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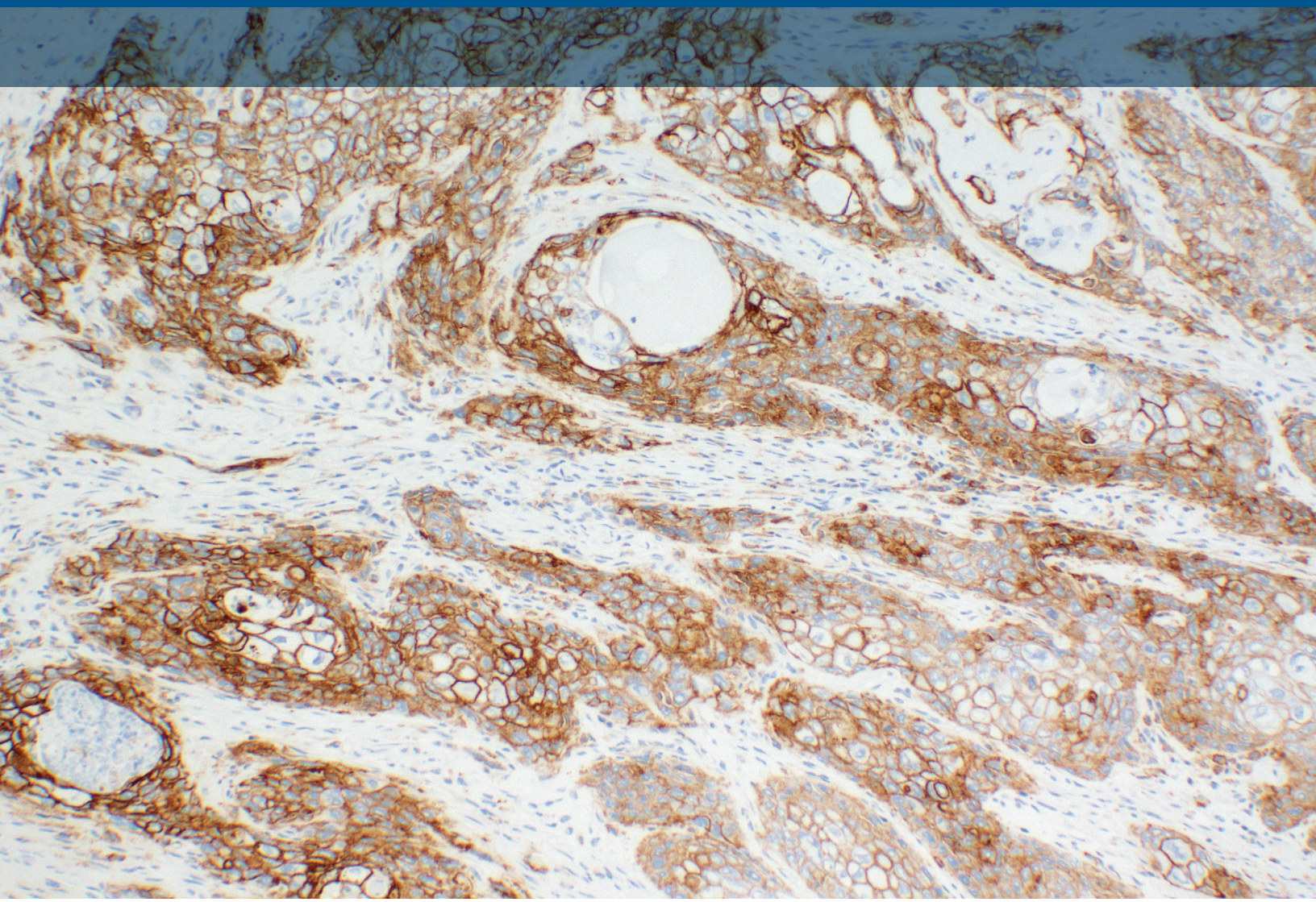
PD-L1 IHC 28-8 pharmDx Interpretation Manual - Urothelial Carcinoma

(Percent Tumor Cell Expression)

FDA-approved for in vitro diagnostic use



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Trusted Answers

Check the OPDIVO® product label for approved indications and expression cutoff values to guide therapy.

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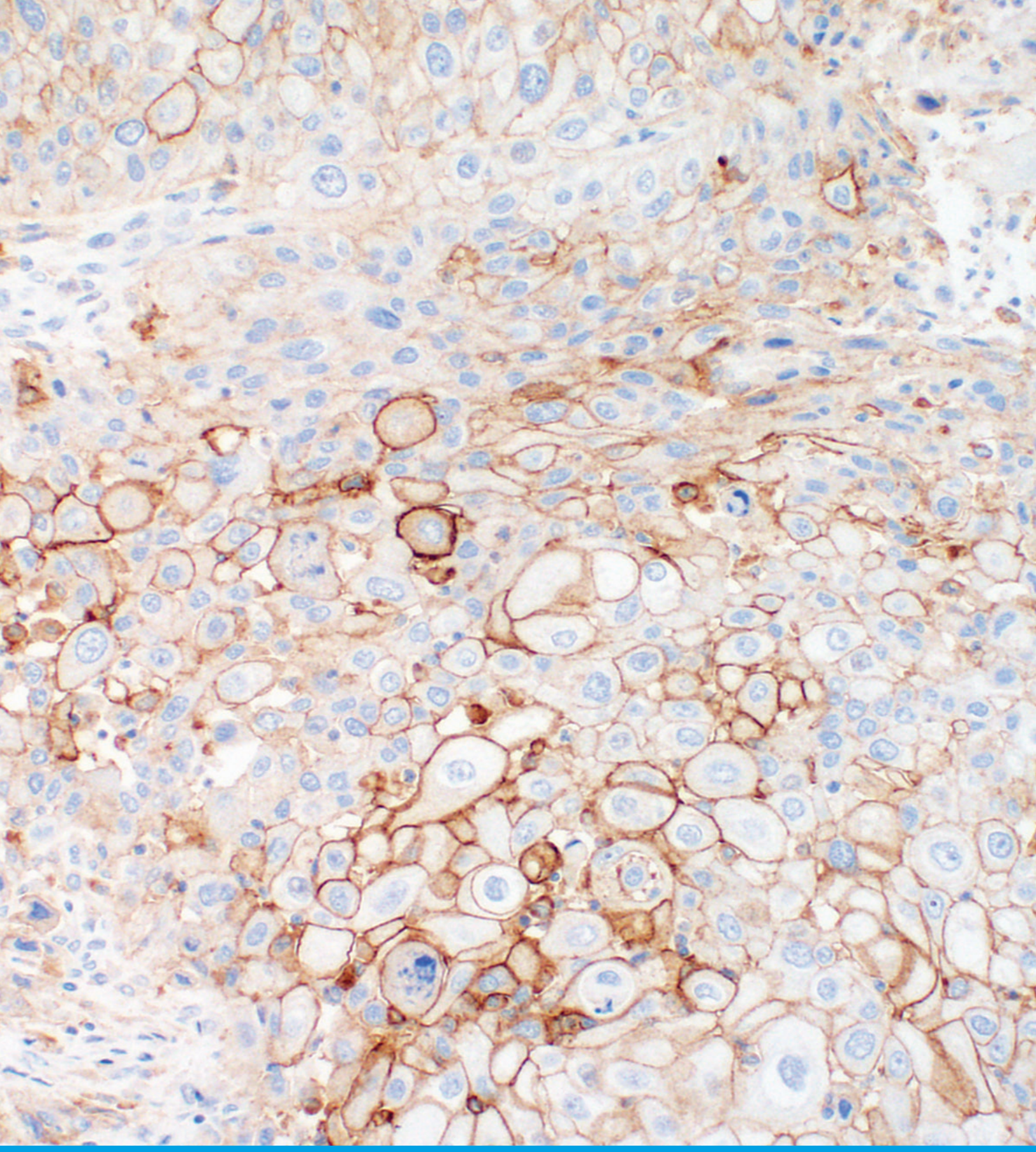


PD-L1 IHC 28-8 pharmDx
Interpretation Manual - UC
(Percent Tumor Cell Expression)

Introduction

Intended Use

For In Vitro Diagnostic Use

PD-L1 IHC 28-8 pharmDx is a qualitative immunohistochemical assay using Monoclonal Rabbit Anti-PD-L1, Clone 28-8 intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) non-small cell lung cancer (NSCLC), squamous cell carcinoma of the head and neck (SCCHN), and urothelial carcinoma (UC) tissues using EnVision FLEX visualization system on Autostainer Link 48.

PD-L1 protein expression is defined as the percentage of evaluable tumor cells exhibiting partial or complete membrane staining at any intensity.

Companion Diagnostic Indication

Tumor Indication	PD-L1 Expression Clinical Cutoff	Intended Use
NSCLC	≥ 1% tumor cell expression	PD-L1 IHC 28-8 pharmDx is indicated as an aid in identifying NSCLC patients for treatment with OPDIVO® (nivolumab) in combination with YERVOY® (ipilimumab).

When used in accordance with approved therapeutic labeling:

PD-L1 expression (≥ 1% or ≥ 5% or ≥ 10% tumor cell expression), as detected by PD-L1 IHC 28-8 pharmDx in non-squamous NSCLC (nsNSCLC) may be associated with enhanced survival from OPDIVO®.

PD-L1 expression (≥ 1% tumor cell expression), as detected by PD-L1 IHC 28-8 pharmDx in SCCHN may be associated with enhanced survival from OPDIVO®.

PD-L1 expression (≥ 1% tumor cell expression), as detected by PD-L1 IHC 28-8 pharmDx in UC may be associated with enhanced response rate and enhanced disease-free survival from OPDIVO®.

See the OPDIVO® and YERVOY® product labels for specific clinical circumstances guiding PD-L1 testing.

PD-L1 expression as detected by PD-L1 IHC pharmDx in UC may be associated with enhanced response rate and enhanced disease-free survival (DFS) from OPDIVO®.

PD-L1 IHC 28-8 pharmDx Interpretation Manual - Overview

This PD-L1 IHC 28-8 pharmDx Interpretation Manual is provided as a tool to help guide pathologists and laboratory technicians to achieve correct and reproducible results in assessing PD-L1 expression in FFPE UC specimens. The goal of this manual is to familiarize you with the requirements for scoring UC specimens stained with PD-L1 IHC 28-8 pharmDx. Photomicrographs of example cases are provided for reference.

PD-L1 IHC 28-8 pharmDx Instructions for Use (IFU) contains guidelines and technical tips for ensuring high quality staining in your laboratory.

Review of this PD-L1 IHC 28-8 pharmDx Interpretation Manual will provide a solid foundation for evaluating UC specimens stained with PD-L1 IHC 28-8 pharmDx. For more details, please refer to the current version of PD-L1 IHC 28-8 pharmDx IFU, or visit www.agilent.com.

Acknowledgment

Assay Interpretation

The clinical interpretation of any staining, or the absence of staining, must be complemented by the evaluation of proper controls. An 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 30.

Photomicrographs

Photomicrographs included in this interpretation manual are urothelial carcinoma (UC) unless otherwise indicated.

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

Note: Tissue samples supplied by BioIVT (Hicksville, New York, USA).

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 a). 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 b). 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

Anti-PD-1 therapy works by blocking the PD-1/PD-L1 interaction between tumor cells and activated T-cells, helping to prevent immunosuppression, thereby enabling cytotoxic T-cells to actively remove tumor cells.

PD-L1 IHC 28-8 pharmDx Detects PD-L1 in UC

PD-L1 upregulation in UC is a biomarker for response to anti-PD-1 therapy. PD-L1 IHC 28-8 pharmDx was the only PD-L1 assay used in the OPDIVO (nivolumab) clinical trial (CheckMate-274) to evaluate the relationship between PD-L1 expression and clinical efficacy.

The Role of the PD-1/PD-L1 Pathway in Cancer

Limiting damage to healthy tissue

Inactivation of T-cells limits damage to healthy tissue.

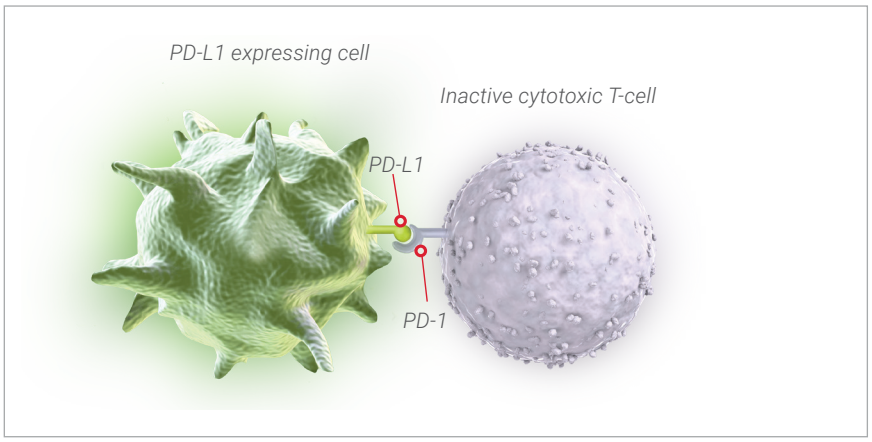


Figure a.

The tumor escapes detection

Inactivation of T-cells reduces tumor cell killing.

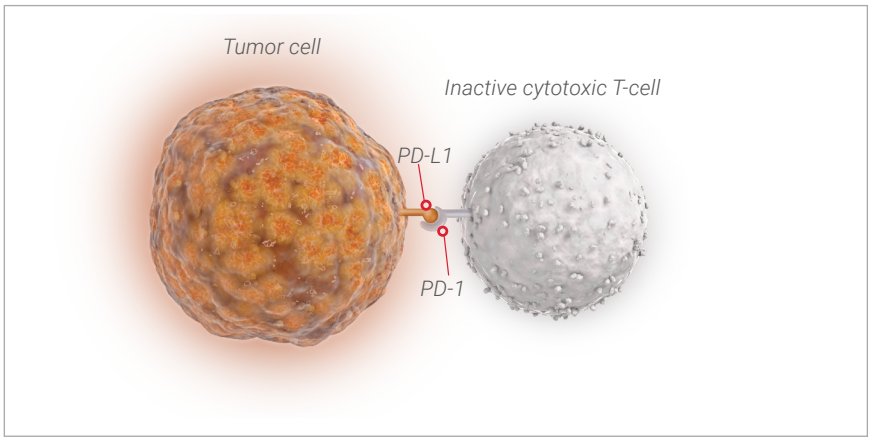


Figure b.

Immuno-oncology therapies harness the immune response to fight tumors

Blocking PD-L1 enables cytotoxic T-cells to actively remove tumor cells.

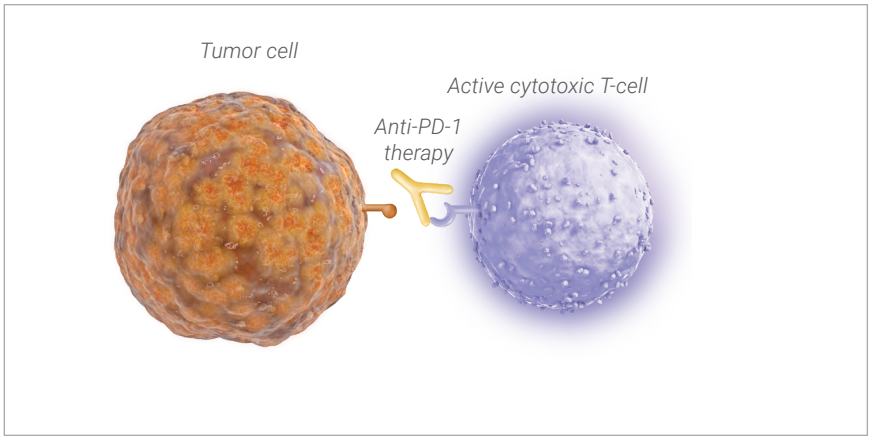


Figure c.

CheckMate-274 Study Data for PD-L1 IHC 28-8 pharmDx for Adjuvant OPDIVO in UC

Detection of PD-L1 expressing tumor cells in UC patient specimens may indicate an enhanced disease-free survival benefit to OPDIVO (nivolumab) treatment for the patient

- The clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in clinical study CA209274 to assess PD-L1 expression in UC patients treated with OPDIVO (nivolumab) versus placebo.
- The study was a phase 3 randomized, double-blind, multi-center study of adjuvant nivolumab versus placebo in subjects with high-risk invasive UC that are at high-risk of recurrence after undergoing radical resection.

A phase 3 randomized, double-blind, multi-center study of adjuvant nivolumab versus placebo in subjects with high-risk invasive UC that are at high-risk of recurrence after undergoing radical resection.

CheckMate-274* **(N=709)**

Study design

The following patients in Study CheckMate-274 were treated with OPDIVO

- With invasive UC that are at high-risk of recurrence after undergoing radical resection.
- Subjects were randomized 1:1 to nivolumab (n=353) or placebo (n=356) and stratified by pathologic nodal status (N+ vs. N0/x with < 10 nodes removed vs. N0 with ≥ 10 nodes removed), tumor PD-L1 expression (≥ 1%, < 1%/indeterminate), and use of cisplatin neo-adjuvant chemotherapy.

Pre-study (baseline) tumor tissue specimens were collected prior to randomization to conduct pre-planned analyses of efficacy according to predefined PD-L1 expression levels.

*Bajorin D. F., Witjes A., et al. Adjuvant Nivolumab versus Placebo in Muscle-Invasive Urothelial Carcinoma. *N. Engl. J. Med.* 2021.

PD-L1 IHC Expression Utilizing 28-8 pharmDx in UC Patients in CheckMate-274

Table 1. Disease-Free Survival in Randomized Subjects with UC- CheckMate-274.

Disease-Free Survival	Placebo (n=356)		Nivolumab (n=353)
All Randomized Subjects			
Events N (%)	204 (57.3)		170 (48.2)
Median DFS (mo) (95% CI mo)[a]	10.84 (8.3, 13.9)		20.76 (16.5, 27.6)
Hazard ratio (% CI)[b]		0.70 (98.2% CI 0.55, 0.90)	
Stratified log-rank p-value[c]		0.0008[d]	
All Randomized Subjects PD-L1 ≥ 1%			
Events N (%)	81 (57)		55 (39)
Median DFS (mo) (95% CI mo)[a]	8.4 (5.6, 21.2)		N.R. (21.2, N.E.)
Hazard ratio (% CI)[b]		0.55 (98.7% CI 0.35, 0.85)	
Stratified log-rank p-value[c]		0.0005[e]	
All Randomized Subjects PD-L1 < 1%[f]			
Events N (%)	120 (57)		114 (54)
Median DFS (mo) (95% CI mo)[a]	11.07 (8.3, 16.7)		16.49 (13.8, 20.8)
Hazard ratio (% CI)[b]		0.82 (95% CI 0.63, 1.06)	
Stratified log-rank p-value[c]		N.A.	

N.R. Not reached, N.E. Not estimable
[a] Based on Kaplan-Meier Estimates
[b] Stratified Cox proportional hazard model. Hazard Ratio is Nivolumab over Placebo
[c] 2 sided p values from stratified regular log-rank test
[d] Log-rank test stratified by prior neo-adjuvant cisplatin, pathological nodal status, PD-L1 status (>=1% versus <1%/indeterminate) as entered in the IRT
[e] Log-rank test stratified by prior neo-adjuvant cisplatin, pathological nodal status as entered in the IRT. Boundary for statistical significance in all randomized patients with PD-L1 ≥1%: p-value <0.01282
[f] Results are based on the PD-L1 negative clinical database, not from the IRT

Study Data for PD-L1 IHC 28-8 pharmDx in UC Patients CheckMate-275

Detection of PD-L1 expressing tumor cells in UC patient specimens may indicate an enhanced response rate benefit to OPDIVO (nivolumab) treatment for the patient.

- The clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in clinical study CA209275 to assess PD-L1 expression in UC patients treated with OPDIVO (nivolumab).
- The study was a phase 2 single arm clinical trial of OPDIVO (nivolumab) in subjects with metastatic or unresectable urothelial cancer who have progressed or recurred following treatment with a platinum agent.

A phase 2 single arm clinical trial OPDIVO (nivolumab) was assessed in subjects with metastatic or unresectable urothelial cancer who have progressed or recurred following treatment with a platinum agent.

CheckMate-275* **(N=270)**

Study design

The following patients in Study CheckMate-275 were treated with OPDIVO

- metastatic urothelial cancer who have progressed or recurred following treatment with a platinum agent.
- unresectable urothelial cancer who have progressed or recurred following treatment with a platinum agent.

Tumor specimens were evaluated prospectively using the PD-L1 IHC 28-8 pharmDx assay at a central laboratory and the results were used to define subgroups for pre-specified analyses.

PD-L1 expression, as detected by PD-L1 IHC 28-8 pharmDx in UC may be associated with enhanced response rate from OPDIVO (nivolumab).

- The frequency of bladder cancer and a tendency for recurrence puts a significant burden on global health care. Standard first-line treatment for metastatic UC involves platinum-based combination chemotherapy. Despite initial responses shown by 40–60% of patients with advanced UC receiving first-line cisplatin-based chemotherapy, disease progression occurs in nearly all patients at a median of about 8 months. There is no global standard of care for patients who progress on or after platinum chemotherapy for advanced disease.
- The clinical utility of PD-L1 IHC 28-8 pharmDx to aid in the assessment of UC patients for OPDIVO (nivolumab) treatment was evaluated in the study CheckMate-275: A phase 2 single arm clinical trial of nivolumab in subjects with metastatic or unresectable urothelial cancer that have progressed or recurred following treatment with a platinum agent.

*Sharma. P., Retz. M., et al. Nivolumab in metastatic urothelial carcinoma after platinum therapy (CheckMate-275): a multicentre, single-arm, phase 2 trial. *The Lancet.* 2017.

The Clinical Value of PD-L1 IHC 28-8 pharmDx Expression in UC Patients CheckMate-275

– As per CheckMate-275 study results, in patients with a PD-L1 expression ≥ 1%, the **Objective Response Rate (ORR)** was **31(17.7, 33.6), and 22(9.7, 21.9)** in patients with PD-L1 expression < 1%.

Table 2. Efficacy Results for study CA209275.
Confirmed ORR in all patients and the two PD-L1 subgroups are summarized in the table below.

Tumor PD-L1 Expression	<1%	≥1%	All Treated Subjects
Total No. of Subjects	N=146	N=124	N=270
Confirmed Objective Response Rate No. of Subjects (95% CI)	22 (9.7, 21.9)	31 (17.7, 33.6)	53 (15.1, 24.9)
Complete Response Rate No. of Subjects (% of Total in PD-L1 expression category)	1 (0.7%)	6 (4.8%)	7 (2.6%)
Partial Response Rate No. of Subjects (% of Total in PD-L1 expression category)	21 (14.4%)	25 (20.2%)	46 (17.0%)
Median Duration of Response* Months (range)	7.6 mos. (3.7+, 12.0+)	NE (1.9+, 12.0+)	10.3 (1.9+, 12.0+)

*Estimated from the Kaplan-Meier Curve

Table 3. Frequency of Tumor PD-L1 Expression in Samples from UC- CA209275.

Tumor PD-L1 Expression	Nivolumab (N=270)
≥1% PD-L1 Expression Subjects	124 (46%)
<1% PD-L1 Expression Subjects	146 (54%)

Clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in CA209275, a phase 2 single arm clinical trial of nivolumab, in which 270 subjects were randomized to receive the drug at 63 sites in 11 countries. Major efficacy outcome measures included confirmed objective response rate (ORR) and duration of response (DOR).

Tumor specimens were collected from UC tumors from either a primary or metastatic site, consistent with the inclusion requirements for the study. Subjects had tumor tissue collected at baseline with the following site proportion:

Baseline UC Specimen Origin - CA209275

Non bladder UC **27%** (73/270)	Visceral metastases **84%** (227/270)

- 27% of nivolumab treated patients had primary, non-bladder UC
- Regardless of primary tumor site, 84% of all treated patients presented with visceral metastases at baseline

Table 4. CheckMate-274 and CheckMate-275 summary**

	CheckMate-274	CheckMate-275
Study Phase	3	2
Brief Title	An Investigational Immuno-Therapy Study of Nivolumab, Compared to Placebo, in Patients with Bladder or Upper Urinary Tract Cancer, Following Surgery to Remove the Cancer	A Study of Nivolumab in Participants with Metastatic or Unresectable Bladder Cancer
Summary	The purpose of this study is to determine the effectiveness and safety of nivolumab compared to placebo in participants who have undergone radical surgery for invasive urothelial cancer	The purpose of this study is to measure the effect of nivolumab (BMS-936558) in reducing tumor size in subjects with metastatic or unresectable bladder cancer
Primary Outcome Measures	Disease-free survival (DFS); Time frame: Approximately 5 years after the first subject is randomized DFS defined as the time between the date of randomization and the date of first recurrence or death (of any cause) whichever occurs first	1. Objective Response Rate (ORR) per BIRC assessment; Time frame: From the date of first dose to the date of objectively documented progression or the date of subsequent therapy, whichever occurs first (assessed up to 14 months) 2. ORR Per BIRC assessment by PD-L1 expression level; Time frame: From the date of first dose to the date of objectively documented progression or the date of subsequent therapy, whichever occurs first (assessed up to 14 months)
Results	As per CheckMate-274 study results, nivolumab treatment resulted in a statistically significant and clinically meaningful improvement in DFS compared to placebo in all randomized subjects: median of 20.76 months (95% CI: 16.49, 27.63) vs 10.84 months (95% CI: 8.25, 13.86) with nivolumab vs placebo, respectively (HR = 0.70 [98.22 CI: 0.55, 0.90]; p = 0.0008)	As per CM-275 study results, with PD-L1 expression ≥ 1%, the ORR was 31 (25.0%, 95% CI: 17.7, 33.6) out of 124 patients. Out of 146 patients with PD-L1 expression < 1%, the ORR was 22 (15.1%, 95% CI: 9.7, 21.9).

**clinicaltrials.gov

PD-L1 IHC 28-8 pharmDx Overview

Code SK005

PD-L1 IHC 28-8 pharmDx contains optimized reagents and protocol required to complete an IHC staining procedure of FFPE tissue sections using Autostainer Link 48 and PT Link Pre-treatment module.

Following incubation with the primary monoclonal antibody PD-L1 or the Negative Control Reagent (NCR), the specimens are incubated with a linker antibody specific to the host species of the primary antibody, and then incubated 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 the 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. Control Slides containing two FFPE human cell lines are provided to aid in validating staining runs.

PD-L1 IHC 28-8 pharmDx staining procedure.

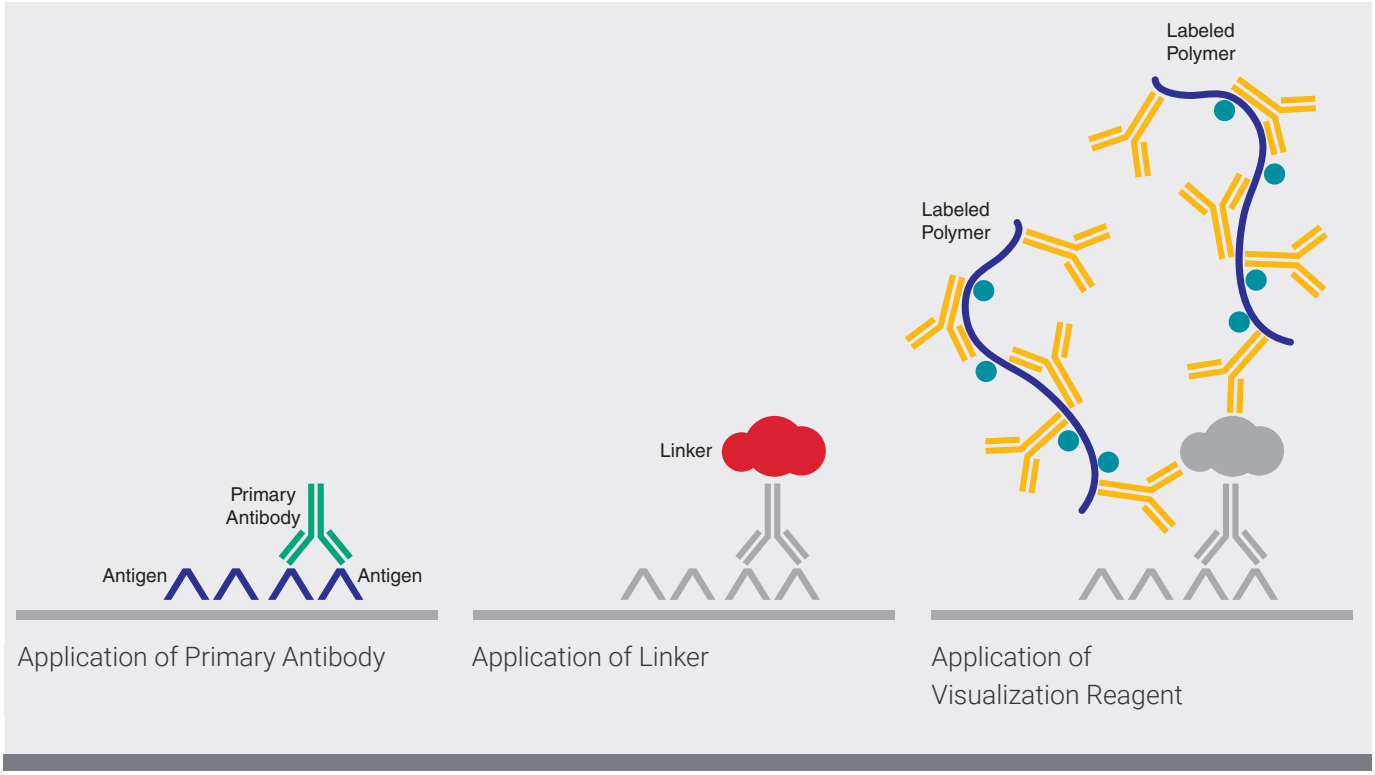


Figure 1a. PD-L1 IHC 28-8 pharmDx staining procedure.

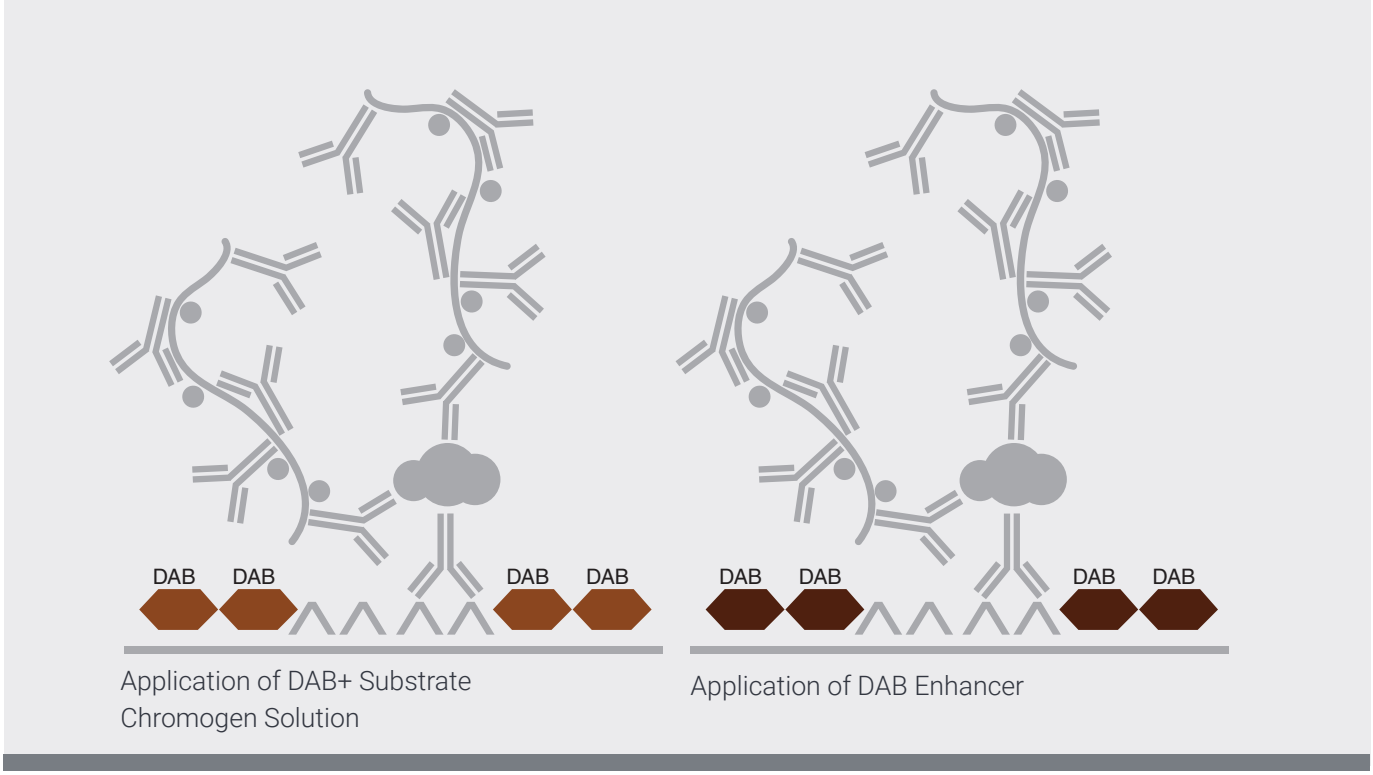


Figure 1b. PD-L1 IHC 28-8 pharmDx staining procedure.

Figure 2. PD-L1 IHC 28-8 pharmDx, component.

All PD-L1 IHC 28-8 pharmDx reagents are to be used on the Autostainer Link 48. All reagents must be used as indicated in the IFU in order for the test to perform as specified.

PD-L1 IHC 28-8 pharmDx contains reagents to perform 50 tests in up to 15 individual runs (see Figure 2).

- EnVision FLEX Target Retrieval Solution, Low pH, 50x
- Peroxidase-Blocking Reagent
- Primary Antibody: Monoclonal Rabbit Anti-PD-L1, Clone 28-8
- Negative Control Reagent
- PD-L1 IHC 28-8 pharmDx Anti-Rabbit LINKER
- Visualization Reagent-HRP
- DAB+ Substrate Buffer
- DAB+ Chromogen
- DAB Enhancer
- PD-L1 IHC 28-8 pharmDx Control Slides

EnVision FLEX Wash Buffer (20x) (Code K8007) and EnVision FLEX Hematoxylin (Code K8008), are required but not included in the kit. Refer to the IFU for a complete list of required materials and equipment.

Technical Considerations for Optimal Performance of PD-L1 IHC 28-8 pharmDx

Optimal staining performance is achieved by adhering to the PD-L1 IHC 28-8 pharmDx protocol. Technical problems relating to the performance of PD-L1 IHC 28-8 pharmDx may arise in two areas; those involving specimen collection and specimen preparation prior to performing the test, as well as problems involving the actual performance of the test itself. Technical problems related to the performance of the test are generally related to procedural deviations and can be controlled and minimized through training and a thorough understanding of the product instructions by the user.

Specimen Collection and Preparation

Specimens must be handled in a way which preserves the tissue for immunohistochemical staining. Tissue should be stained and interpreted as close to the time of biopsy as possible. The stability of PD-L1 immunoreactivity in tissue blocks has not been assessed. Tissue may be susceptible to loss of PD-L1 immunoreactivity with age. Confirm appropriate intact tumor morphology and the presence of sufficient tumor cells for evaluation. Use recommended methods of tissue processing for all specimens.

Control Tissue

Differences in processing and embedding in the user's laboratory may produce significant variability in results. Include positive and negative control tissue in each staining run, in addition to the PD-L1 IHC 28-8 pharmDx Control Slide.

Control tissue should be biopsy/surgical specimens, fixed, processed, and embedded as soon as possible in the same manner as the patient sample(s). Control tissue must represent one of the approved tumor indications for PD-L1 IHC 28-8 pharmDx as listed in the Intended Use. 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. The ideal negative control tissue should demonstrate no staining.

Tissue Processing

FFPE tissues are suitable for use. Specimens should be blocked into a thickness of 3 mm or 4 mm, fixed in 10% Neutral Buffered Formalin (NBF), and dehydrated and cleared in a series of alcohols and xylene, followed by infiltration with melted paraffin. The paraffin temperature should not exceed 60 °C. An ischemia time from excision to fixation start time of less than 30 minutes followed by immersion in NBF for 24–48 hours is recommended.

The use of PD-L1 IHC 28-8 on decalcified tissue has not been validated and is not recommended.

Cut tissue specimens into sections of 4–5 µm. After sectioning, mount tissues on FLEX IHC microscope slides, Code K8020, or Superfrost Plus charged slides and place in a 58 ± 2 °C oven for 1 hour.

To preserve antigenicity, UC tissue sections, once mounted on slides, should be stored in the dark at 2–8 °C, or room temperature up to 25 °C, and stained within 4 months of sectioning. Slide storage and handling conditions should not exceed 25 °C at any point post-mounting to ensure tissue integrity and antigenicity.

PD-L1 IHC 28-8 pharmDx Staining Procedure

The PD-L1 IHC 28-8 pharmDx reagents and instructions have been designed for optimal performance. Further dilution of the reagents, alteration of incubation times, temperatures, or instruments may give erroneous results.

Reagent Storage

Store all components of PD-L1 IHC 28-8 pharmDx, including Control Slides, in the dark at 2–8 °C when not in use on Autostainer Link 48. Do not use after the expiration date printed on the outside package.

Reagent Preparation

Equilibrate all components to room temperature (20–25 °C) prior to immunostaining.

EnVision FLEX Target Retrieval Solution, Low pH

Prepare a sufficient quantity of EnVision FLEX 1x Target Retrieval Solution, Low pH by diluting EnVision FLEX Target Retrieval Solution, Low pH (50x) 1:50 using reagent-quality water; the pH of EnVision FLEX 1x Target Retrieval Solution should be 6.1 ± 0.2. One 30 mL bottle of Target Retrieval Solution, Low pH (50x), diluted 1:50 will provide 1.5 L of EnVision FLEX 1x reagent, sufficient to fill one PT Link tank, which will treat up to 24 slides per use. Discard EnVision FLEX 1x Target Retrieval Solution after three uses and no longer than 5 days after dilution. Note the EnVision FLEX Target Retrieval Solution, low pH (50x) is a red-colored solution.

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

EnVision FLEX Wash Buffer (20x)

Prepare a sufficient quantity of 1x EnVision FLEX Wash Buffer by diluting Wash Buffer (20x) 1:20 using reagent-quality water for the wash steps. Store unused 1x solution at 2–8 °C for no more than one month. Discard buffer if cloudy in appearance. Refer to the User Guide for your Autostainer Link 48 for further information. EnVision FLEX Wash Buffer (20x) is available as Code K8007.

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 does 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 as per the guidelines above. Adding excess DAB+ Chromogen to the DAB+ Substrate Buffer results in deterioration of the positive signal.

Control to Assess Staining Quality

Each slide contains sections of two pelleted, FFPE cell lines: NCI-H226** with positive PD-L1 protein expression (originating from human lung squamous cell carcinoma with positive PD-L1 protein expression) and MCF-7 with negative PD-L1 protein expression (originating from human breast adenocarcinoma with negative PD-L1 protein expression).

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

Staining Protocol

Program slides by selecting PD-L1 IHC 28-8 pharmDx staining protocol from the options in the DakoLink drop-down menu. All of the required steps and incubation times for staining are preprogrammed in the DakoLink software. Print and attach slide labels to each slide.

Deparaffinization, Rehydration, and Target Retrieval

Use PT Link, Code PT100/PT101/PT200, to perform the Deparaffinization, Rehydration, and Target Retrieval 3-in-1 procedure.

- Set Pre-heat 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 EnVision FLEX Target Retrieval Solution, Low pH, working solution to cover the tissue sections.
- Pre-heat the Target Retrieval Solution, Low pH to 65 °C.
- Immerse Autostainer racks containing mounted, FFPE tissue sections into the pre-heated Target Retrieval Solution, Low pH (1x working solution) in PT Link tank. Incubate for 20 minutes at 97 °C.

- As soon as the target retrieval incubation time 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 EnVision FLEX Wash Buffer 1x working solution.
- Leave Autostainer rack with slides in room temperature 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.
- The instrument performs the staining and counterstaining procedures by applying the appropriate reagent, monitoring the incubation time, and rinsing slides between reagents. Counterstaining using EnVision FLEX Hematoxylin, Code K8008, for 7 minutes, is included in the staining protocol. Do not allow slides to dry prior to mounting.

Mounting

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

PD-L1 IHC 28-8 pharmDx Technical Checklist

Customer Name/Institution: _____

Name and Title: _____

Autostainer Link 48 Serial Number: _____ Software Version: _____

		Yes	No
1.	Regular preventive maintenance is performed on the Autostainer Link 48 and PT Link?	☐	☐
2.	PD-L1 IHC 28-8 pharmDx is used before the expiration date printed on the outside of the box?	☐	☐
3.	All PD-L1 IHC 28-8 pharmDx components, including Control Slides, are stored in the dark at 2–8 °C?	☐	☐
4.	All PD-L1 IHC 28-8 pharmDx components, including Control Slides, are equilibrated to room temperature (20–25 °C) prior to immunostaining?	☐	☐
5.	Appropriate positive and negative control tissues are identified?	☐	☐
6.	Tissues are fixed in neutral buffered formalin?	☐	☐
7.	Tissues are infiltrated with melted paraffin, at or below 60 °C?	☐	☐
8.	Tissue sections of 4–5 µm are mounted on FLEX IHC Microscope Slides, or Superfrost Plus charged slides?	☐	☐
9.	UC specimens are stained within 4 months of sectioning when stored in the dark at 2–8 °C or at room temperature up to 25 °C?	☐	☐
10.	EnVision FLEX Target Retrieval Solution (pH 6.1±0.2), Low pH is prepared properly?	☐	☐
11.	EnVision FLEX Wash Buffer is prepared properly?	☐	☐
12.	DAB+ Substrate-Chromogen Solution is prepared properly?	☐	☐
13.	The Deparaffinization, Rehydration, and Target Retrieval 3-in-1 procedure is followed, using PT Link?	☐	☐
14.	Slides remain wet with buffer while loading and prior to initiating run on Autostainer Link 48?	☐	☐
15.	The PD-L1 IHC 28-8 pharmDx protocol is selected on Autostainer Link 48?	☐	☐
16.	Slides are counterstained with EnVision FLEX Hematoxylin?	☐	☐
17.	Do you have all the necessary equipment to perform the PD-L1 IHC 28-8 pharmDx test according to the protocol? If not, specify what is missing in the comments below.	☐	☐

If you answered “No” to any of the above, consult with your local Agilent Technical Support Representative for assistance.

Additional Observations or Comments:

_____

_____

_____

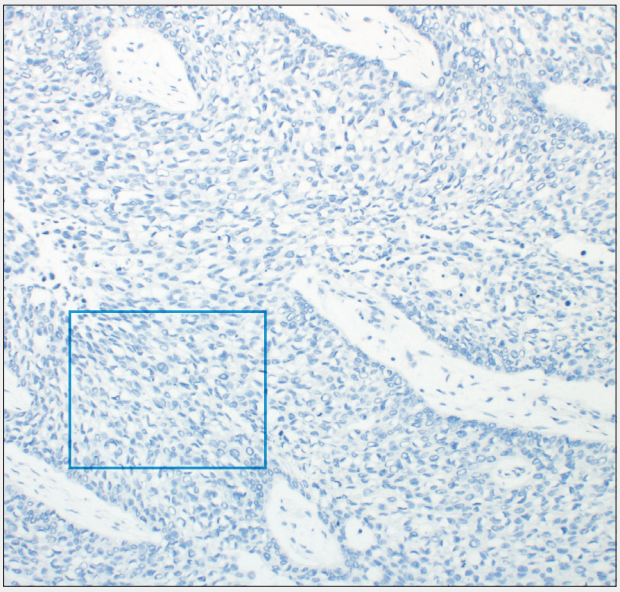
Guidelines for Scoring PD-L1 IHC 28-8 pharmDx in UC

Agilent emphasizes that scoring of PD-L1 IHC 28-8 pharmDx must be performed in accordance with the guidelines established in the IFU, within the context of best practices and the pathologist's experience.

This assay was validated for invasive UC tissue samples and not for lesions with foci of dysplasia or carcinoma in situ. Hematoxylin and eosin (H&E) stained slides should accompany each PD-L1 stained sample to allow proper assessment of invasive carcinoma, carcinoma in situ, and adjacent normal epithelium.

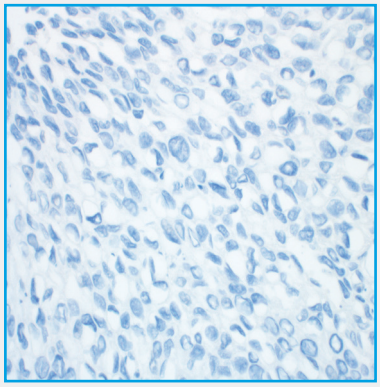
The percentage of stained viable tumor cells in the specimen determines PD-L1 IHC 28-8 pharmDx result. Scoring guidelines and reporting recommendations are presented in Figure 3. See page 30 for an example of a pathology report form for PD-L1 IHC 28-8 pharmDx.

< 1% of the viable tumor cells exhibit complete circumferential or partial linear plasma membrane staining at any intensity.

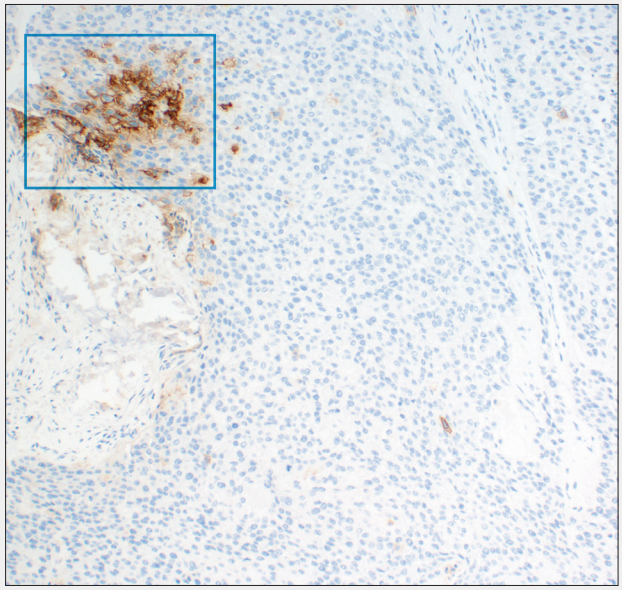


PD-L1 expression

< 1%



≥ 1% of the viable tumor cells exhibit complete circumferential or partial linear plasma membrane staining at any intensity.



PD-L1 expression

≥ 1%

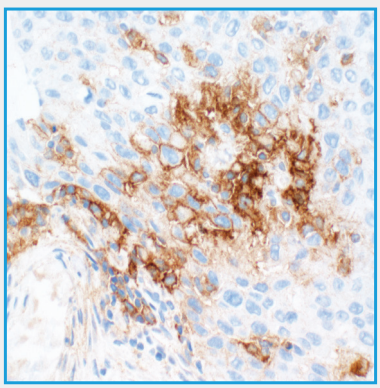


Figure 3. Guidelines for scoring and reporting PD-L1 IHC pharmDx results.

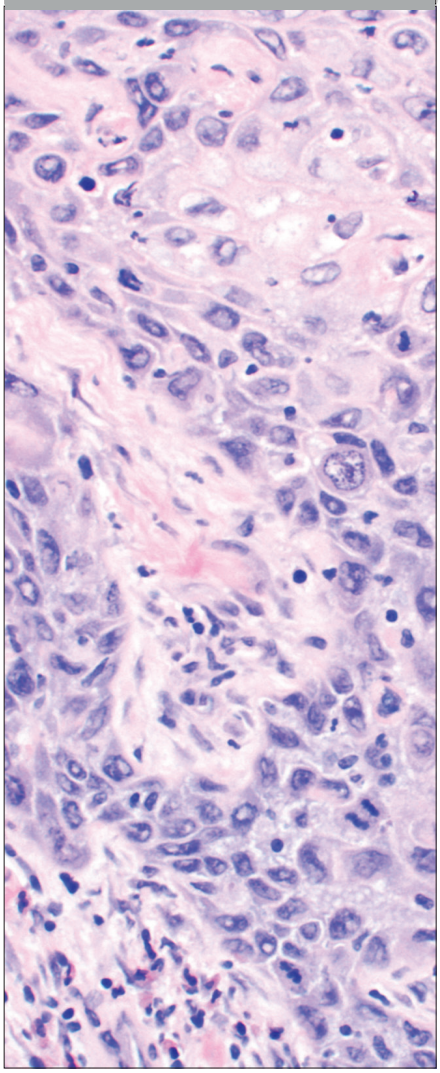
Recommended Slide Order for Interpretation of PD-L1 IHC 28-8 pharmDx

The following flow of slide review is recommended when conducting interpretation of PD-L1 IHC 28-8 pharmDx.
Refer to the detailed description on pages 24–27.

1

Patient Specimen stained with H&E
Histology and Preservation Quality

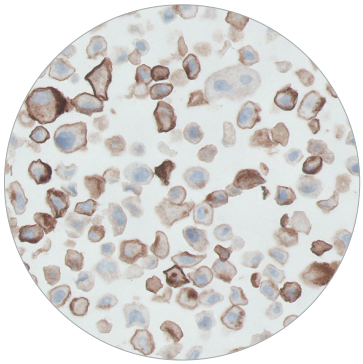
Acceptable



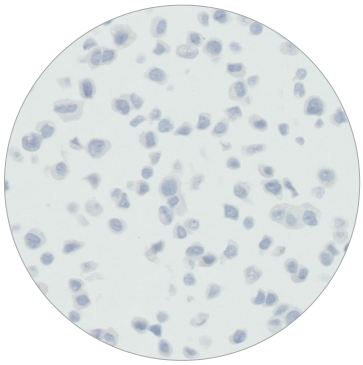
2

PD-L1 IHC 28-8 pharmDx Control Slide
Stained with PD-L1 Primary Antibody

Acceptable



PD-L1 Positive Control

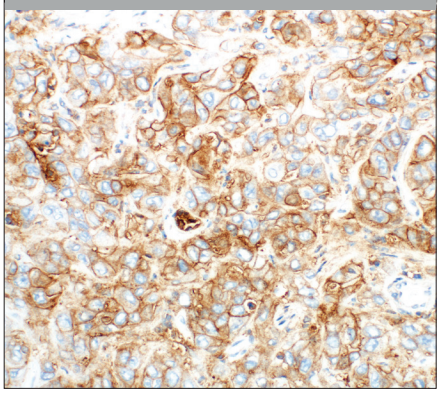


PD-L1 Negative Control

3A

Positive Control Tissue
Stained with PD-L1 Primary Antibody

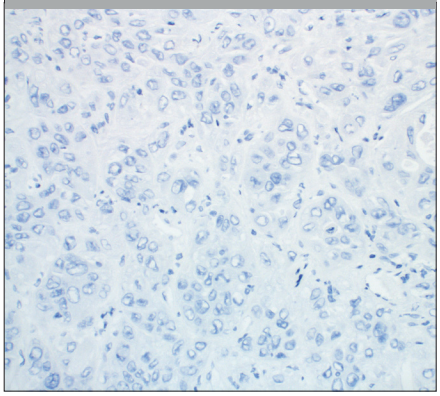
Acceptable



3B

Positive Control Tissue
Stained with Negative Control Reagent

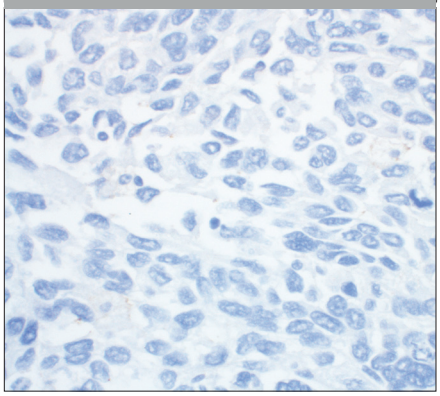
Acceptable



4A

Negative Control Tissue
Stained with PD-L1 Primary Antibody

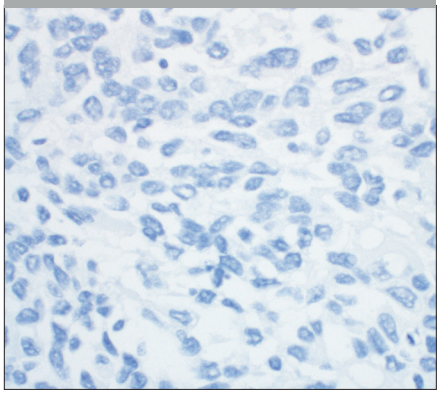
Acceptable



4B

Negative Control Tissue
Stained with Negative Control Reagent

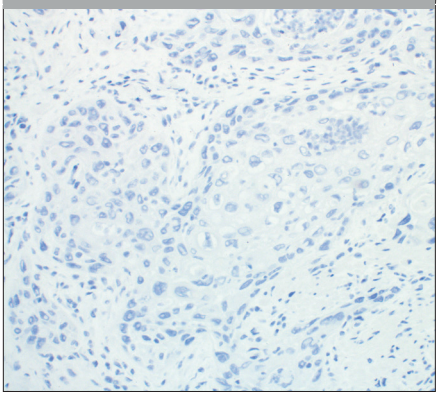
Acceptable



5

Patient Specimen
Stained with Negative Control Reagent

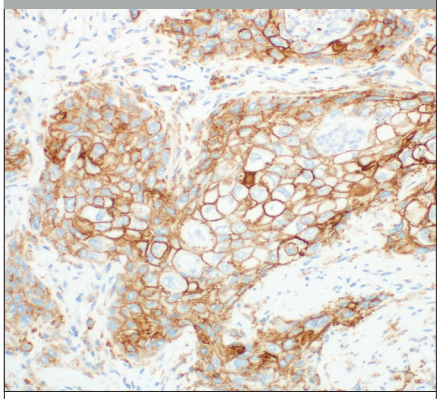
Acceptable



6

Patient Specimen
Stained with PD-L1 Primary Antibody

Acceptable



≥ 100 viable tumor cells should be present for scoring.

Include when scoring:

- Score viable tumor cells exhibiting complete circumferential or partial linear plasma membrane staining at any intensity
- Determine the percentage of stained viable tumor cells in the entire specimen

Exclude from scoring:

- Cytoplasmic staining
- Immune cells
- Normal cells
- Necrotic cells
- in situ carcinoma (dysplasia)

Recommendations for Interpretation of PD-L1 IHC 28-8 pharmDx in UC

PD-L1 IHC 28-8 pharmDx evaluation must be performed by a pathologist using a bright field microscope. Before examining the patient specimen for PD-L1 staining, it is important to examine the hematoxylin and eosin (H&E) and controls first, to assess staining quality. Examine a serial section of the patient specimen stained with H&E for histology and preservation quality. Then, examine the PD-L1 IHC 28-8 pharmDx Control Slide, the positive and negative control tissue slides, and the patient specimen slide stained with with the Negative Control Reagent (NCR) to assess reagent performance.
Lastly, examine the patient specimen stained with Primary Antibody to assess the staining of viable tumor cells.

PD-L1 staining is defined as complete circumferential or partial linear plasma membrane staining at any intensity. Cytoplasmic staining, if present, is not included in the score. Non-malignant cells and immune cells (e.g., such as infiltrating lymphocytes or macrophages) may also stain with PD-L1; however, these should not be included in the scoring for the determination of PD-L1 percent tumor cell expression.

For the determination of PD-L1 percent tumor cell expression positive control tissue slides and negative control tissue slides should be supplied by the laboratory. Only the PD-L1 IHC pharmDx Control Cell Line Slide is provided in the kit.

Patient Specimen Stained with H&E

An hematoxylin & eosin (H&E) stained section is required for the evaluation of histology and preservation quality.

PD-L1 IHC 28-8 pharmDx and the H&E staining should be performed on a serial section from the same paraffin block of the specimen.

PD-L1 IHC 28-8 pharmDx Control Slide

Examine the PD-L1 IHC 28-8 pharmDx Control Slide to ascertain that reagents are functioning properly. Each slide contains sections of cell pellets with positive and negative PD-L1 expression, see Figure 4. If any staining of the Control Slide is not satisfactory, all results with the patient specimens should be considered invalid. Do not use the Control Slide as an aid in the interpretation of patient results.

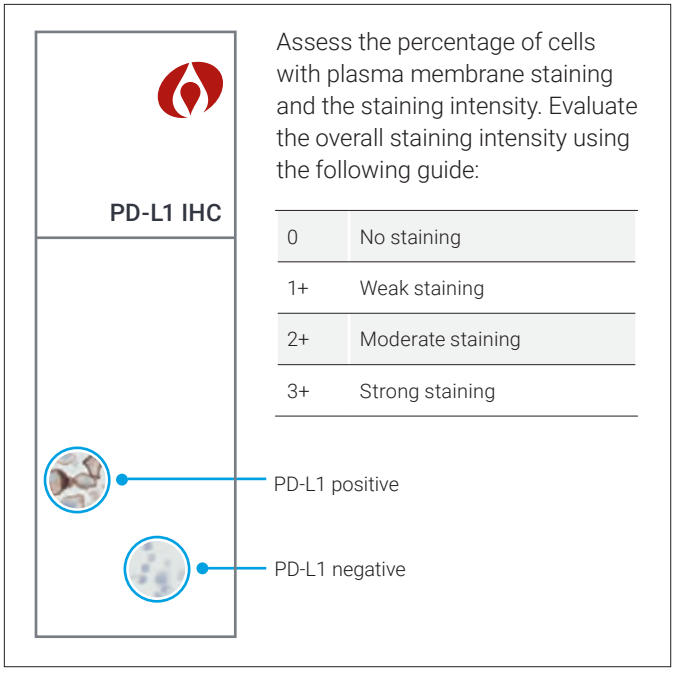


Figure 4. Each Control Slide contains sections of cell pellets with positive and negative PD-L1 expression.

For the PD-L1 positive cell pellet on the Control Slide, the following staining is acceptable, see Figure 5:

- Plasma membrane staining of ≥ 80% of cells
- ≥ 2+ average staining intensity of cells with membrane staining
- Non-specific staining < 1+ intensity

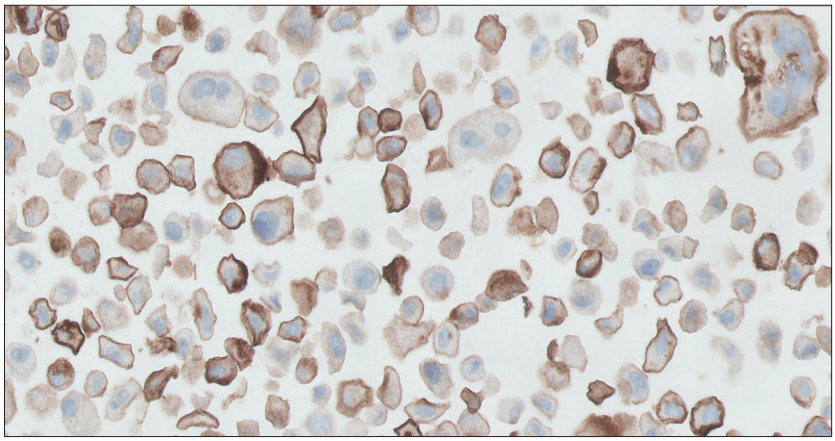


Figure 5. Acceptable Positive PD-L1 Control.

For the PD-L1 negative cell pellet on the Control Slide, the following staining is acceptable, see Figure 6:

- No specific staining
- Non-specific staining < 1+ intensity

Staining of a few cells in the negative pellet on the Control Slide may occasionally be observed. The presence of ≤ 10 cells with distinct plasma membrane staining, and/or cytoplasmic staining with ≥ 1+ intensity within the boundaries of the negative cell pellet is acceptable.

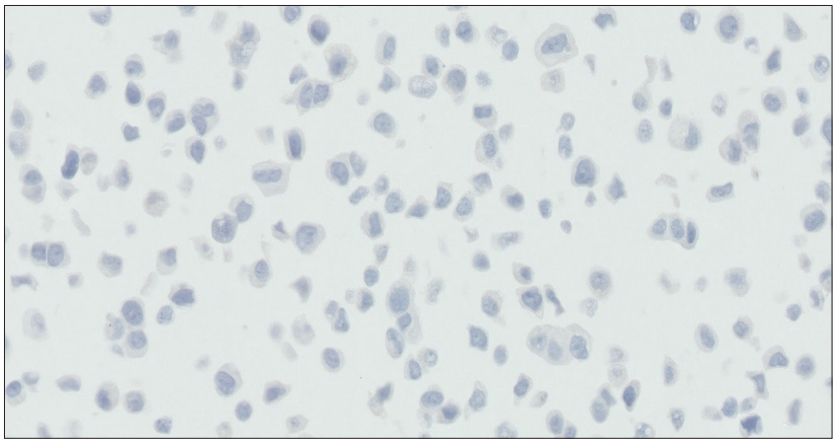


Figure 6. Acceptable Negative PD-L1 Control.

Positive Control Tissue Slides

Examine the positive control tissue slides (Primary Antibody, NCR) to ascertain if tissues are correctly prepared, and reagents are functioning properly. Any non-specific staining should be of ≤ 1+ staining intensity. Exclude necrotic or nonviable tumor cells from the evaluation. If the staining of positive control tissues is not satisfactory, all results with the patient specimens should be considered invalid. Do not use control tissue as an aid in the interpretation of patient results.

Negative Control Tissue Slides

Examine the negative control tissue slides (Primary Antibody, NCR) to confirm that there is no unintended staining. Any non-specific staining should be ≤ 1+ staining intensity. If plasma membrane staining of malignant cells occurs in the negative control tissue, all results with the patient specimens should be considered invalid. Do not use control tissue as an aid in the interpretation of patient results.

Patient Specimen Stained with Negative Control Reagent

Examine the patient specimen stained with NCR to ascertain that reagents are functioning properly. The absence of plasma membrane staining of viable tumor cells is satisfactory and non-specific staining should be ≤ 1+ staining intensity. If any staining is not satisfactory, results with the patient specimen should be considered invalid.

The NCR indicates non-specific staining and allows better interpretation of the patient specimen stained with the Primary Antibody.

Patient Specimen Stained with Primary Antibody

Staining should be assessed within the context of any non-specific staining of the patient specimen stained with NCR. A minimum of 100 viable tumor cells should be present in the PD-L1 stained patient slide in order to perform an evaluation.

1	At 4x objective magnification, carefully examine the tumor areas of the entire specimen. All areas with viable tumor cells on the specimen should be evaluated. Exclude non-malignant cells, necrotic cells, and cellular debris. Cytoplasmic staining, if present, should be disregarded.
2	Use the 10–20x objective magnifications to determine the percentage of viable tumor cells expressing PD-L1 membranous staining. The 40x objective can be used for confirmation if needed. Tumor cells are considered to be PD-L1 positive if they exhibit either partial linear or complete circumferential staining of the plasma membrane at any intensity. Non-malignant cells and immune cells (e.g., infiltrating lymphocytes or macrophages) may also stain with PD-L1 but must be excluded.
3	Record if the specimen has PD-L1 percent tumor cell expression < 1% or ≥ 1%. When determining the percentage of stained tumor cells in the entire specimen, the numerator is the number of stained viable tumor cells and the denominator is the total number of viable tumor cells in the specimen.

$$\%\ \text{PD-L1 expression} = \frac{\#\ \text{PD-L1 staining tumor cells}}{\text{Total \# viable tumor cells}} \times 100$$

Tips and Special Considerations

- Include the entire specimen for evaluation of PD-L1 percent tumor cell expression
- Use higher magnifications to confirm cell types and areas absent of staining
- Be careful not to overlook weak 1+ staining, which can be missed at 4x and 10x
- Disregard cytoplasmic staining
- Necrotic tissue may stain but should be excluded
- Exclude any non-malignant cells and immune cells
- Granular staining must demonstrate a perceptible and convincing membrane pattern

Non-evaluable Specimens

The specimen should be considered non-evaluable if there are fewer than 100 viable tumor cells. A different section from the same block or another block from the same patient may be required to present a sufficient quantity of viable tumor cells for PD-L1 IHC 28-8 pharmDx evaluation.

Indeterminate Specimen

The tumor cell membrane has been hampered for reasons attributed to the biology of the tumor tissue sample rather than improper sample preparation. For example, high cytoplasmic staining of the tumor cells can hamper the scoring of the membrane staining. An additional cut section or section from another block of the same patient may be required for PD-L1 IHC 28-8 pharmDx evaluation.

PD-L1 IHC 28-8 pharmDx Suggested Scoring Methods for Calculating PD-L1 Percent Tumor Cell Expression

Agilent offers two different examples of scoring techniques that may be used when assessing stained specimens exhibiting different staining patterns.

Example 1: Calculating PD-L1 percent tumor cell expression in a specimen with a small PD-L1 staining tumor area

At lower objective magnification, assess the entire specimen for the presence of PD-L1 staining in viable tumor cells at any intensity. Any non-malignant and immune cells staining PD-L1 positive must be excluded.

- In this example, assume the number of tumor cells is equally distributed in the tumor and that there are a total of 1,000 viable tumor cells in the entire specimen.
- 10% of the tumor area has staining, 90% of the tumor area has no staining.

At a higher objective magnification, carefully examine PD-L1 staining tumor area (blue circle in Figure 7). PD-L1 positive staining is defined as complete circumferential and/or partial linear plasma membrane staining of tumor cells at any intensity.

- 50 out of 100 viable tumor cells are staining PD-L1 positive in the single region of the tumor area (Method 1) which may also be described as 50% PD-L1 positive in a single region representing 10% of the tumor area (Method 2).

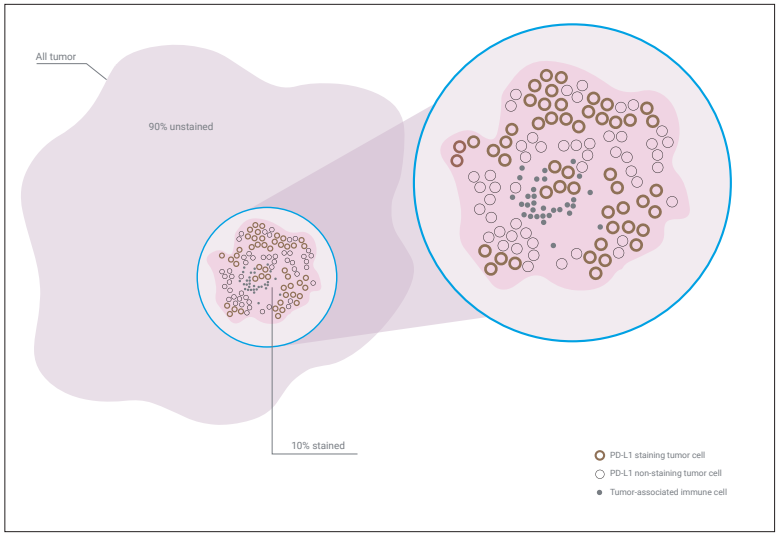


Figure 7. Example of a tumor with a small PD-L1 staining area.

Determine the overall percentage of PD-L1 staining tumor cells for the entire specimen as shown:

Method 1

$$\frac{\text{50 tumor cells staining PD-L1 positive}}{\text{1,000 viable tumor cells}} \times 100 = \text{5\% tumor cell expression}$$

Method 2

$$\frac{50\% \times 10\%}{100} = \text{5\% tumor cell expression}$$

Example 2: Calculating PD-L1 percent tumor cell expression in a specimen with heterogeneous staining

At lower objective magnification, assess the entire specimen for the presence of PD-L1 staining in viable tumor cells at any intensity. Visually divide the tumor area into regions. Any non-malignant and immune cells staining PD-L1 positive must be excluded.

- The tumor area is divided into four equivalent quadrants in Figure 8.

At a higher objective magnification, assess and calculate the percentage of PD-L1 staining tumor cells in each quadrant. PD-L1 positive staining is defined as complete circumferential and/or partial linear plasma membrane staining of tumor cells at any intensity.

- The percentage of PD-L1 staining tumor cells for each of the four respective quadrants are 80%, 30%, 50% and 100%.

Figure 8. Example with heterogenous PD-L1 staining area.

Determine the overall percentage of PD-L1 staining tumor cells for the entire specimen:

$$\frac{(80\% + 30\% + 50\% + 100\%)}{4 \text{ quadrants}} = 65\% \text{ tumor cell expression}$$

Reporting Results

Suggested information to include when reporting results with PD-L1 IHC 28-8 pharmDx in UC

PD-L1 IHC 28-8 pharmDx, Code SK005

Summary of Sample Tested:

Date of Run: _____ PD-L1 IHC 28-8 pharmDx Lot: _____

Staining Run Log ID: _____ Specimen ID: _____

Patient Identifier: _____

Type of Service: IHC Stain with Manual Interpretation _____

Other: _____

Type of Tissue: _____

Additional Tests Performed with PD-L1 IHC 28-8 pharmDx: _____

PD-L1 IHC 28-8 pharmDx Controls Results:

PD-L1 IHC 28-8 Control Slide:	Pass ☐	Fail ☐
Positive Control Tissue Slides:	Pass ☐	Fail ☐
Negative Control Tissue Slides:	Pass ☐	Fail ☐
Patient Specimen, Negative Control Reagent:	Pass ☐	Fail ☐

PD-L1 Results: Detection of PD-L1 expressing tumor cells in UC patient specimens may indicate an enhanced response rate or disease-free survival benefit from OPDIVO (nivolumab) treatment for the patient.

Viable Tumor Cells Present: ☐ ≥ 100 cells ☐ Not evaluable

☐ PD-L1 percent tumor cell expression is < 1%
Percent of UC cells with complete circumferential and/or partial linear membrane PD-L1 staining is < 1%.

☐ PD-L1 percent tumor cell expression is ≥1%
Percent of UC cells with complete circumferential and/or partial linear membrane PD-L1 staining is ≥ 1%.

Other Comments to Treating Physician: _____

Note: PD-L1 IHC 28-8 pharmDx was validated for invasive UC tissue samples and not for lesions with foci of dysplasia or carcinoma in situ*. An H&E stained slide should accompany each PD-L1 stained sample to allow a proper assessment of invasive carcinoma, carcinoma* in situ*, and adjacent normal epithelium.*

PD-L1 IHC 28-8 pharmDx Immunostaining Examples in UC

The following images present examples of UC tumor samples stained with PD-L1 IHC 28-8 pharmDx.

An example of urothelial carcinoma of the bladder stained with PD-L1 IHC 28-8 pharmDx. The staining shows a range of PD-L1 expression. This specimen would be appropriate to use as a positive control specimen for detection of subtle changes in assay sensitivity. Note the partial linear (**red arrow**) and complete circumferential (**black arrow**) plasma membrane staining.

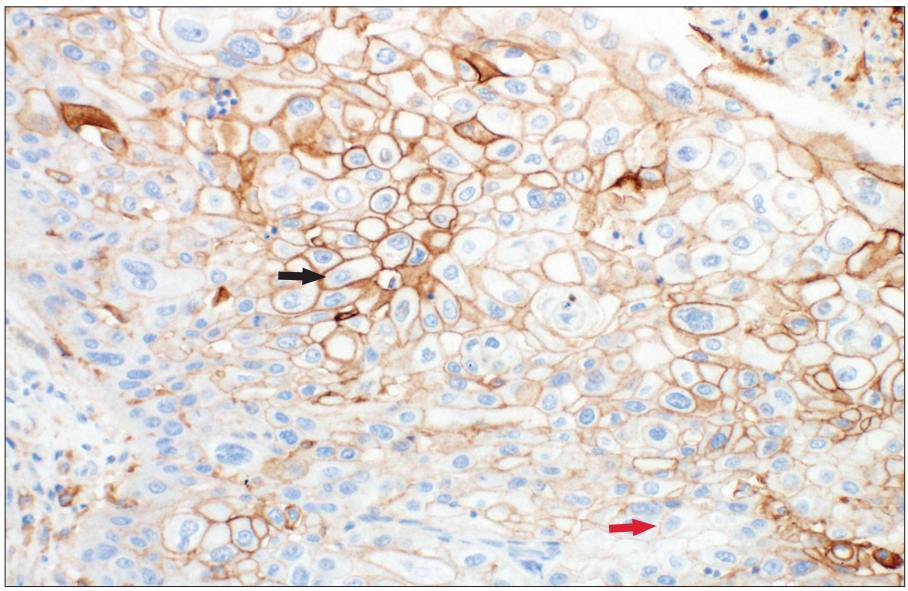


Figure 9. 20x magnification.

Urothelial carcinoma of the bladder
PD-L1 percent tumor cell expression < 1%.

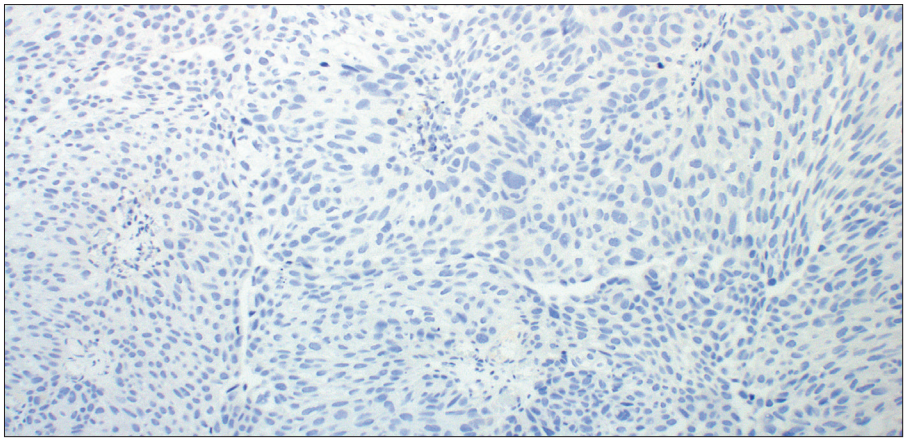


Figure 10. 10x magnification.

Urothelial carcinoma of the bladder
PD-L1 percent tumor cell expression ≥1%.

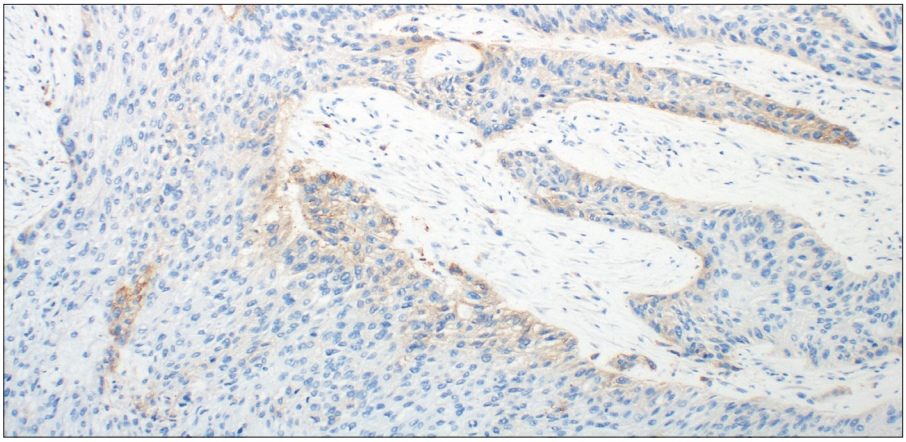


Figure 11. 10x magnification.

Urothelial carcinoma of the bladder demonstrating >1%, moderate PD-L1 percent tumor cell expression.

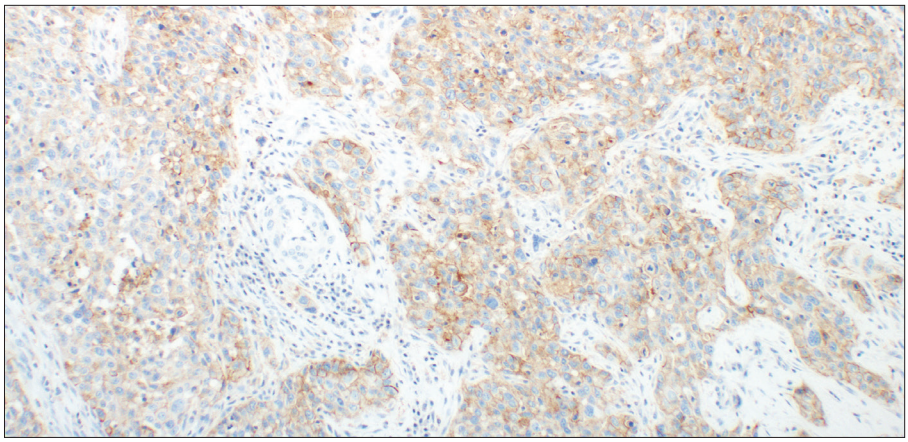


Figure 12. 10x magnification.

Urothelial carcinoma of the bladder demonstrating >1%, high PD-L1 percent tumor cell expression.

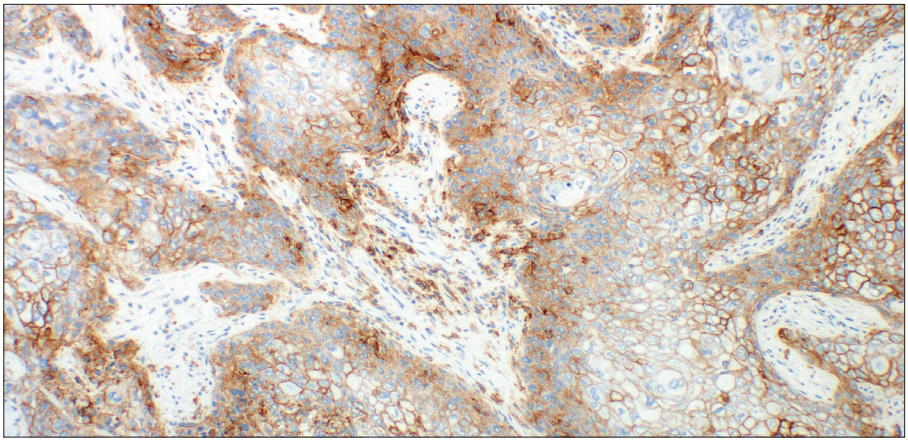


Figure 13. 10x magnification.

Urothelial carcinoma of the bladder showing strong staining of intra-tumoral associated immune cells (**red arrows**), while the tumor cells are negative (**black arrows**) for PD-L1.
Note the staining of intra-tumoral mononuclear inflammatory cells (histiocytes and lymphocytes) are not included in determining the PD-L1 percent tumor cell expression.

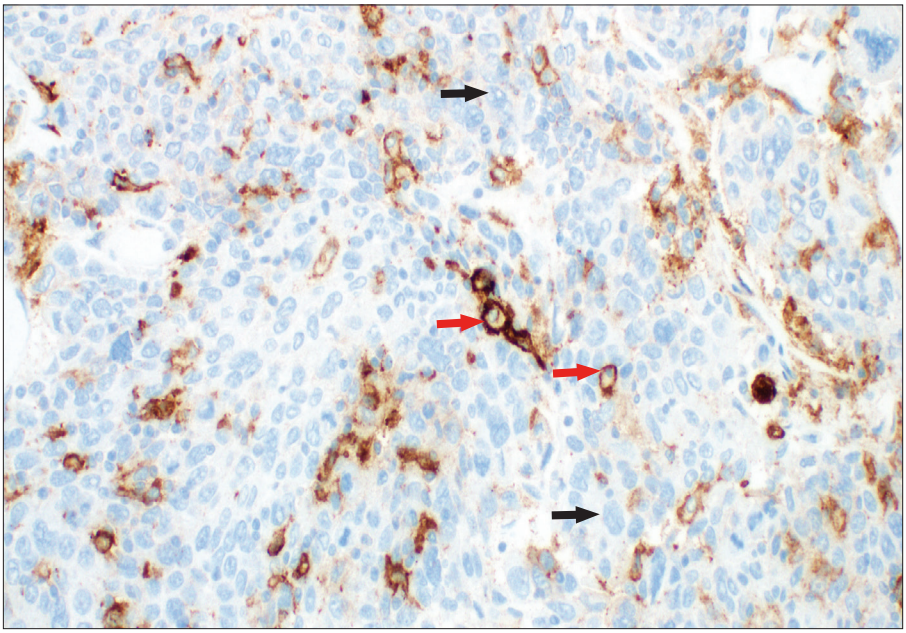


Figure 14. 20x magnification.

Urothelial carcinoma of the bladder showing PD-L1 staining of peri-tumoral (**red arrows**) associated immune cells and tumor cells (**black arrows**). Note the staining of peri-tumoral mononuclear inflammatory cells (histiocytes and lymphocytes) are not included in determining the PD-L1 percent tumor cell expression.

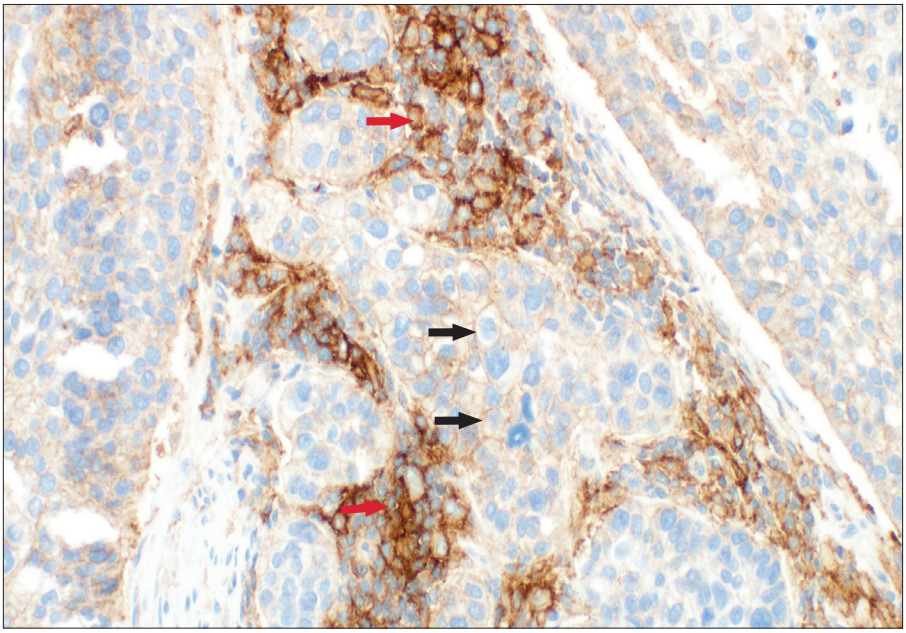


Figure 15. 20x magnification.

H&E stain case of UC of the bladder showing in situ component.

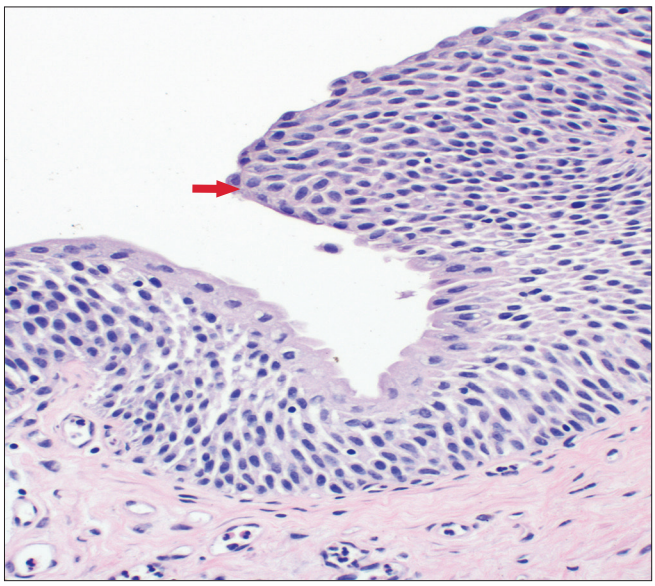


Figure 16a. 20x magnification.

Urothelial carcinoma of the bladder showing negative staining for PD-L1 in the dysplasia/in-situ component (**red arrow**). When scoring PD-L1 percent tumor cell expression, the in-situ component is not included in the denominator. Only the invasive component is evaluated when determining percent tumor cell expression.

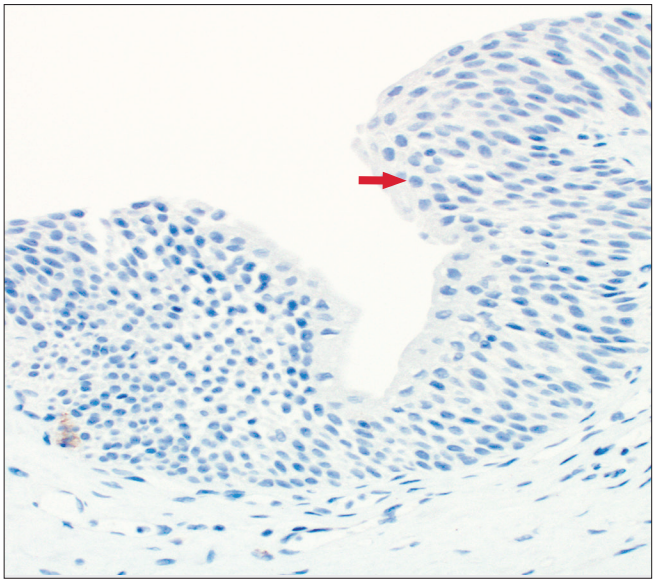


Figure 16b. 20x magnification.

H&E stain of UC of the bladder showing normal urothelium.

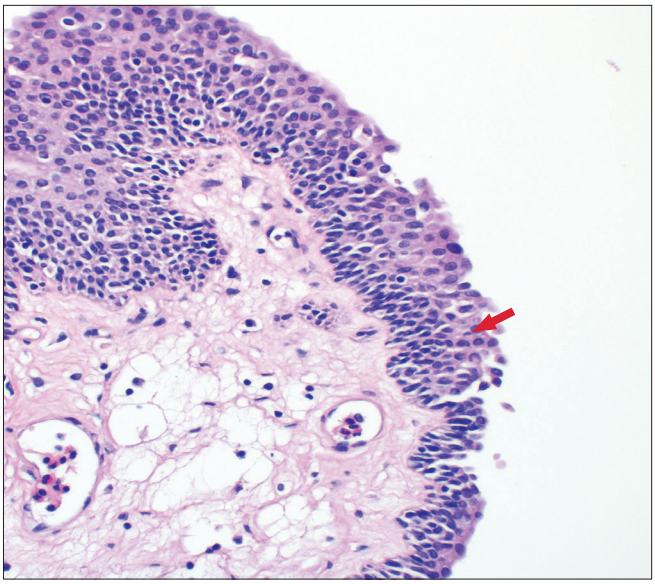


Figure 17a. 20x magnification.

Urothelial carcinoma of the bladder showing normal urothelium (**red arrow**) staining for PD-L1. When determining the PD-L1 percent tumor cell expression for the specimen stained normal urothelium, dysplasia, and in-situ carcinoma components are not included in the numerator, and the entire normal urothelium, dysplasia and in-situ carcinoma components are not included in the denominator. Only the invasive component is evaluated when determining percent tumor cell expression.

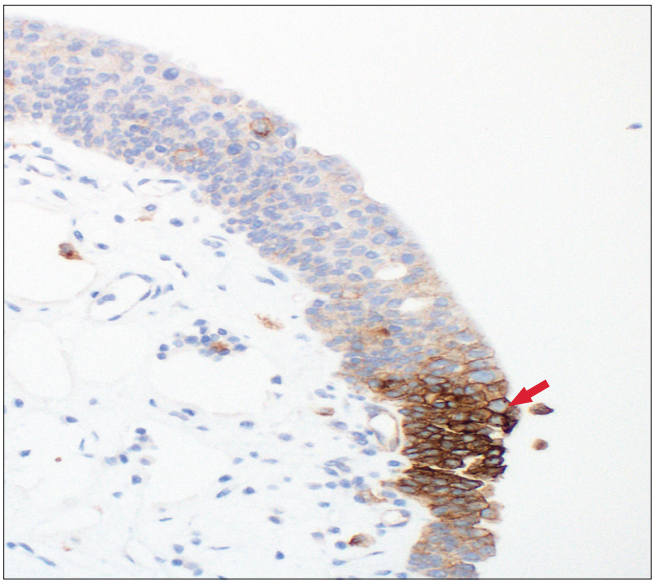


Figure 17b. 20x magnification.

Challenging Cases for UC PD-L1 IHC 28-8 pharmDx

Non-specific staining

Non-specific staining is defined as any off-target staining of the specimen and is often diffuse in pattern. It is caused by several factors. These factors include, but are not limited to, pre-analytic fixation and processing of the specimen, incomplete removal of paraffin from sections, and incomplete rinsing of slides.

The use of fixatives other than 10% NBF may be a source of non-specific staining.

Possible causes of non-specific staining

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

The non-specific staining present on the negative control tissue specimen is useful in determining the level of non-specific staining in the same patient tissue specimen stained with PD-L1. All specimens must have ≤ 1+ non-specific staining.

Immune cells

Intense staining of inflammatory cell infiltrate in the tumor may occur. Inflammatory cells are not included in determining the percent tumor cell expression.

Necrosis

Necrotic tissue may show non-specific staining and should not be included in scoring percent tumor cell expression.

Urothelial carcinoma of the bladder. Tumor cells with granular staining (**black arrows**) in the cytoplasm and no linear membrane staining should not be included in scoring.

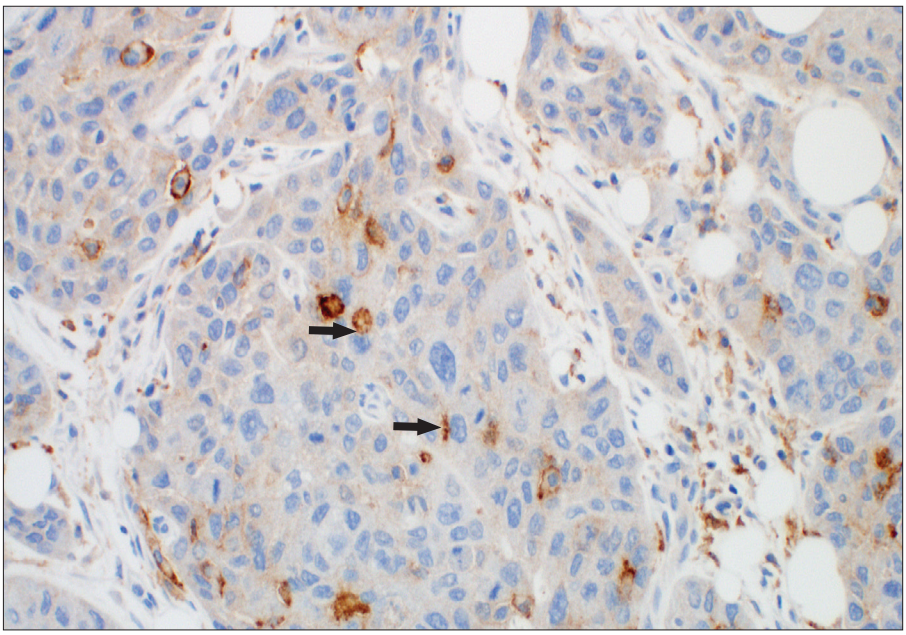


Figure 18. 20x magnification.

Urothelial carcinoma of the bladder. Cells with linear membrane staining that is distinguishable from cytoplasmic staining are included in scoring.

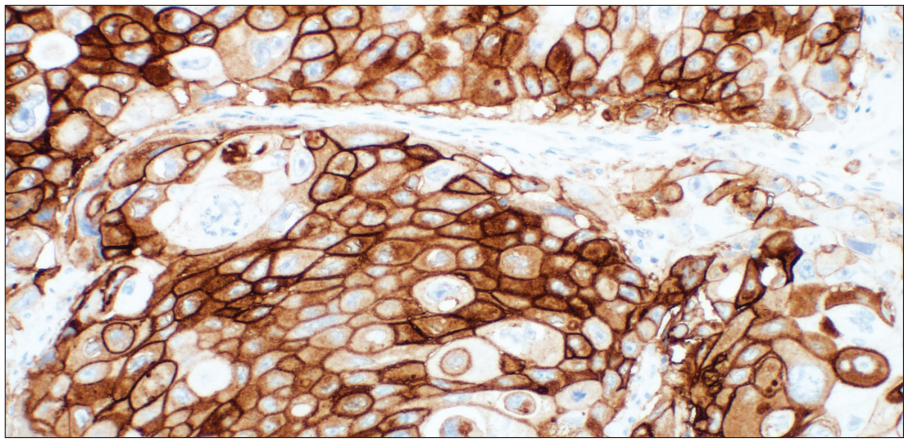


Figure 19. 20x magnification.

Urothelial carcinoma of the bladder. This example may be considered an indeterminate case if the excess cytoplasmic staining hampers scoring. Linear membrane staining of the tumor is observed (**black arrow**), however cytoplasmic staining is excessive in much of the specimen (**red arrow**).

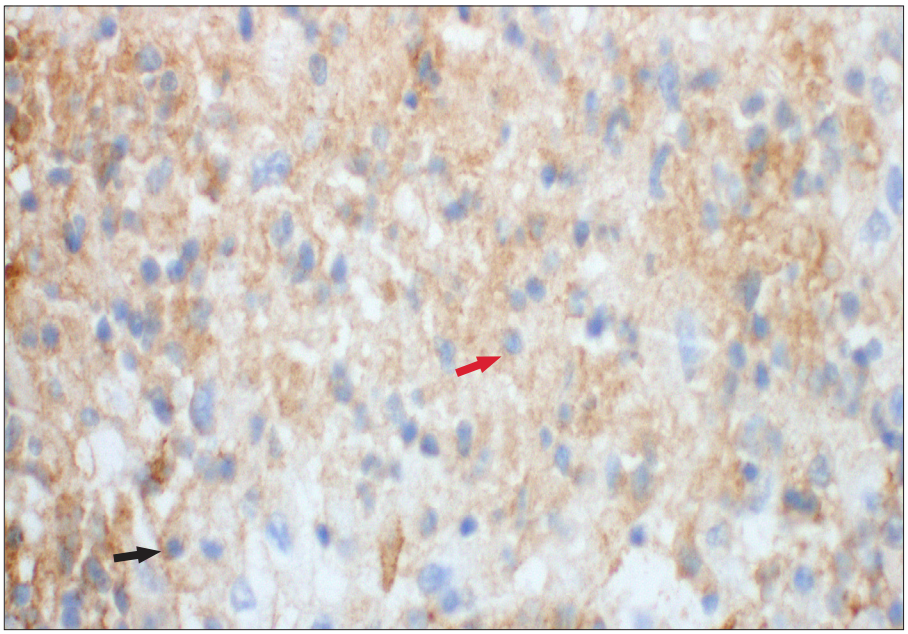


Figure 20. 40x magnification.

Urothelial carcinoma of the bladder. Note plasma membrane staining of foreign body giant cells (FBGC) (**black arrows**) next to tumor cells. FBGCs are created from the fusion of macrophages reacting to foreign material extruded by tumor cells into the stroma. These FBGCs can be misidentified as tumor in the PD-L1 stained slide, but can be recognized as non-tumor in the H&E stained slide. Exclude FBGCs in the scoring of the tumor.

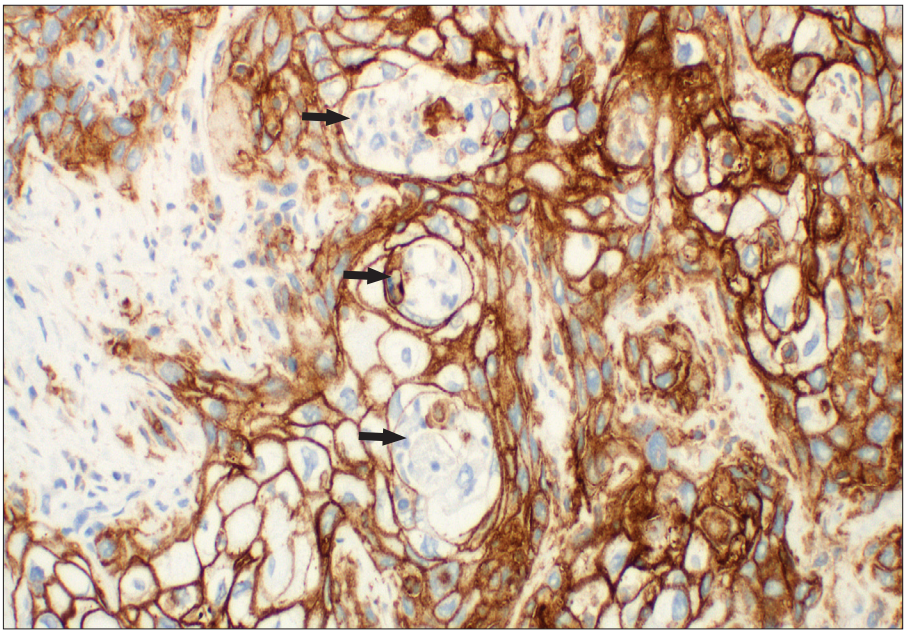


Figure 21. 20x magnification.

Urothelial carcinoma of the bladder. Necrotic tissue may show non-specific staining and should not be included in scoring percent tumor cell expression of the tumor. Care should be taken to only include viable tumor cells for scoring and not necrotic regions. If the specimen is excessively necrotic, the specimen is considered not evaluable. A minimum of 100 viable tumor cells should be present for evaluating the specimen. If the specimen is excessively necrotic and contains <100 viable tumor cells, the specimen is considered non-evaluable (NE).

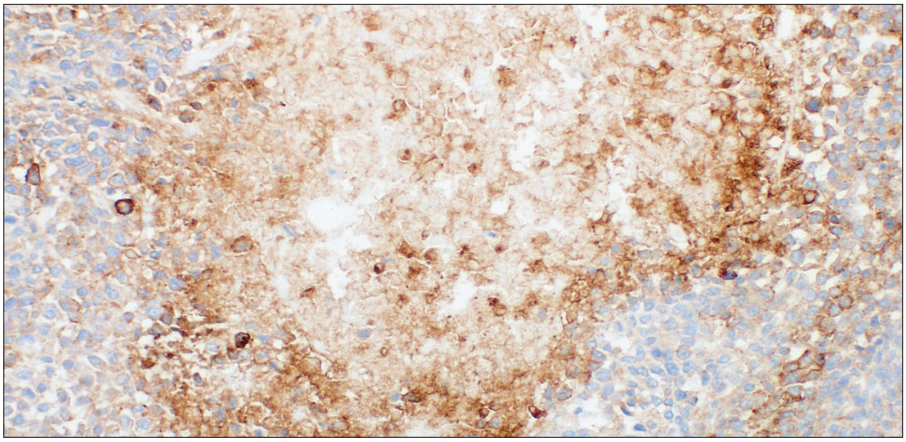


Figure 22. 20x magnification.

Urothelial carcinoma of the bladder with squamous differentiation (**black arrows**). Note, in order to confirm squamous differentiation, intracellular keratin, intercellular bridges, or keratin pearls (**red arrow**) are expected to be present and can be determined by using H&E stain.

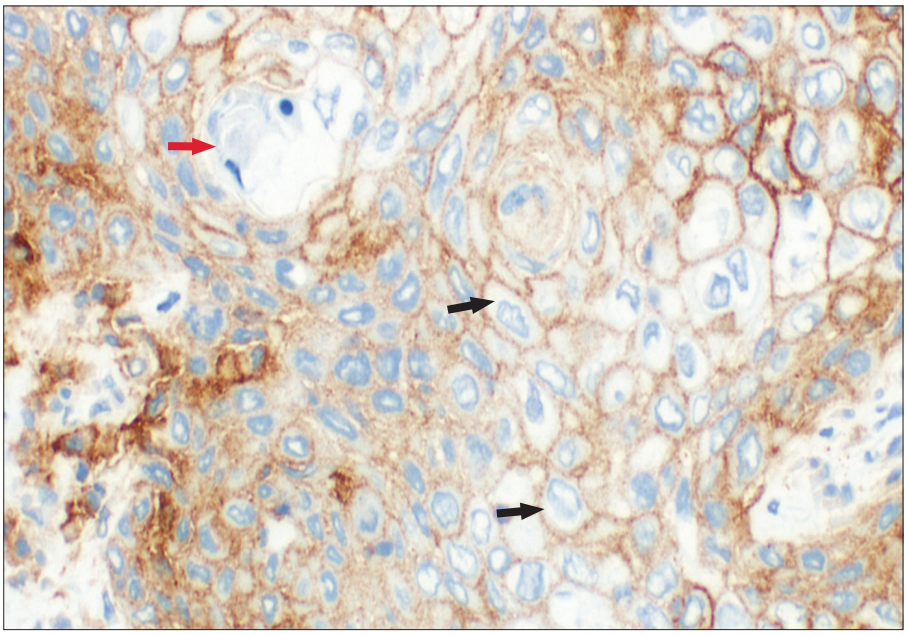


Figure 23. 20x magnification.

PD-L1 staining observed in urothelial carcinoma of the bladder. When scoring for percent tumor cell expression, the percentage is determined by the number of stained cells and not area. Note in this example, the presence of staining in smaller tightly packed basaloid cells (**red arrow**) which take up less area than an equal number of well-differentiated staining cells (**black arrow**).

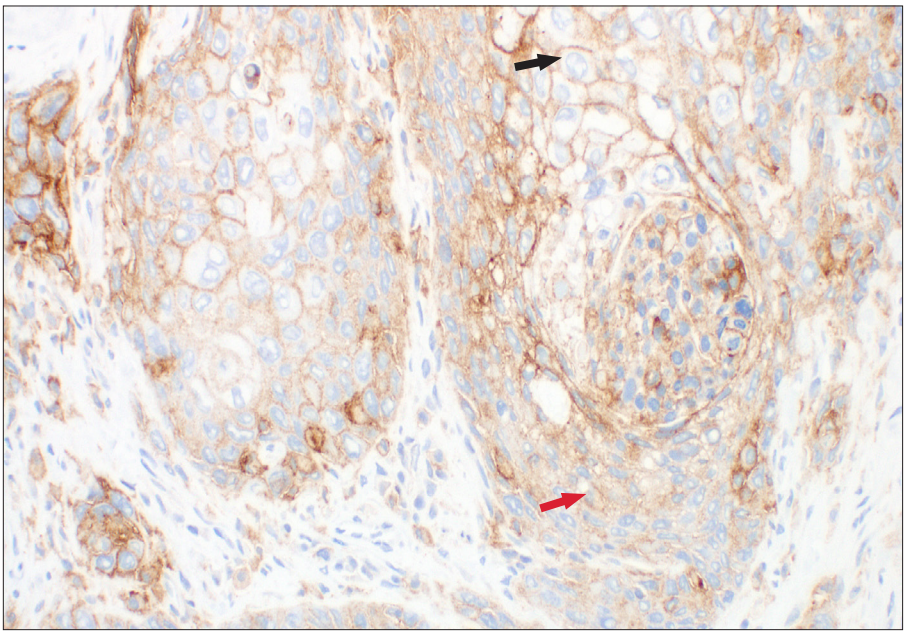


Figure 24. 20x magnification.

Troubleshooting Guide for PD-L1 IHC 28-8 pharmDx

Problem	Probable Cause	Suggested Action
1. No staining of control or specimen slides	1a. Programming error	1a. Verify that the SK005 PD-L1 IHC 28-8 pharmDx program was selected for programming of slides
	1b. Lack of reaction with DAB+ Substrate - Chromogen Solution (DAB)	1b. Verify that DAB+ Substrate-Chromogen Solution was prepared properly
	1c. Sodium azide in wash buffer	1c. Use only EnVision FLEX Wash Buffer, Code K8007
	1d. Degradation of Control Slide	1d. Check kit expiration date and kit storage conditions on outside of package
2. Weak staining of specimen slides	2a. Inappropriate fixation method used	2a. Ensure that only neutral buffered formalin fixative and approved fixation methods are used
	2b. Insufficient reagent volume applied	2b. Check size of tissue section and reagent volume applied
	2c. Inappropriate wash buffer used	2c. Use only EnVision FLEX Wash Buffer, Code K8007
3. Weak staining of specimen slides or the positive cell line on the Agilent-supplied Control Slide	3a. Inadequate target retrieval	3a. Verify that the 3-in-1 pre-treatment procedure was correctly performed
	3b. Inappropriate wash buffer used	3b. Use only EnVision FLEX Wash Buffer, Code K8007
4. Excessive non-specific staining of slides	4a. Paraffin incompletely removed	4a. Verify that the 3-in-1 pre-treatment procedure was correctly performed
	4b. Slides dried while loading onto the Autostainer Link 48	4b. Ensure slides remain wet with buffer while loading and prior to initiating run
	4c. Non-specific binding of reagents to tissue section	4c. Check for proper fixation of the specimen and/or the presence of necrosis
	4d. Inappropriate fixation method used	4d. Ensure that only neutral buffered formalin fixative and approved fixation methods are used
	4e. Inadequate mixing of wash buffer	4e. Ensure wash buffer is properly mixed
5. Tissue detached from slides	5a. Use of incorrect microscope slides	5a. Use FLEX IHC Microscope Slides, (Code K8020), or Superfrost Plus slides
	5b. Inadequate preparation of specimens	5b. Cut sections should be placed in a 58 ± 2 °C oven for 1 hour prior to staining
6. Excessively strong specific staining	6a. Inappropriate fixation method used	6a. Ensure that only approved fixatives and fixation methods are used
	6b. Inappropriate wash buffer used	6b. Use only EnVision FLEX Wash Buffer, Code K8007
7. 1x EnVision FLEX Target Retrieval Solution is cloudy in appearance when heated	7. When heated the 1x EnVision FLEX Target Retrieval Solution turns cloudy in appearance	7. This is normal and does not influence staining

Problem	Probable Cause	Suggested Action
8. 1x EnVision FLEX Target Retrieval Solution does not meet pH specifications	8a. pH meter is not calibrated correctly	8a. Ensure pH meter is calibrated per manufacturer's recommendations. After re-calibration, re-test the pH of 1x EnVision FLEX Target Retrieval Solution. Do not modify the pH of 1x EnVision FLEX Target Retrieval Solution. If the pH is outside the acceptable range (6.1 ± 0.2), discard 1x EnVision FLEX Target Retrieval Solution. Prepare new 1x EnVision FLEX Target Retrieval Solution. Check the pH of the new 1x EnVision FLEX Target Retrieval Solution
	8b. Inferior quality water is used to dilute the EnVision FLEX Target Retrieval Solution concentrate	8b. Ensure that distilled or deionized water is used to prepare 1x Target Retrieval Solution
	8c. Incorrect Target Retrieval Solution is used	8c. Ensure that the correct EnVision Flex Target Retrieval Solution specified in “Materials Provided” and “Reagent Preparation” sections of the IFU is used

If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please contact Agilent Pathology Support for further assistance. Additional information on staining techniques and specimen preparation can be found in the Education Guide: Immunohistochemical Staining Methods (Taylor C. R. and Rudbeck L. Education Guide: Immunohistochemical Staining Methods – Sixth Edition. Dako, Carpinteria, California. 2013; available from Agilent).

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Published in the USA, November 30, 2021
29458 D69027 2021NOV01

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