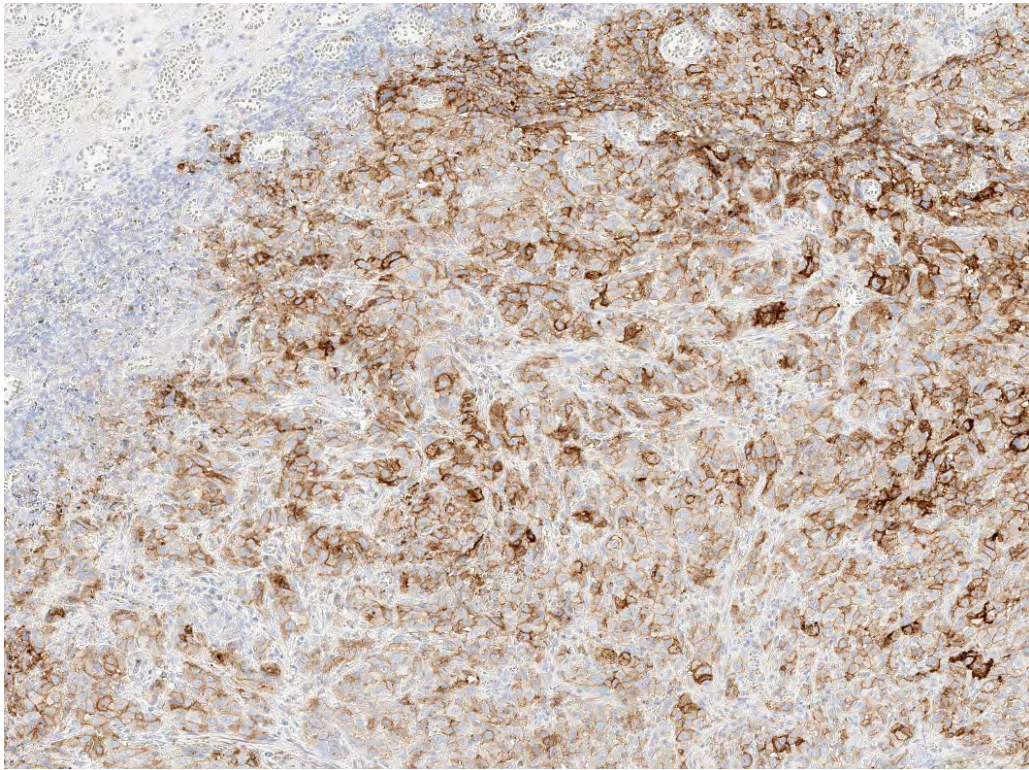
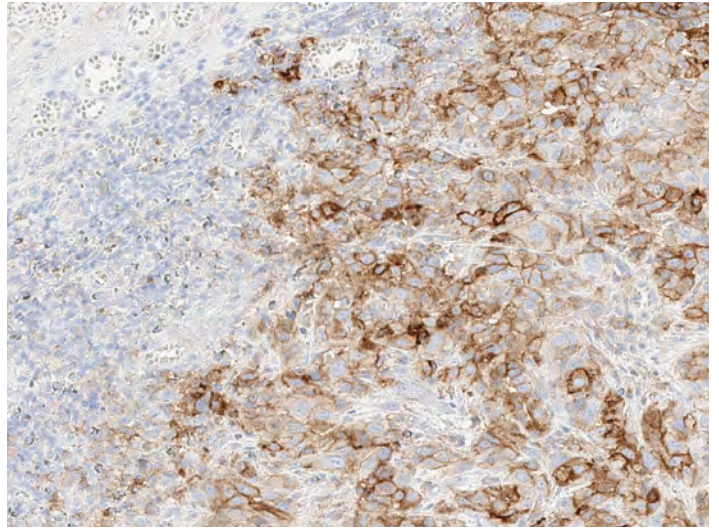
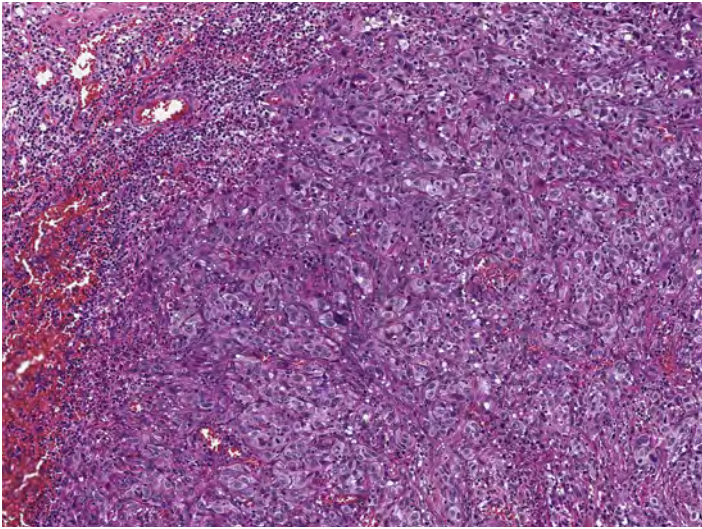


**Tumor Cell Case 6:** Tumor cells with 100% staining, Immune cells with 20% staining. (IC+) (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)

## Immune Cell Cases

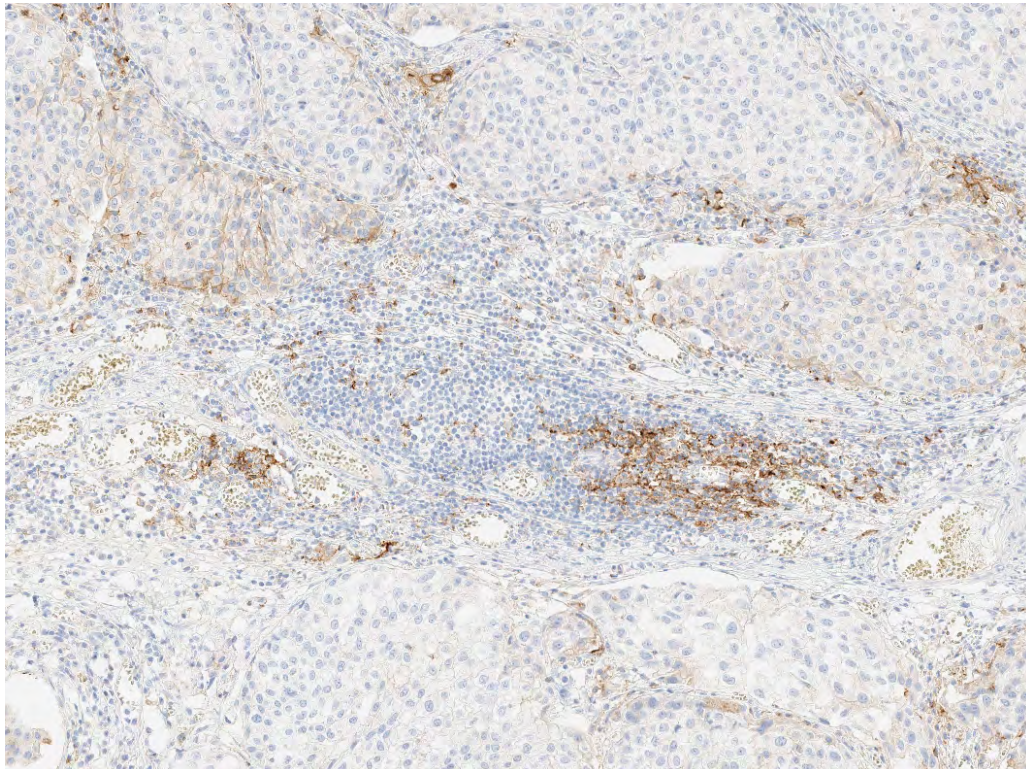
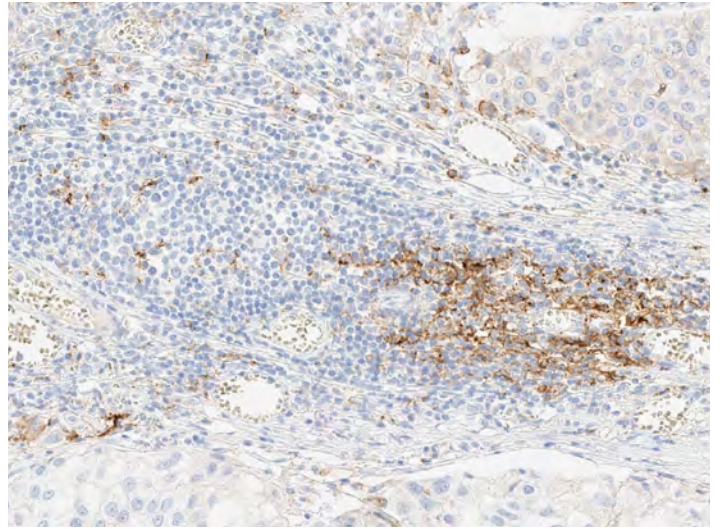
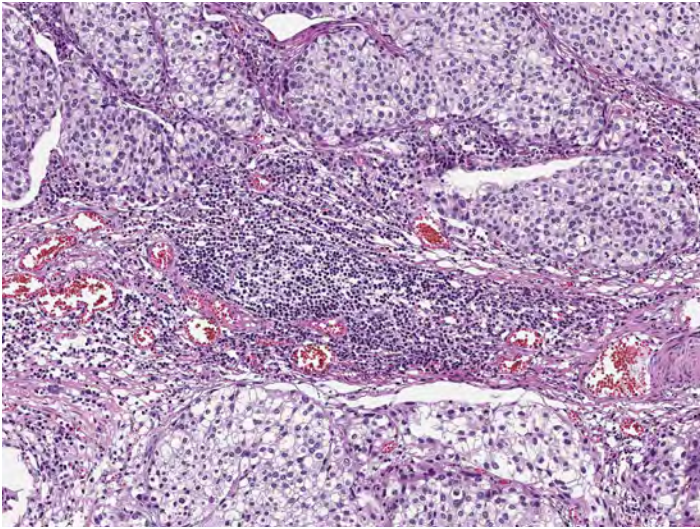


**Immune Cell Case 1:** Immune cells with 0% staining (IC+), Tumor cells with 0% staining. (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)

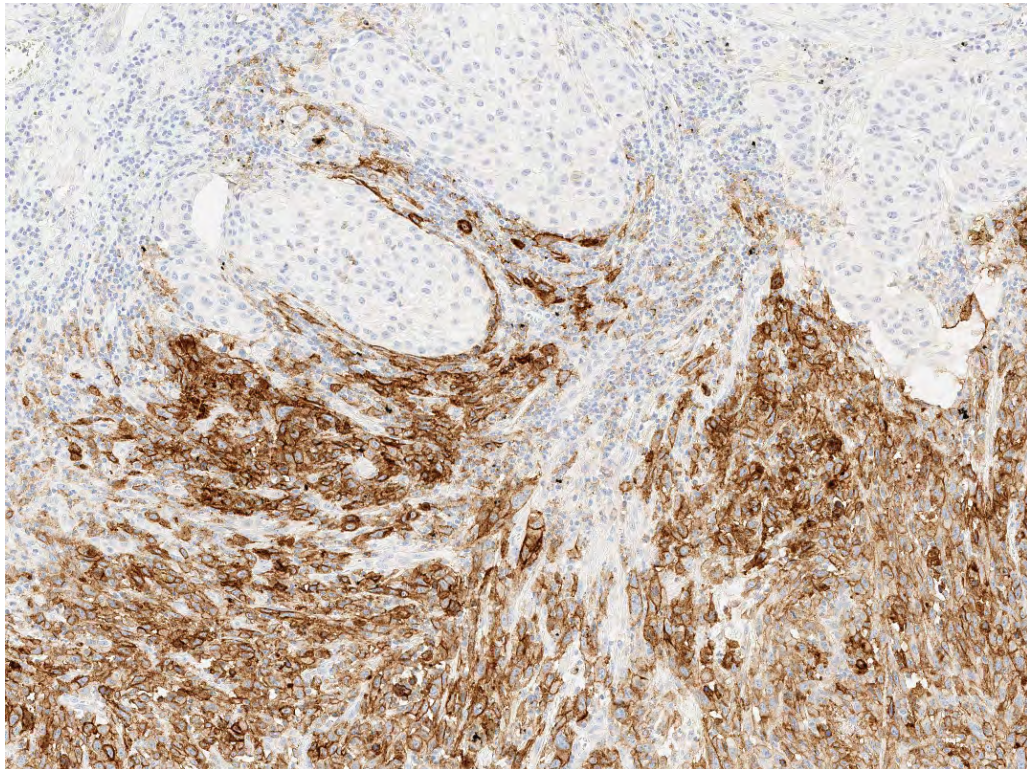
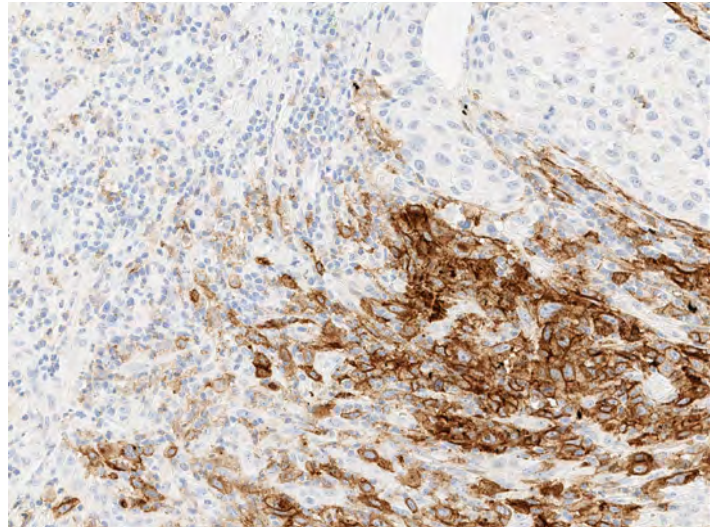
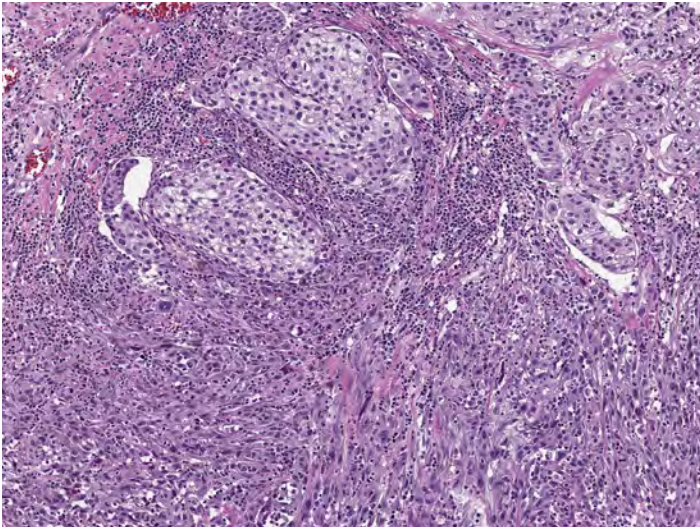


**Immune Cell Case 2:** Immune cells with 10% staining (IC+), Tumor cells with 80% staining. (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)

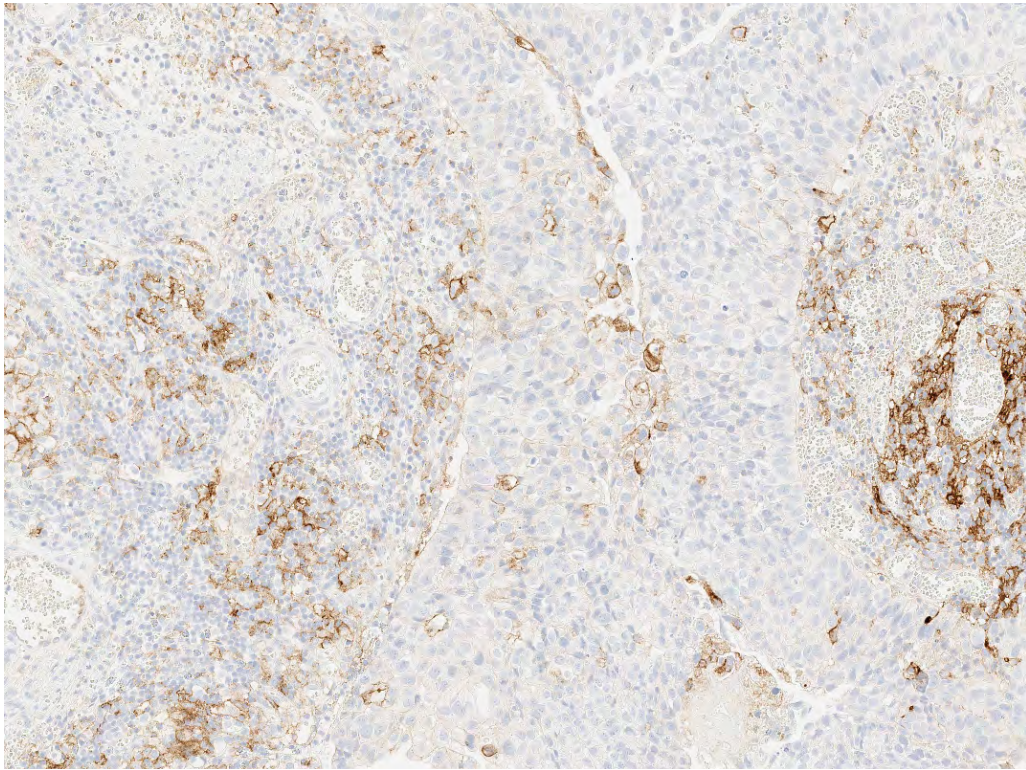
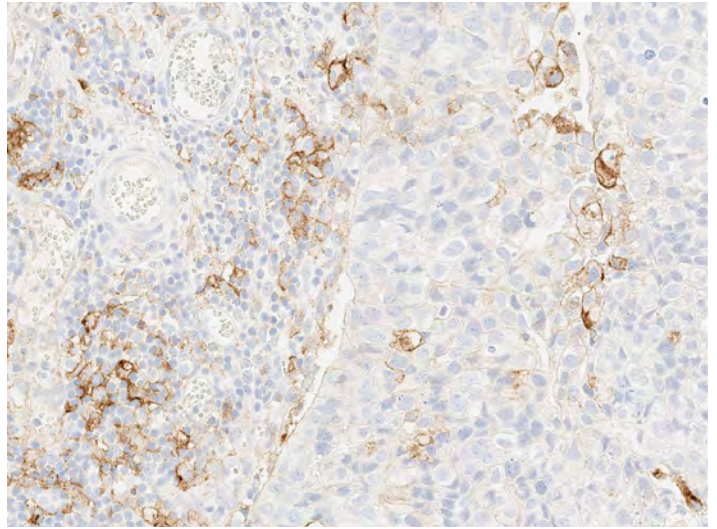
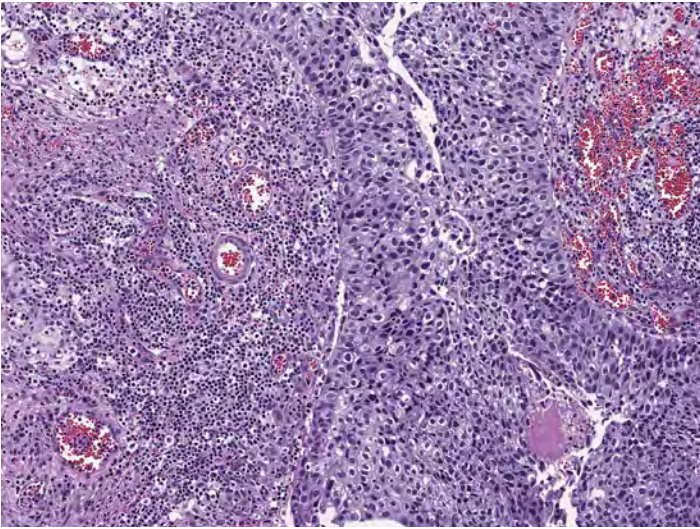




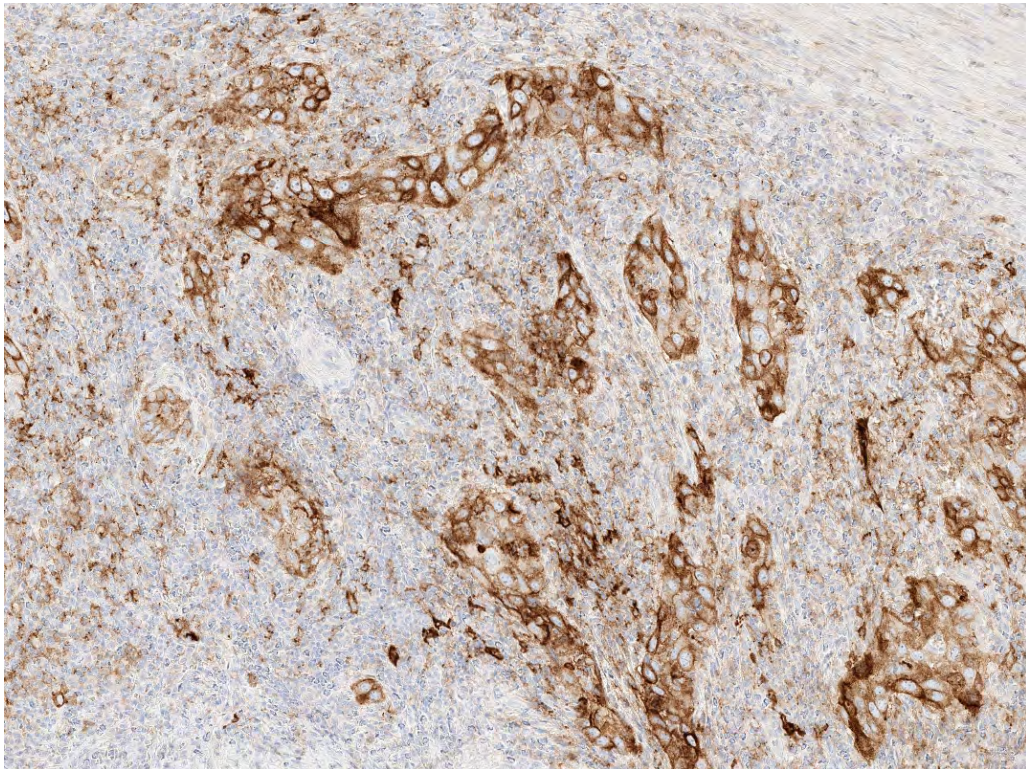
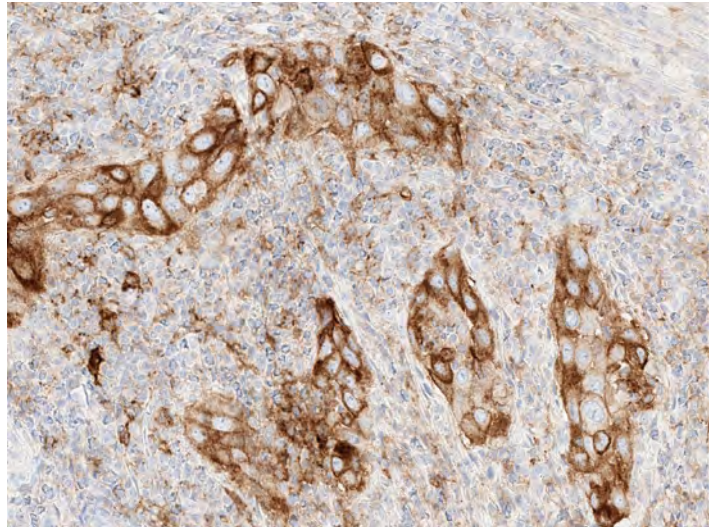
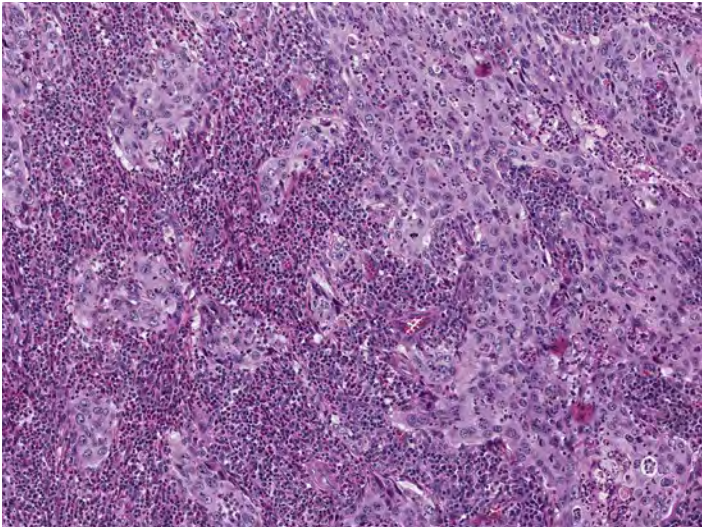
**Immune Cell Case 3:** Immune cells with 30% staining (IC+), Tumor cells with 10% staining. (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)



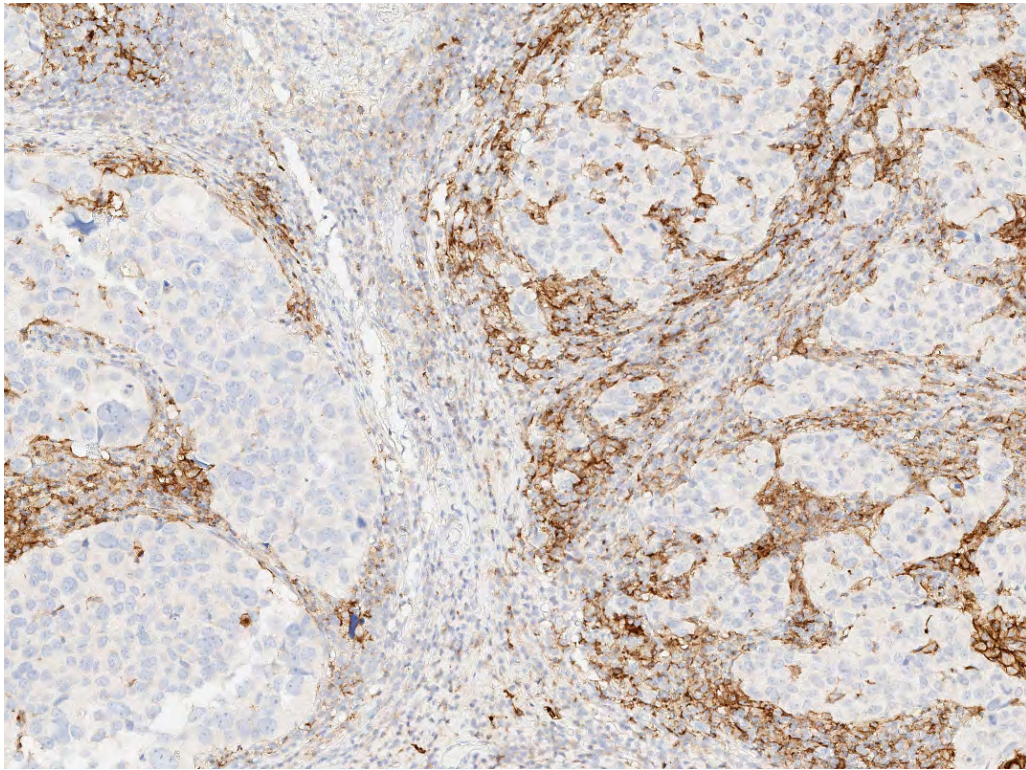
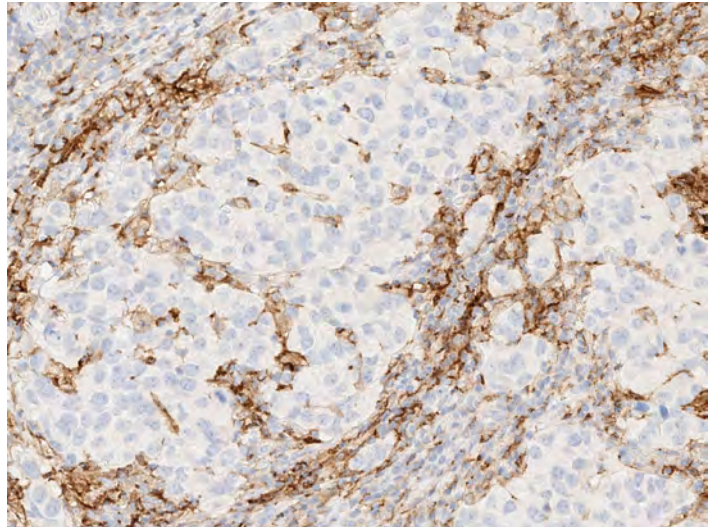
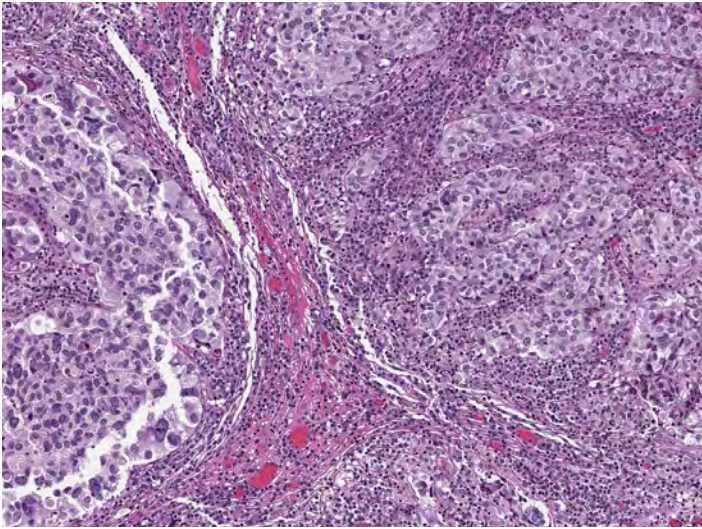
**Immune Cell Case 4:** Immune cells with 25% staining (IC+), Tumor cells with 100% staining. (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)



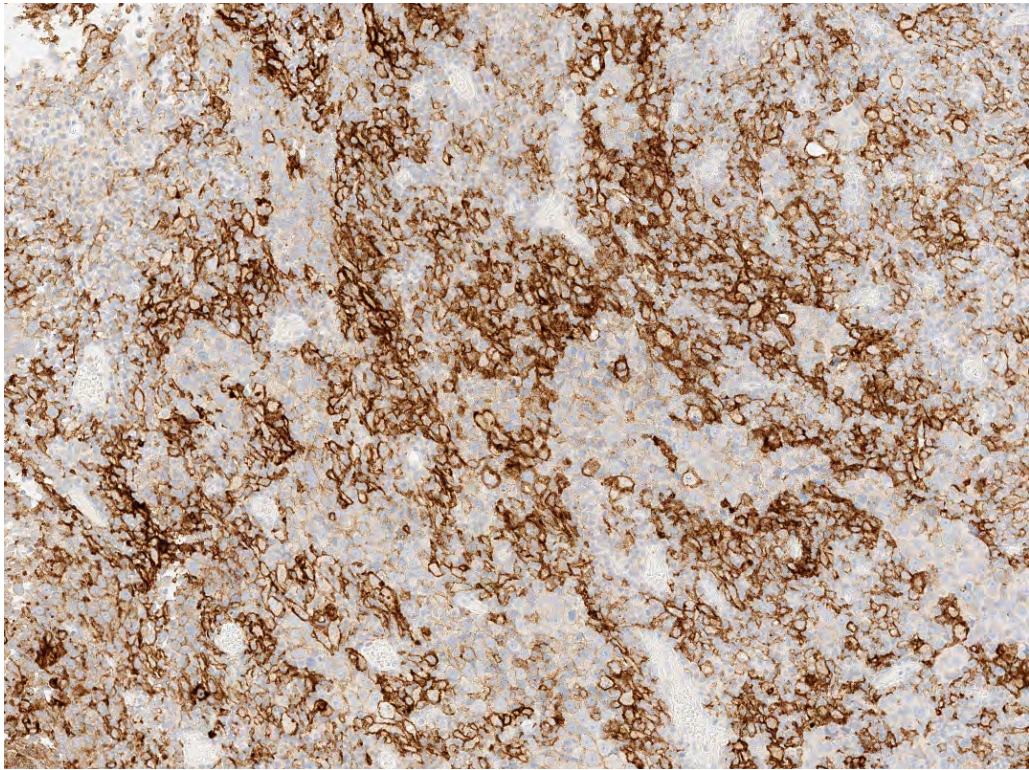
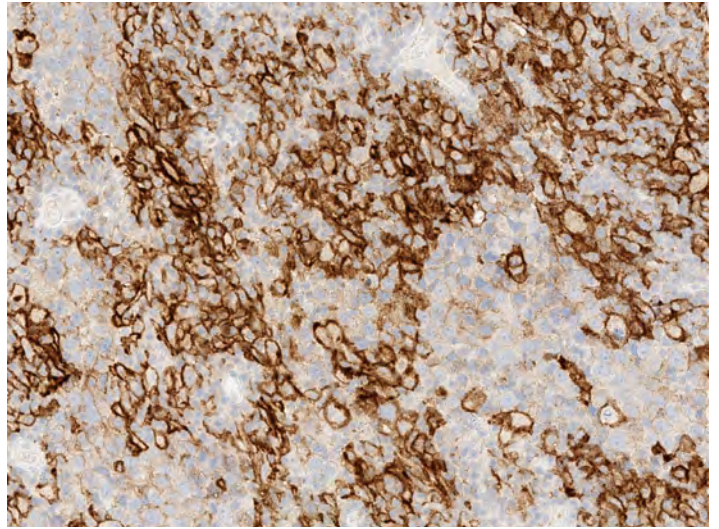
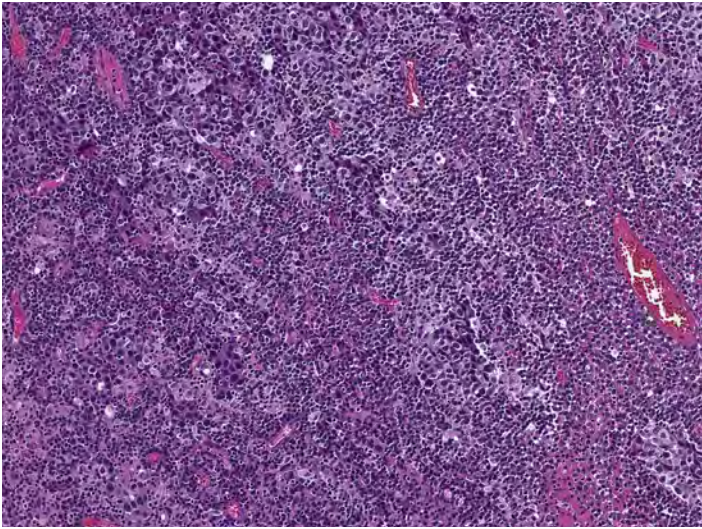
**Immune Cell Case 5:** Immune cells with 40% staining (IC+), Tumor cells with 10% staining. (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)



**Immune Cell Case 6:** Immune cells with 50% staining (IC+), Tumor cells with 100% staining. (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)



**Immune Cell Case 7:** Immune cells with 60% staining (IC+), Tumor cells with 0% staining. (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)



**Immune Cell Case 8:** Immune cells with 90% staining (IC+), Tumor cells with 100% staining. (Top row H&E 10X, PD-L1 20X. Bottom row PD-L1 10X.)

# Challenging Cases

Cases are given a PD-L1 score according to percentage of tumor cells with membrane staining and tumor-associated immune cells. Various staining patterns and morphologic features may make interpretation and quantification of tumor membrane staining difficult.

Some cases may be particularly challenging due to the following issues:

- **Weak Tumor Cell Membrane vs Weak Tumor Cell Cytoplasmic Staining**

Some specimens may exhibit weak cytoplasmic staining of the tumor cells that may be confused at low power with weak tumor cell membrane staining. For this reason when evaluating PD-L1 IHC stained slides, weak staining should be confirmed with examination at higher powers to distinguish between tumor cell membranous and cytoplasmic staining.

- **Strong Immune Cell Staining Overlapping with Tumor Cell Staining**

Some tumors may contain an extensive inflammatory component both surrounding the tumor and infiltrating within the tumor. In instances when significant staining is seen for both tumor and immune cells, it can be challenging to differentiate and quantify the PD-L1 IHC staining between the two cell populations. The presence of immune cells infiltrating the tumor should be confirmed using the H&E slide. The pattern of PD-L1 staining and nuclear morphology are utilized to help attribute expression to immune cells (punctate staining, small uniform nucleus) and tumor cells (linear membrane staining, large irregular nucleus).

- **Borderline PD-L1 (SP263) Status**

Some cases are near the cut-off between a PD-L1 High and Low/negative status. These cases are particularly challenging to estimate the percent of target cells with staining. For these borderline cases it may be helpful to view the case at a magnification that enables the entire tumor area to be assessed. Consultation with a trained colleague may also be helpful.

- **Cases with Multiple Tissue Fragments**

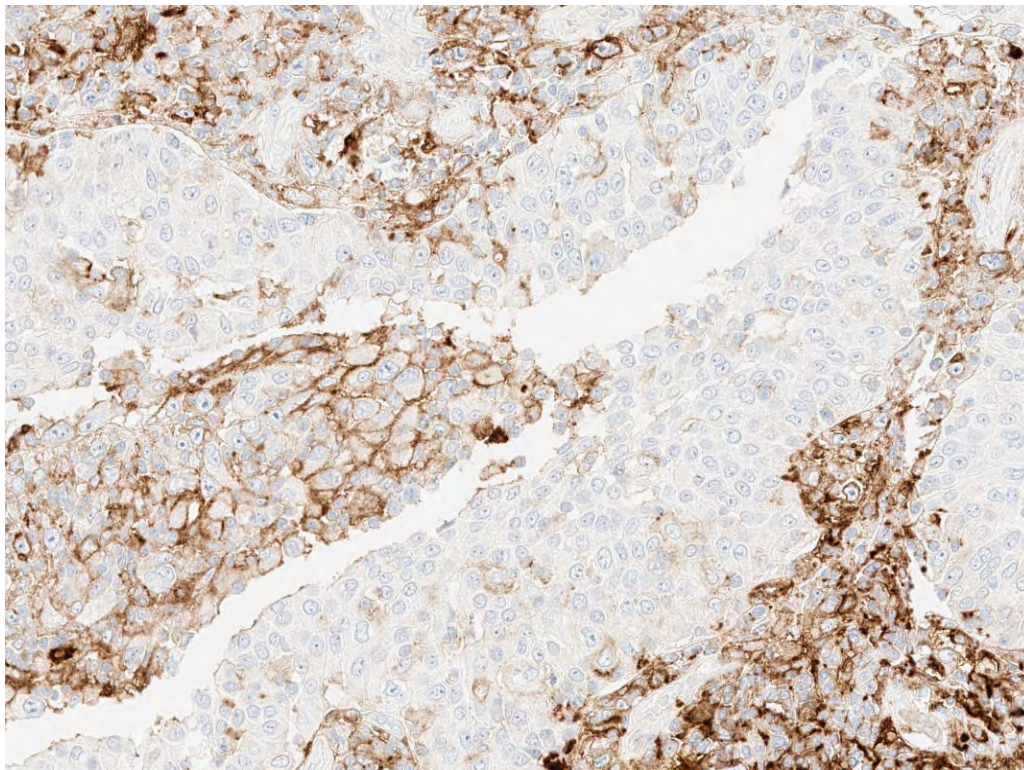
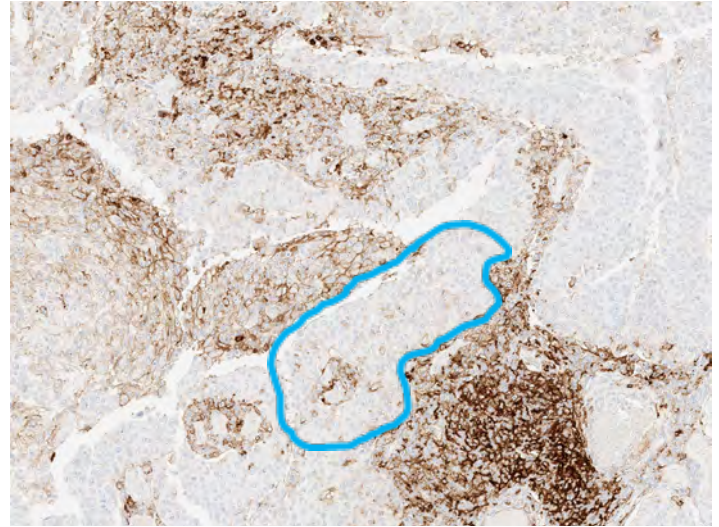
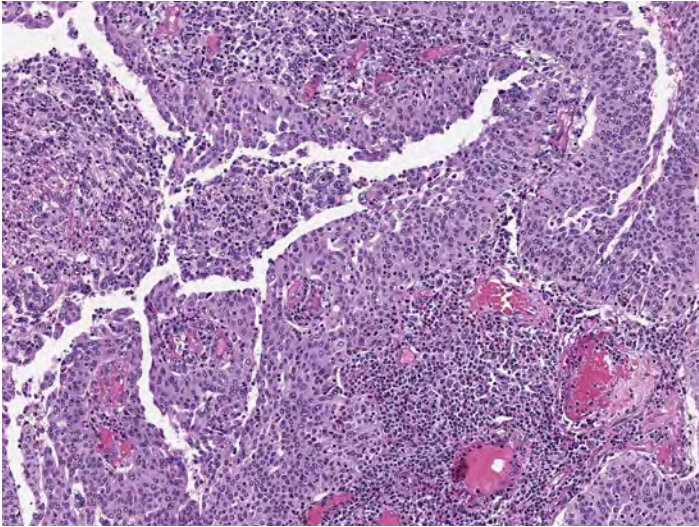
Urothelial carcinoma samples resulting from transurethral resections of the bladder (TURB) can result in challenging cases complicated by multiple fragments of tissue scattered across the slide with varying amounts of necrosis, crush/cautery artifact, viable tumor cells and/or tumor-associated immune cells. A fragment-by-fragment examination approach with individual percent staining estimates followed by an overall average can help in the evaluation.

- **Obscuring Endogenous Material**

Occasionally in urothelial carcinoma samples endogenous material, such as melanin pigment or hemosiderin, may obscure and interfere with interpretation of PD-L1 IHC staining of tumor and immune cells. Comparison of the negative isotype control slide with the PD-L1 stained slide can aid in differentiating between biomarker staining and endogenous material. Non-specific staining can also be seen on the PD-L1 stained slide in areas of necrosis.

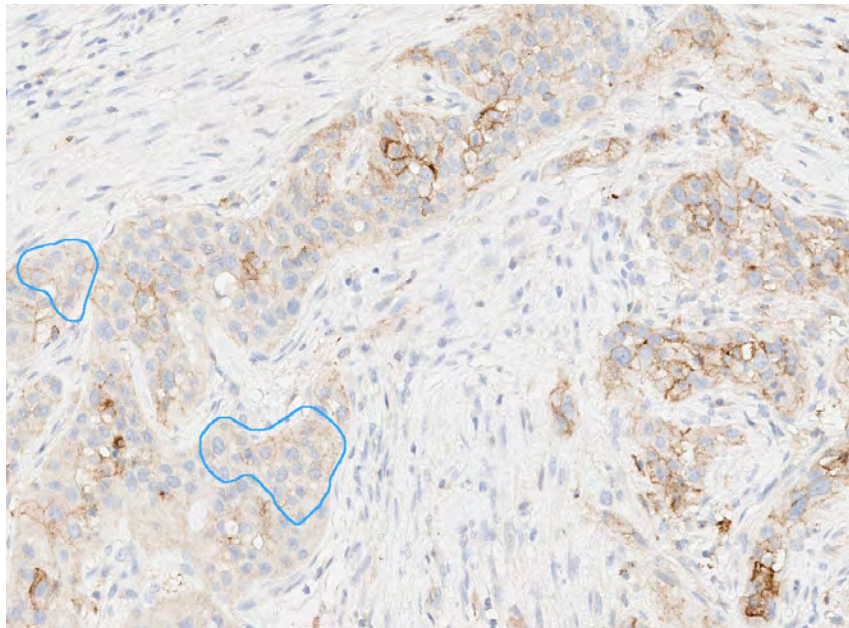
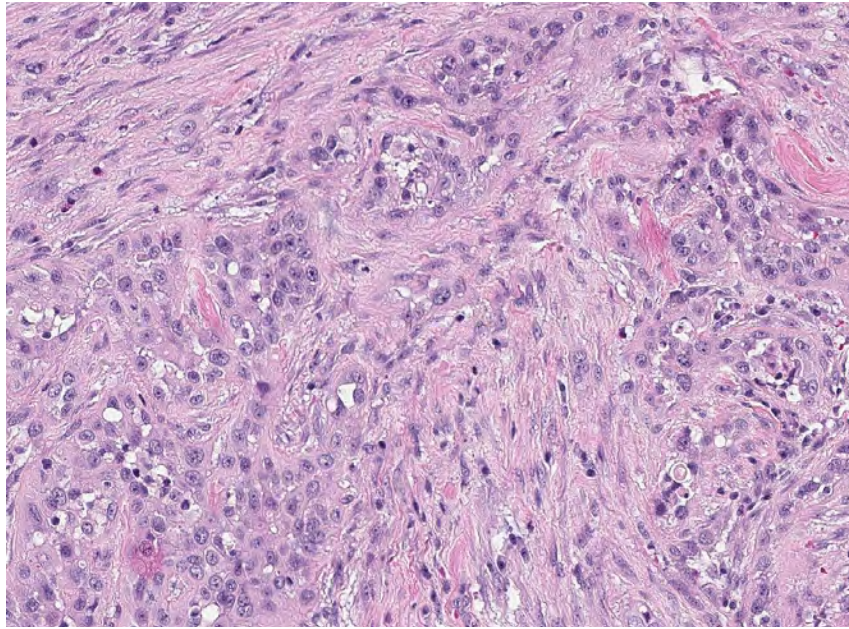
Some challenging cases are shown.

## Challenging cases: Weak Tumor Membrane vs Cytoplasmic Staining



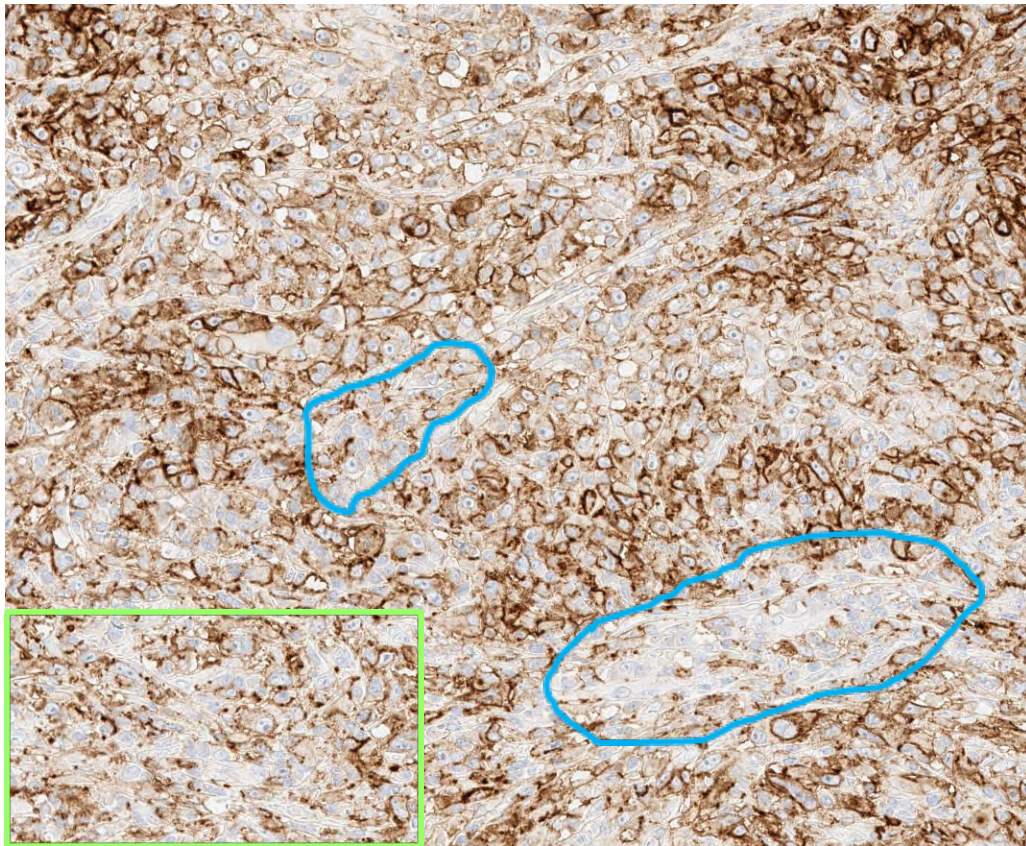
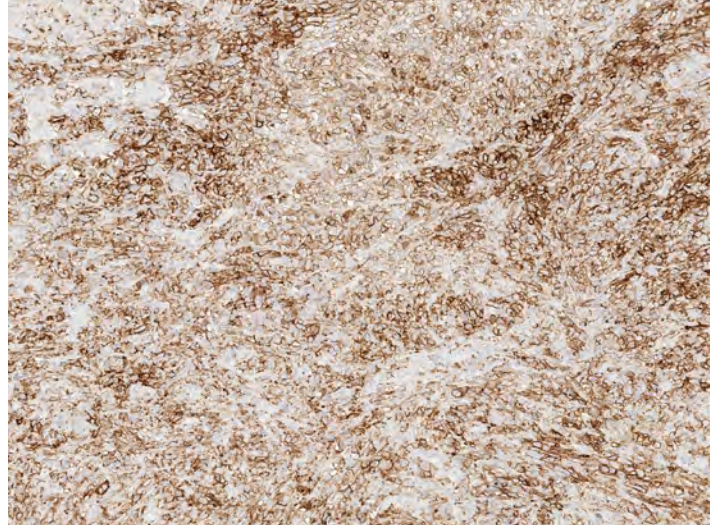
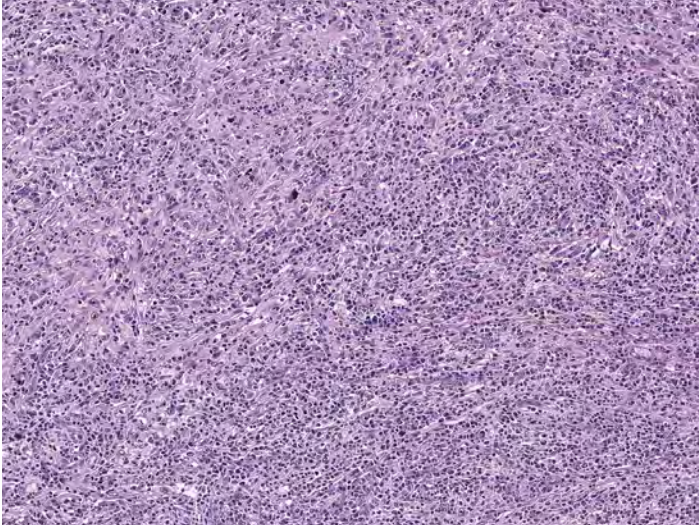
**Challenging Case 1:** The area outlined in blue (10X PD-L1 image, upper right) contains weak tumor cell staining that is difficult to distinguish. Examination at higher power (20X) shows there is scattered weak tumor membrane staining. For 10X image: Tumor cells with 40% staining, Immune cells with 80% staining. (IC+) (Top row H&E 10X, PD-L1 10X. Bottom row PD-L1 20X.)



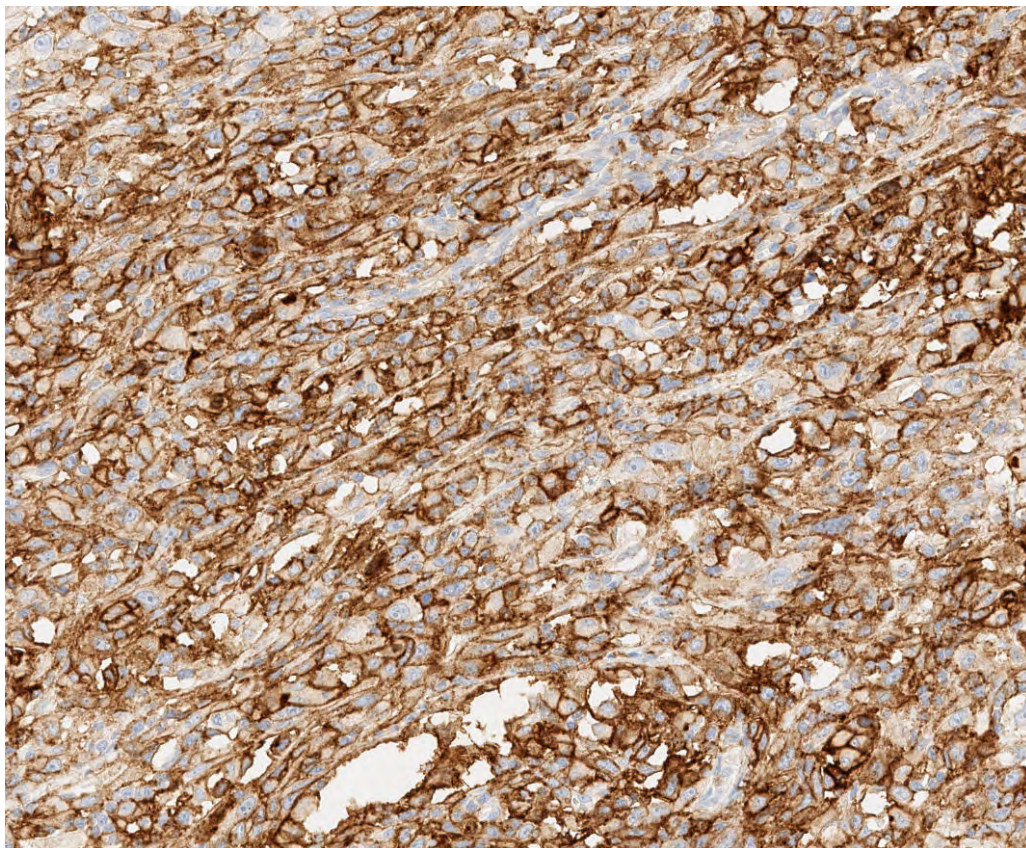
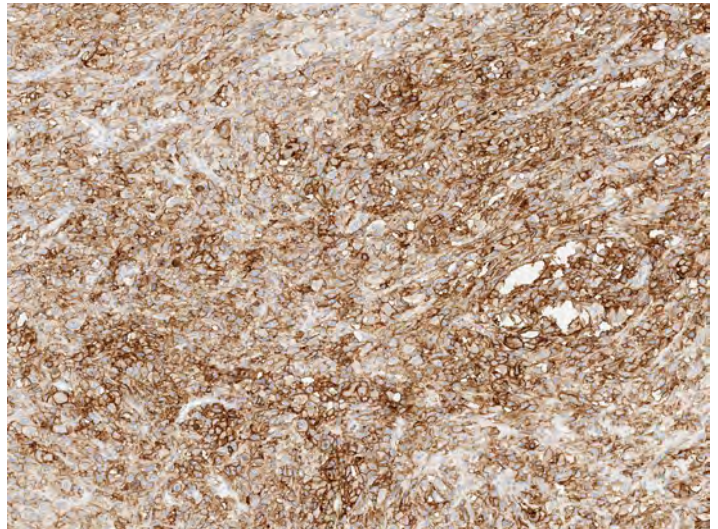
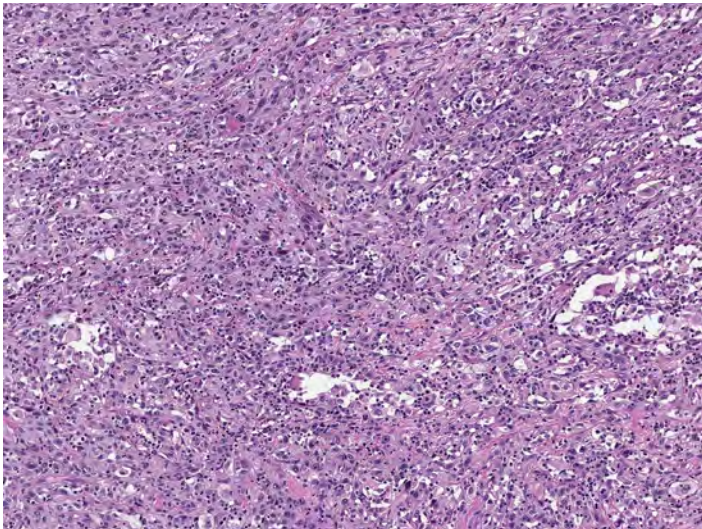


**Challenging Case 2:** Tumor cells with moderate or stronger membrane staining are readily identified within the right half of this field. Careful examination of the tumor demonstrates cells with weak membrane expression (blue outlines) that should also be counted towards the total percentage of membrane staining.

## Challenging cases: Strong Immune Cell Staining Overlapping with Tumor Cell Staining

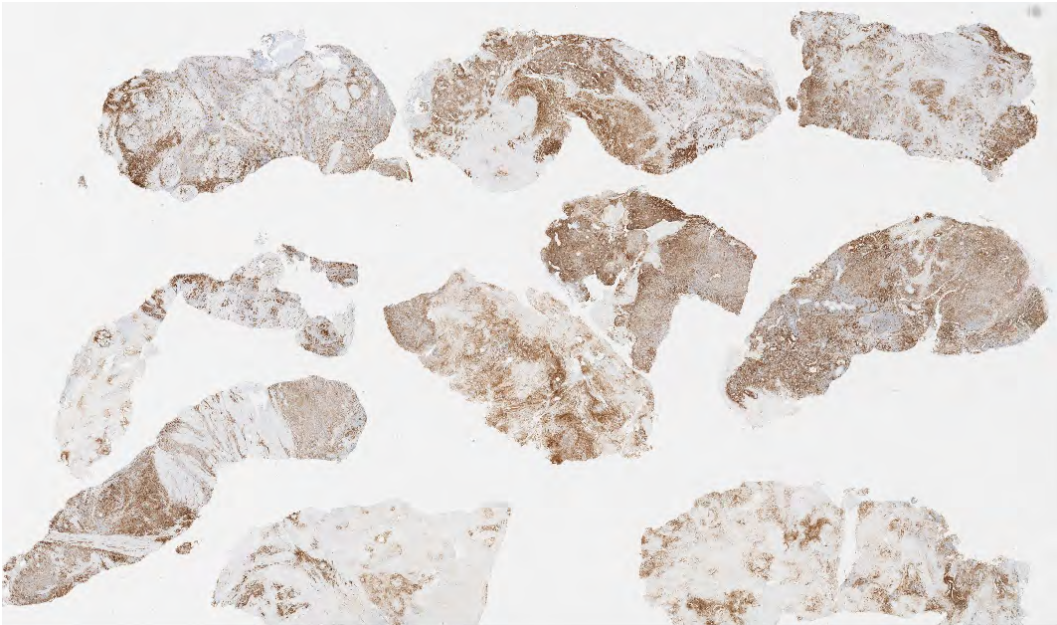
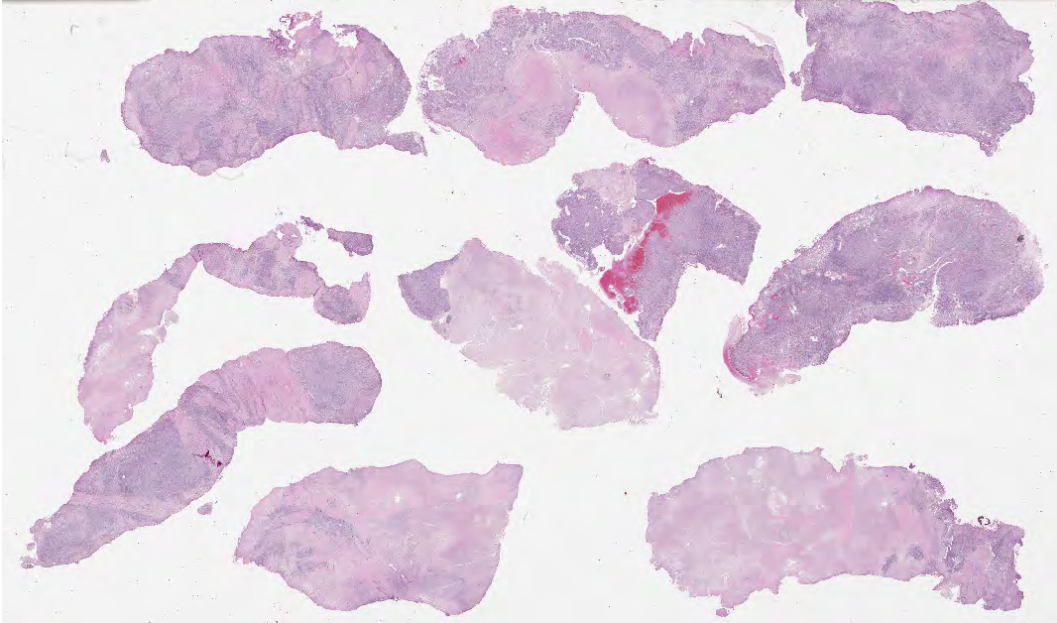


**Challenging Case 3:** TC and IC intermixed together with both expressing PD-L1. In areas with strong TC staining, IC can only be evaluated where discernible from tumor cell staining. The different patterns of IC staining (punctate pattern) as well as nuclear morphology can be used to identify PD-L1 expression. The blue outlines contain areas with discernible immune cell staining with the green outlined area having scattered punctate immune cell staining. Tumor cells with 100% staining, Immune cells with 25% staining (IC+) (Top row H&E 10X, PD-L1 10X. Bottom row PD-L1 20X.)



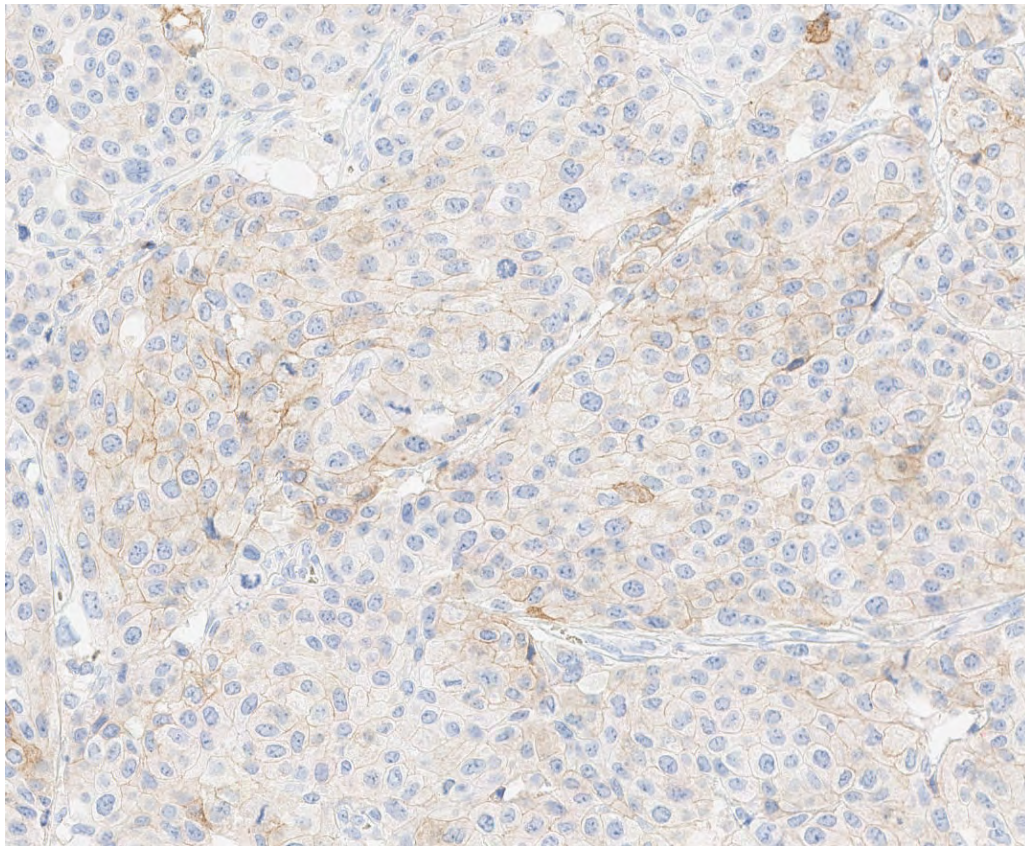
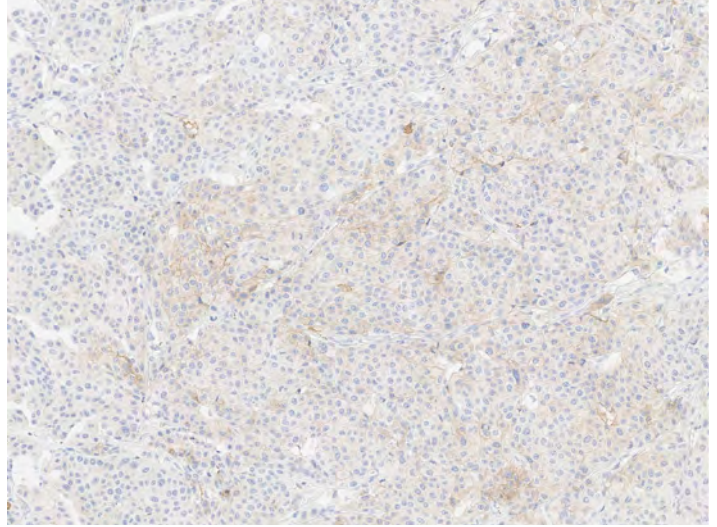
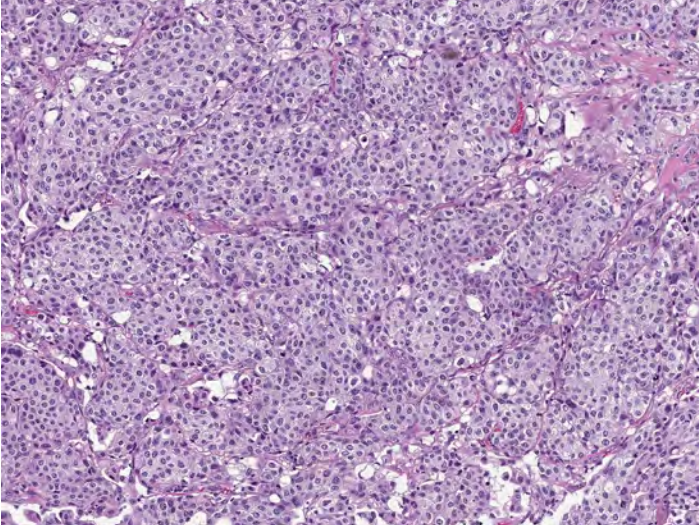
**Challenging Case 4:** TC and IC intermixed together with TC membrane staining more distinct and IC difficult to evaluate and can only be scored along the periphery of the tumor. Tumor cells with 100% staining. (Top row H&E 10X, PD-L1 10X. Bottom row PD-L1 20X.)

**Challenging cases: Cases with Multiple Tissue Fragments**

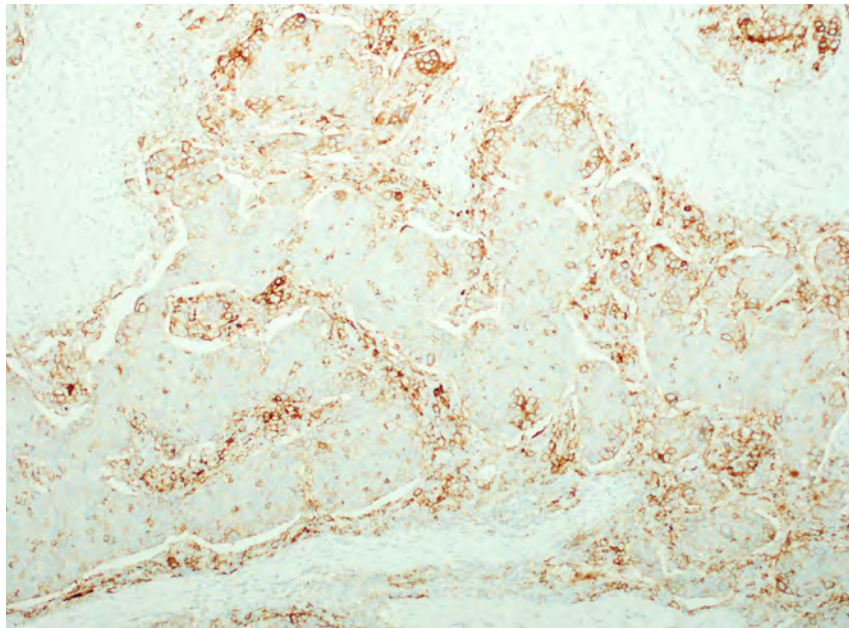
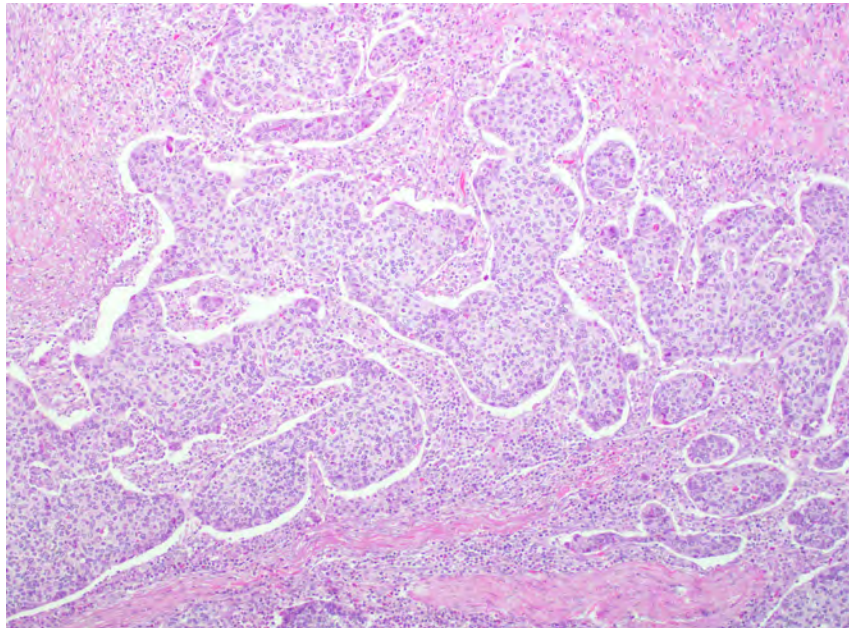


**Challenging Case 5:** TURB specimen contains multiple pieces with crush artifact, blood clot and necrosis which shows non-specific staining on the IHC slide(1X).

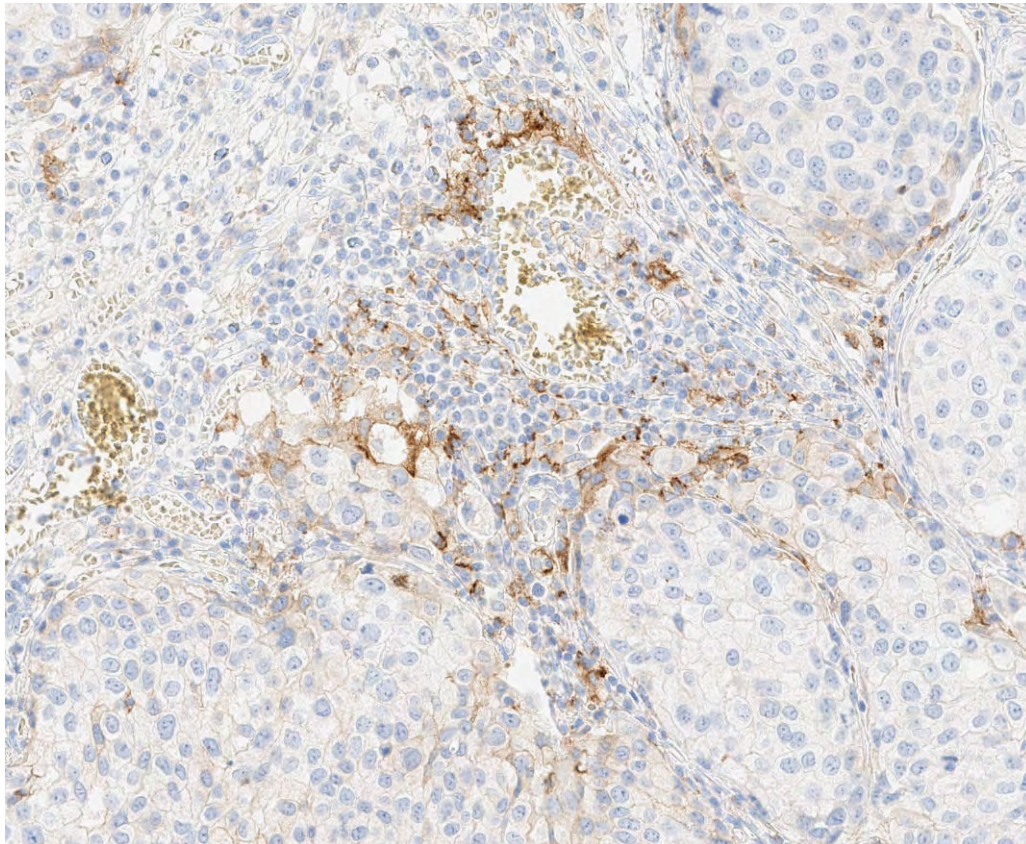
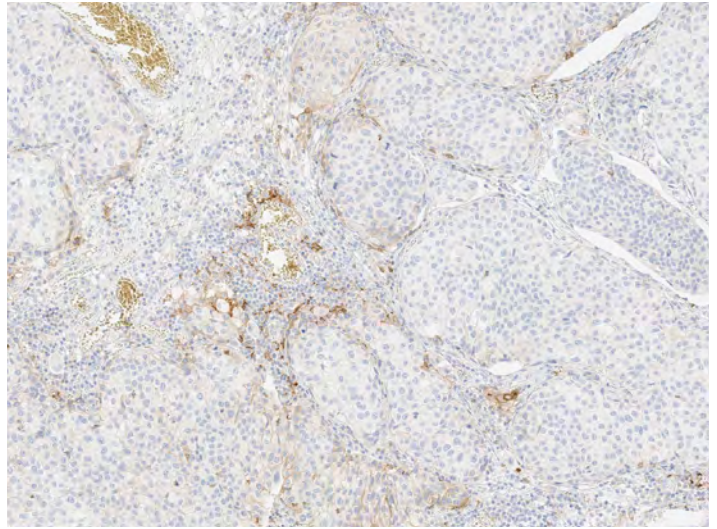
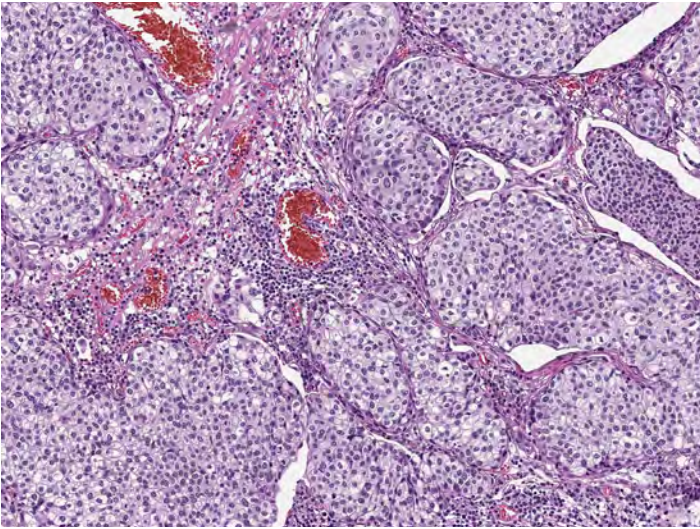
## Challenging cases: Borderline PD-L1 Status



**Challenging Case 6:** Weak tumor cell staining seen at 10X magnification is difficult to distinguish between membrane and cytoplasmic staining. Examination at higher power (20X) reveals tumor cells with 20% weak, delicate membranous staining.

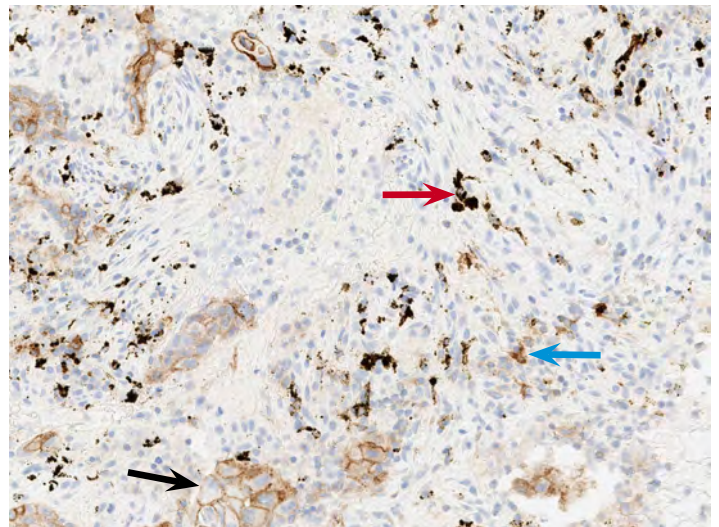
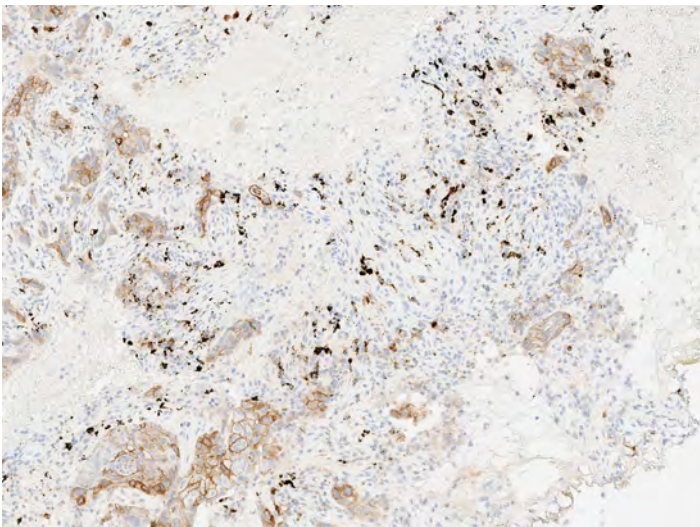
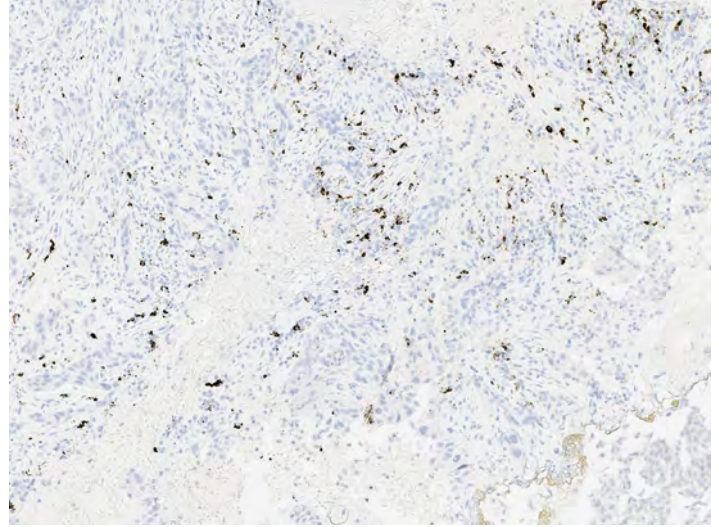
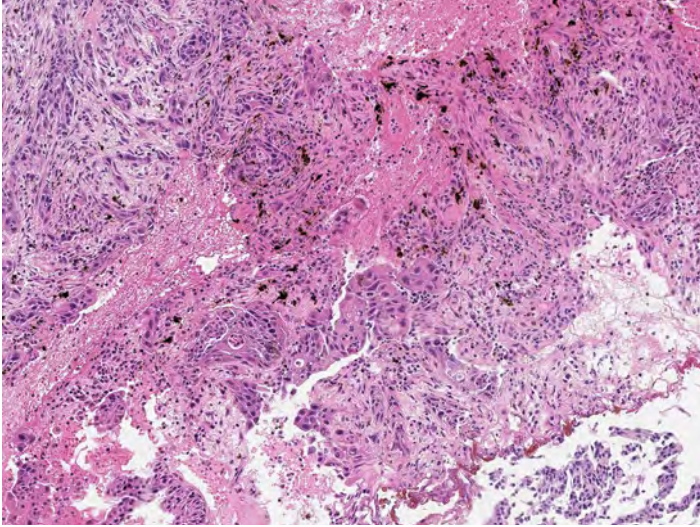


**Challenging Case 7:** Borderline TC PD-L1 expression with 25% tumor cell membrane staining (10X)



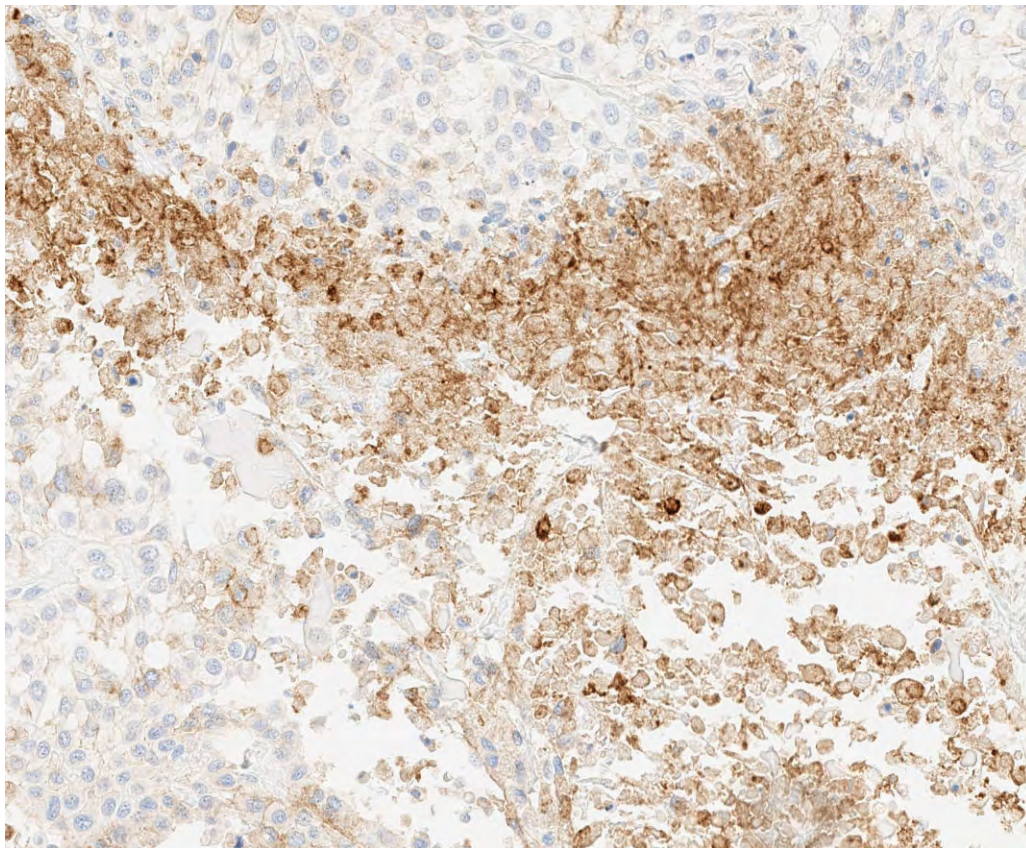
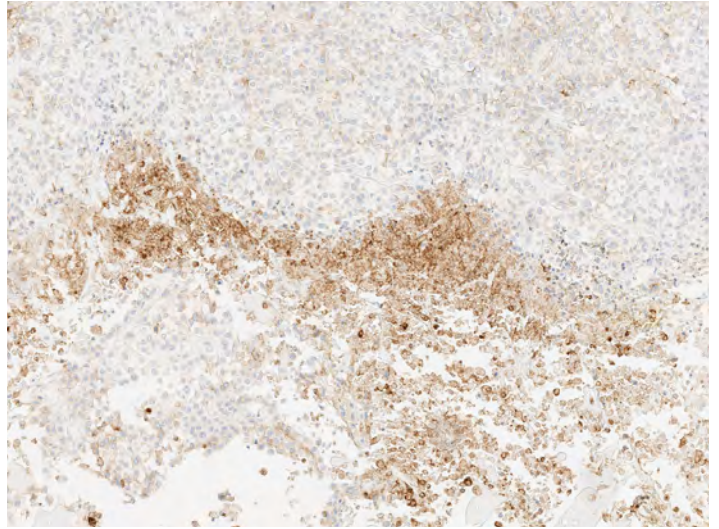
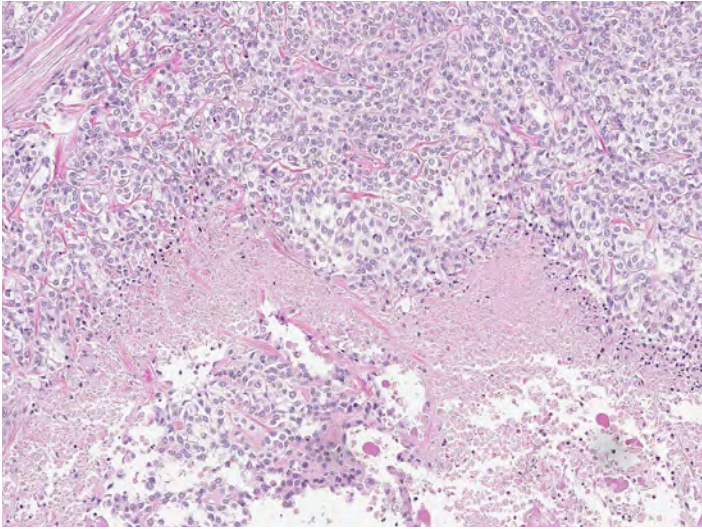
**Challenging Case 8:** Borderline IC PD-L1 expression with an IC+ of 20% (20X). (Top row H&E 10X, PD-L1 10X. Bottom row PD-L1 20X.)

## Challenging cases: Obscuring Endogenous Material



**Challenging Case 9:** Case contains anthracotic pigment (red arrow) overlapping with punctate immune cell staining (blue arrow) as well as tumor cell membrane staining (black arrow) (20X). (Top row H&E 10X, Neg Rb 10X. Bottom row PD-L1 10X.)





**Challenging Case 10:** Large necrotic areas can have non-specific DAB staining which may be confused with immune cell staining. (Top row H&E 10X, PD-L1 10X. Bottom row PD-L1 20X.)

# Impact of Pre-Analytical Conditions on VENTANA PD-L1 (SP263) Assay Staining

## Fixative Recommendations to Achieve Optimal Staining Results with the VENTANA PD-L1 (SP263) Assay

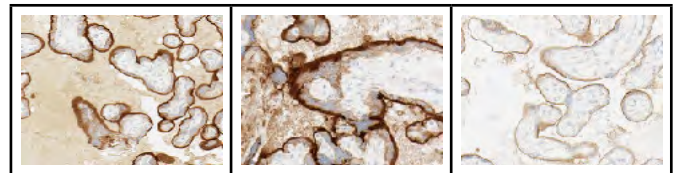
Ventana recommends fixation in 10% NBF for 6-72 hours. Acceptable fixatives and fixation times are outlined in blue.

VENTANA PD-L1 (SP263) Assay Staining of Placenta Tissue Across Fixatives and Fixation Times						
Time Point (Hrs)	Fixative					
	10% NBF	Zinc Formalin	PREFER fixative**	AFA**	Alcoholic Formalin**	95% Ethanol**
1*						
6						
12						
24						
48						
72						

### NOTES

\*One hour fixation time is not recommended for all fixative types

\*\*Use of PREFER fixative and 95% Ethanol (weaker staining) or alcoholic fixatives (high background) is not recommended. See additional higher magnification images to the right.



## Impact of Tissue Thickness on Assay Staining

Ventana recommends tissue thickness of 4-5 microns for use with the Assay.

## Cut Slide Stability

Sections approximately 4-5 microns in thickness should be cut and mounted on positively charged glass slides. Slides should be stained within 6 months of sectioning.

## References

1. Chalasani V, Chin JL, Izawa JI. Histologic variants of urothelial bladder cancer and nonurothelial histology in bladder cancer. *Can Urol Assoc J.* 2009;3(6 Suppl 4):S193-198.
2. Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0. Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. <http://globocan.iarc.fr>. Published 2013-12-12. Updated 2014-01-09. Accessed 2014-02-02.
3. American Cancer Society. *Cancer Facts & Figures 2015*. Atlanta: American Cancer Society; 2015.
4. Howlader N, Noone AM, Krapcho M, et al. (eds). *SEER Cancer Statistics Review (CSR), 1975-2012*. National Cancer Institute. [http://seer.cancer.gov/csr/1975\\_2012/](http://seer.cancer.gov/csr/1975_2012/). Published 2015-04-23. Updated 2015-11-18. Accessed 2016-04-19.
5. Keir ME, Butte MJ, Freeman GJ, et al. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 2008;26:677-704.
6. Blank C, Mackensen A. Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion. *Cancer Immunol Immunother.* 2007;56(5):739-745.
7. Butte MJ, Keir ME, Phamduy TB, et al. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity.* 2007;27(1):111-122.
8. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med.* 1999;5(12):1365-1369.
9. Massard C, Gordon MS, Sharma S, et al. Safety and efficacy of durvalumab (MEDI4736), an anti-programmed cell death ligand-1 immune checkpoint inhibitor, in patients with advanced urothelial bladder cancer. *J Clin Oncol.* 2016; 34(26):3119-25.
10. Patel SP, Kurzrock R. PD-L1 Expression as a Predictive Biomarker in Cancer Immunotherapy. *Mol Cancer Ther.* 2015;14(4) 847-856.



Ventana Medical Systems, Inc.  
1910 E. Innovation Park Drive  
Tucson, Arizona 85755  
USA  
+1 520 887 2155  
+1 800 227 2155 (USA)

[www.ventana.com](http://www.ventana.com)

©2017 Ventana Medical Systems, Inc.  
All rights reserved.

VENTANA, BENCHMARK, OPTIVIEW, and the  
VENTANA logo are trademarks of Roche. All other  
trademarks are the property of their respective  
owners.

1014738US Rev A



**VENTANA**

**Empowering | Innovation**

---

