Urothelial Carcinoma (UC)
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Introduction

Intended Use in Urothelial Carcinoma
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-squamous non-small cell lung cancer (NSCLC), squamous cell carcinoma of the head and neck (SCCHN), urothelial carcinoma (UC), and melanoma 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, as defined by the specific tumor indication staining interpretation guidelines in the instructions for use (IFU).

How to Use the PD-L1 IHC 28-8 pharmDx Interpretation Manual
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. 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 IFU contain 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 provided or visit www.agilent.com.

PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in non-squamous NSCLC and SCCHN may be associated with enhanced survival from OPDIVO® (nivolumab). PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in urothelial carcinoma may be associated with enhanced response rate from OPDIVO.

PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in melanoma may be used as an aid in the assessment of patients for whom OPDIVO (nivolumab) and YERVOY® (ipilimumab) combination treatment is being considered.

The included photomicrographs are UC unless otherwise noted. OPDIVO is a trademark of Bristol-Myers Squibb Company.
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.

**The tumor escapes detection**

Inactivation of T cells reduces tumor cell killing.

**Immuno-oncology therapies harness the immune response to fight tumors**

Blocking PD-L1 enables cytotoxic T cells to actively remove tumor cells.
The Clinical Value of PD-L1 IHC 28-8 pharmDx Expression in Urothelial Carcinoma

Detection of PD-L1 expressing tumor cells in urothelial carcinoma (UC) patient specimens may indicate an enhanced response rate benefit to OPDIVO (nivolumab) treatment for the patient.\(^1\)

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).\(^1\)

The study was a phase II 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.\(^1\)

In study CA209275, Objective Response Rate (ORR) based on PD-L1 expression was evaluated using PD-L1 IHC 28-8 pharmDx and is summarized below. Median time to response was 1.9 months (range: 1.6-7.2).

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

**Table 1: Efficacy Results for study CA209275**

Confirmed ORR in all patients and the two PD-L1 subgroups are summarized in the table below.

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

*Estimated from the Kaplan-Meier Curve*
PD-L1 expression, as determined by PD-L1 IHC 28-8 pharmDx in UC, may be associated with enhanced response rate from OPDIVO (nivolumab).

Worldwide, bladder cancer is the 7th most common cancer in men and 17th most common cancer in women, resulting in approximately 165,000 deaths in 2012. The frequency of bladder cancer and tendency for recurrence puts a significant burden on global health care. Standard first-line treatment for metastatic UC involves platinum based combination chemotherapy. Despite 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 CA209275: A phase II single arm clinical trial of nivolumab in subjects with metastatic or unresectable urothelial cancer who have progressed or recurred following treatment with a platinum agent.

Study Design:

- The following patients in Study CA209275 were treated with OPDIVO\(^{(1)}\)
  - 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
Clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in CA209275, a phase II 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

<table>
<thead>
<tr>
<th>Non-bladder UC</th>
<th>Visceral metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>27% (73/270)</td>
<td>84% (227/270)</td>
</tr>
</tbody>
</table>

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

### Table 2. Frequency of Tumor PD-L1 Expression in Samples from UC - CA209275

<table>
<thead>
<tr>
<th>Tumor PD-L1 Expression</th>
<th>Nivolumab (N=270)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1% PD-L1 Expression Subjects</td>
<td>124 (45.9%)</td>
</tr>
<tr>
<td>&lt;1% PD-L1 Expression Subjects</td>
<td>146 (54.1%)</td>
</tr>
</tbody>
</table>
PD-L1 IHC 28-8 pharmDx Overview
Code SK005

PD-L1 IHC 28-8 pharmDx contains optimized reagents and the protocol required to complete an IHC staining procedure of FFPE tissue sections using Autostainer Link 48 and the PT Link Pretreatment Module (see Figure 1). Following incubation with the primary monoclonal antibody PD-L1 or the Negative Control Reagent, the specimens are incubated with a PD-L1 IHC 28-8 pharmDx purified Rabbit Linker antibody specific to the host species of the Primary Antibody, and then are 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 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 is then counterstained and coverslipped. Results are interpreted using a light microscope. Control Slides containing two formalin-fixed, paraffin-embedded human cell lines are provided to aid in validating staining runs.

Figure 1: PD-L1 IHC 28-8 pharmDx staining procedure.
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 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 IFU for a complete list of required materials and equipment.

Figure 2: PD-L1 IHC 28-8 pharmDx, components.

All PD-L1 IHC 28-8 pharmDx reagents are to be performed on Autostainer Link 48. All reagents must be used as indicated in the IFU in order for the test to perform as specified.
Optimal staining performance is achieved by adhering to the PD-L1 IHC 28-8 pharmDx protocol. The following are tips for optimizing staining performance. Technical problems related to the performance of PD-L1 IHC 28-8 pharmDx may arise; those involving specimen collection, specimen preparation prior to performing the test and problems involving the actual performance of the test itself. Technical problems of the test can be minimized with a thorough understanding of the product instructions by the user.

Specimen Collection and Processing
Specimens must be handled in a way which preserves the tissue for immunohistochemical staining. Tissue should be stained and interpreted as close to time of biopsy as possible. 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 PD-L1 IHC 28-8 pharmDx Control Slides.

Select positive and negative control tissue from fresh UC specimens. Fix, process, and embed the control tissue in the same manner as patient specimens. Control tissue processed differently from the patient specimen validates reagent performance only and does not verify tissue preparation. The ideal positive control tissue shows weak to moderate PD-L1 expression. The variety of different cell types present in most tissue sections offers internal negative control sites; this should be verified by the user. A suggested UC-negative control tissue is one that shows no staining in tumor cells but possesses stained immune cells such as macrophages and lymphocytes.

Tissue Processing
Formalin-fixed, paraffin-embedded tissues are suitable for use. Block specimens into a thickness of 3 mm or 4 mm, fix in 10% Neutral Buffered Formalin (NBF), and dehydrate and clear in a series of alcohols and xylene, followed by infiltration with melted paraffin. An ischemia time from excision to fixation start time of less than 30 minutes followed by immersion in 10% neutral buffered formalin for 24-48 hours is recommended. The paraffin temperature should not exceed 60 °C. Decalcified tissue has not been validated on PD-L1 IHC 28-8 pharmDx 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 Fisherbrand Superfrost Plus charged slides and place in a 58 ± 2 °C oven for 1 hour. To preserve antigenicity, 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
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 materials 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.

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

EnVision FLEX Target Retrieval Solution, Low pH
Dilute EnVision FLEX Target Retrieval Solution, Low pH (50x) 1:50 using distilled or deionized water (reagent-quality water). One 30 mL bottle of concentrate provides 1.5 L of working solution which is sufficient to fill one PT Link Pretreatment Module tank and will treat up to 24 slides per use. The pH of the working solution should be 6.1 ± 0.2. Discard Low pH working solution after three uses. Do not use after 5 days following dilution.

EnVision FLEX Wash Buffer, Code K8007
Dilute EnVision FLEX Wash Buffer (20x) 1:20 using distilled or deionized water (reagent-quality water). Store unused working solution at 2-8 °C for no more than one month. Wash buffer can also be stored for up to 7 days at 25 °C. Discard if cloudy in appearance.
**DAB+ Substrate-Chromogen Solution**

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

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

**Controls to Assess Staining Quality**

Control slides are recommended to determine that PD-L1 IHC 28-8 pharmDx results (generated by the system containing reagents, instrument hardware and software) are valid and the reagents are functioning properly. For each staining run include the following control slides:

- One PD-L1 IHC 28-8 pharmDx Control Slide stained with the Primary Antibody in each staining run.
- Two positive control tissue slides (one stained with Primary Antibody and the other stained with Negative Control Reagent) for each set of test conditions.
- Two negative control tissue slides (one stained with Primary Antibody and the other stained with Negative Control Reagent).
- Lastly, for each patient specimen stained with Primary Antibody, include a sequential section of patient specimen stained with Negative Control Reagent.

**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 Pretreatment Module to perform a deparaffinization, rehydration and target retrieval 3-in-1 procedure.

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

Start the Autostainer to run the PD-L1 IHC 28-8 pharmDx protocol. The instrument performs the staining and counterstaining procedures by applying the appropriate reagent, monitoring the incubation time and rinsing slides between reagents. Counterstain slides using EnVision FLEX Hematoxylin. Do not allow slides to dry prior to mounting.

**Mounting**

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

**Example of Adequate Dehydration Procedure**

<table>
<thead>
<tr>
<th>Step</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% EtOH</td>
<td>1</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>1</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>1</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>1</td>
</tr>
<tr>
<td>Xylene</td>
<td>3</td>
</tr>
<tr>
<td>Xylene</td>
<td>3</td>
</tr>
</tbody>
</table>

Ensure that slides do not dry between the end of the Autostainer run and mounting procedure. Xylene may be substituted with Histoclear solution.
PD-L1 IHC 28-8 pharmDx Technical Checklist

Customer Name / Institution: __________________________________________________________

Name and Title: ____________________________________________________________________

Autostainer Link 48 Serial Number: ___________________________ Software Version: __________

Regular preventive maintenance is performed on Autostainer Link 48? Yes ☐ No ☐

All the necessary equipment is available to perform PD-L1 IHC 28-8 pharmDx according to protocol? Yes ☐ No ☐

PD-L1 IHC 28-8 pharmDx is used before the expiration date printed on the outside of the box? Yes ☐ No ☐

All PD-L1 IHC 28-8 pharmDx components, including Control Slides, are stored in the dark at 2-8 °C? Yes ☐ No ☐

All PD-L1 IHC 28-8 pharmDx components, including Control Slides, are equilibrated to room temperature (20-25 °C) prior to immunostaining? Yes ☐ No ☐

Appropriate positive and negative control tissue from UC are identified? Yes ☐ No ☐

Tissues are fixed in neutral buffered formalin? Yes ☐ No ☐

Tissues are infiltrated with melted paraffin, at or below 60 °C? Yes ☐ No ☐

Tissue sections of 4-5 µm are mounted on FLEX IHC Microscope Slides or Fisherbrand Superfrost Plus charged slides? Yes ☐ No ☐

Specimens are oven-dried at 58 ± 2 °C for 1 hour? Yes ☐ No ☐

Specimens are stained within 4 months of sectioning when stored in the dark at 2-8 °C or room temperature up to 25 °C? Yes ☐ No ☐

EnVision FLEX Target Retrieval Solution, Low pH is prepared properly? pH of 1x Target Retrieval Solution should be 6.1 ± 0.2? Yes ☐ No ☐

EnVision FLEX Wash Buffer is prepared properly? Yes ☐ No ☐

DAB+ Substrate-Chromogen Solution is prepared properly? Yes ☐ No ☐

The Deparaffinization, Rehydration and Target Retrieval 3-in-1 procedure is followed, using PT Link? Yes ☐ No ☐

Slides remain wet with buffer while loading and prior to initiating run on Autostainer Link 48? Yes ☐ No ☐

PD-L1 IHC 28-8 pharmDx protocol is selected on Autostainer Link 48? Yes ☐ No ☐

Slides are counterstained with EnVision FLEX Hematoxylin? Yes ☐ No ☐

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

Additional observations or comments: __________________________________________________

__________________________________________________________________________

__________________________________________________________________________

__________________________________________________________________________

__________________________________________________________________________
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. 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 20 for an example of a pathology report form for PD-L1 IHC 28-8 pharmDx.

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

<table>
<thead>
<tr>
<th>&lt; 1% of the viable tumor cells exhibit complete circumferential or partial linear plasma membrane staining at any intensity.</th>
<th>≥ 1% of the viable tumor cells exhibit complete circumferential or partial linear plasma membrane staining at any intensity.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-L1 expression &lt; 1%</td>
<td>PD-L1 expression ≥ 1%</td>
</tr>
</tbody>
</table>
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 detailed description on pages 18-19.

1. Patient Specimen stained with H&E
   Histology and preservation quality

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

3A. Positive Control Tissue
    Stained with PD-L1 Primary Antibody

3B. Positive Control Tissue
    Stained with Negative Control Reagent

PD-L1 Positive Control

PD-L1 Negative Control
≥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)
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 PD-L1 IHC 28-8 pharmDx Control Slide, followed by the positive and negative control tissue slides, stained with Negative Control Reagent and Primary Antibody for each set of test conditions. Lastly, examine the patient specimen stained with Negative Control Reagent and Primary Antibody to assess the percentage staining of viable tumor cells.

PD-L1 staining is defined as complete circumferential and/or partial linear plasma membrane staining at any intensity. Only the PD-L1 IHC 28-8 pharmDx Control Slide is provided in the PD-L1 IHC 28-8 pharmDx kit. Positive control tissue slides and negative control tissue slides should be supplied by the laboratory. Laboratory provided positive and negative control tissue may be included on the same slide as the patient specimen.

1. **Patient Specimen Stained with H&E**
   An 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 serial sections from the same paraffin block of the specimen.

   Foci of dysplasia and carcinoma in situ are excluded from scoring. An accompanying H&E section allows for the proper assessment of invasive carcinoma, carcinoma in situ, and adjacent normal epithelium.

2. **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 interpretation of patient results.

   - **PD-L1 positive cell pellet on the Control Slide**
     - At least 80% of the cells contain plasma membrane staining of at least 2+ average staining intensity
     - Any background staining is of less than 1+ staining intensity

   - **PD-L1 negative cell pellet on the Control Slide**
     - No plasma membrane staining
     - Any background staining is of less than 1+ staining intensity

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

   - Staining of a few cells in the negative pellet on the Control Slide may occasionally be observed. The presence of 10 or fewer cells with distinct plasma membrane staining, or cytoplasmic staining with ≥ 1+ intensity within the boundaries of the negative cell pellet are acceptable.
3. Positive Control Tissue Slides
Examine the positive UC control tissue slides (one stained with Primary Antibody and the other with Negative Control Reagent) to ascertain if tissues are correctly prepared and reagents are functioning properly. Any background staining should be of ≤ 1+ staining intensity. Exclude necrotic or degenerated cells from evaluation. If 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 interpretation of patient results.

4. Negative Control Tissue Slides
Examine the negative UC control tissue slides (one stained with Primary Antibody and the other with Negative Control Reagent) to confirm that there is no unintended staining. Any background staining should be of ≤ 1+ staining intensity. If unwanted specific 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 interpretation of patient results.

5. Patient Specimen Stained with Negative Control Reagent
The Negative Control Reagent indicates non-specific background staining and allows better interpretation of patient specimen stained with the Primary Antibody. Examine the patient specimen stained with Negative Control Reagent to identify non-specific background staining. Staining by the Negative Control Reagent must not show positive membrane staining and non-specific background should be ≤ 1+. If staining is not satisfactory, results with the patient specimen should be considered invalid.

6. Patient Specimen Stained with Primary Antibody
Staining should be assessed within the context of any non-specific background staining of the patient specimen stained with Negative Control Reagent. A minimum of 100 viable tumor cells should be present in the PD-L1 stained patient specimen slide to determine the percentage of stained cells.

**Tips and Special Considerations**
- Include the entire specimen for evaluation of PD-L1 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

**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 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.

![Acceptable Positive PD-L1 Control](image)

![Acceptable Negative PD-L1 Control](image)
Reporting Results

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 proper assessment of invasive carcinoma, carcinoma in situ, and adjacent normal epithelium.

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 Slides:  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 urothelial carcinoma patient specimens may indicate an enhanced response rate benefit from OPDIVO (nivolumab) treatment for the patient.^[1^]

Viable Tumor Cells Present  ☐ ≥ 100 cells  ☐ Not Evaluable

☐ **PD-L1 expression < 1%:**
Percent of UC cells with complete circumferential and/or partial linear membrane PD-L1 staining is < 1%

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

% Percent Expression PD-L1 Tumor Cells: ___________________

Other Comments to Treating Physician: ___________________________
The following images present examples of UC tumor samples stained with PD-L1 IHC 28-8 pharmDx.

Figure 7: 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. 20x magnification.
**Figure 8:** Urothelial carcinoma of the bladder. PD-L1 expression < 1%. 10x magnification.

**Figure 9:** Urothelial carcinoma of the bladder. PD-L1 expression ≥1%. 10x magnification.
Figure 10: Urothelial carcinoma of the bladder demonstrating >1%, moderate PD-L1 expression. 10x magnification.

Figure 11: Urothelial carcinoma of the bladder demonstrating >1%, high PD-L1 expression. 10x magnification.
Figure 12: 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 positivity. Note the staining of the intra-tumoral histiocytes are not included in determining the percent PD-L1 positive score. 20x magnification.

Figure 13: Urothelial carcinoma of the bladder showing PD-L1 positive staining of peri-tumoral (red arrows) associated immune cells and tumor cells (black arrows). Note the staining of the peri-tumoral histiocytes are not included in determining the percent PD-L1 positive score. 20x magnification.
Figure 14A: H&E stain of a case of urothelial carcinoma of the bladder showing in situ component.

Figure 14B: Urothelial carcinoma of the bladder showing in situ component (red arrow) is not staining for PD-L1 in this case. When scoring for percent positivity, the in situ component is not included in the denominator for determining the PD-L1 percentage score for the specimen. Only the invasive component is evaluated. 20x magnification.

Figure 15A: H&E stain of a case of urothelial carcinoma of the bladder showing in situ component.

Figure 15B: Urothelial carcinoma of the bladder showing in situ component (red arrow) staining for PD-L1 in this case. When determining the PD-L1 percentage score for the specimen, the positively stained in situ component is not included in the numerator, and the entire in situ component is not included in the denominator. Only the invasive component is evaluated. 20x magnification.
Challenging Cases for UC PD-L1 IHC 28-8 pharmDx

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

The use of fixatives other than 10% neutral buffered formalin may be a source of background staining.

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

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

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

Necrosis
Necrotic tissue may show non-specific staining and should not be included in scoring percent positivity of the tumor.

Figure 16: Urothelial carcinoma of the bladder. Granular staining [black arrow] in the cytoplasm of tumor cells with no positive linear membrane staining is not considered positive. 20x magnification.
Figure 17: Urothelial carcinoma of the bladder. Positive linear membrane staining of the tumor is observed and distinguishable from the cytoplasmic staining. 20x magnification.

Figure 18: Urothelial carcinoma of the bladder. This example may be considered an indeterminate case if the excess cytoplasmic staining hampers scoring. Positive linear membrane staining of the tumor is observed (black arrow), however cytoplasmic staining is excessive in much of the specimen (red arrow). 40x magnification.
Figure 19: 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. 20x magnification.

Figure 20: Urothelial carcinoma of the bladder. Necrotic tissue may show non-specific staining and should not be included in scoring percent positivity 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 not evaluable. 20x magnification.
Figure 21: 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. 20x magnification.

Figure 22: PD-L1 positive staining observed in urothelial carcinoma of the bladder. When scoring for percent positivity, percentage is determined by the number of positively 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 positively staining cells with squamous differentiation (black arrow). 20x magnification.
## Troubleshooting Guide for PD-L1 IHC 28-8 pharmDx

<table>
<thead>
<tr>
<th>Problem</th>
<th>Probable Cause</th>
<th>Suggested Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1</strong> No staining of control or specimen slides</td>
<td>1a Programming error</td>
<td>Verify that the SK005 PD-L1 IHC 28-8 pharmDx protocol was selected for programming of slides</td>
</tr>
<tr>
<td></td>
<td>1b Lack of reaction with DAB+ Substrate-Chromogen Solution</td>
<td>Verify that DAB+ Substrate-Chromogen Solution was prepared properly</td>
</tr>
<tr>
<td></td>
<td>1c Sodium azide in wash buffer</td>
<td>Use only Dako Wash Buffer, Code K8007</td>
</tr>
<tr>
<td></td>
<td>1d Degradation of Control Slide</td>
<td>Check kit expiration date and kit storage conditions on outside of package</td>
</tr>
<tr>
<td><strong>2</strong> Weak staining of specimen slides</td>
<td>2a Inappropriate fixation method used</td>
<td>Ensure that only approved fixatives and fixation methods are used</td>
</tr>
<tr>
<td></td>
<td>2b Insufficient reagent volume applied</td>
<td>Check size of tissue section and reagent volume applied</td>
</tr>
<tr>
<td></td>
<td>2c Inappropriate wash buffer used</td>
<td>Use only Dako Wash Buffer, Code K8007</td>
</tr>
<tr>
<td></td>
<td>2d Reagents used when cold</td>
<td>Allow reagents to come to room temperature (20-25 °C) prior to staining</td>
</tr>
<tr>
<td><strong>3</strong> Weak staining of specimen slides or of the positive cell line on the Dako-provided Control Slide</td>
<td>3a Inadequate target retrieval</td>
<td>Verify that the 3-in-1 pre-treatment procedure was correctly performed</td>
</tr>
<tr>
<td></td>
<td>3b Inappropriate wash buffer used</td>
<td>Use only Dako Wash Buffer, Code K8007</td>
</tr>
<tr>
<td><strong>4</strong> Excessive background staining of slides</td>
<td>4a Paraffin incompletely removed</td>
<td>Verify that the 3-in-1 pre-treatment procedure was correctly performed</td>
</tr>
<tr>
<td></td>
<td>4b Slides dried while loading onto Autostainer Link 48</td>
<td>Ensure slides remain wet with buffer while loading and prior to initiating run</td>
</tr>
<tr>
<td></td>
<td>4c Non-specific binding of reagents to tissue section</td>
<td>Check for proper fixation of the specimen and/or the presence of necrosis</td>
</tr>
<tr>
<td></td>
<td>4d Inappropriate fixation method used</td>
<td>Ensure that only neutral buffered formalin fixative and approved fixation methods are used</td>
</tr>
<tr>
<td><strong>5</strong> Tissue detached from slides</td>
<td>5a Use of incorrect microscope slides</td>
<td>Use Dako FLEX IHC Microscope Slides (Code K8020), or Fisherbrand Superfrost Plus slides</td>
</tr>
<tr>
<td></td>
<td>5b Inadequate preparation of specimens</td>
<td>Cut sections should be placed in a 58 ± 2 °C oven for one hour prior to staining</td>
</tr>
<tr>
<td><strong>6</strong> Excessively strong specific staining</td>
<td>6a Inappropriate fixation method used</td>
<td>Ensure that only approved fixatives and fixation methods are used</td>
</tr>
<tr>
<td></td>
<td>6b Inappropriate wash buffer used</td>
<td>Use only Dako Wash Buffer, Code K8007</td>
</tr>
<tr>
<td></td>
<td>6c Temperature of staining reagents too high</td>
<td>It is recommended that reagents are equilibrated to 20-25 °C prior to staining</td>
</tr>
<tr>
<td><strong>7</strong> Target Retrieval Solution is cloudy in appearance when heated</td>
<td>7 When heated the Target Retrieval Solution turns cloudy in appearance</td>
<td>This is normal and does not influence staining</td>
</tr>
<tr>
<td><strong>8</strong> Artifacts in specimen giving a bubbling staining appearance</td>
<td>8 Inadequate slide dehydration procedure prior to slide mounting</td>
<td>Ensure adequate slide dehydration procedure was used. An example of dehydration procedure is given in this manual</td>
</tr>
</tbody>
</table>
Bibliography


- Moch H, Humphrey PA, Ulbright TM. WHO Classification of Tumours of the Urinary System and Male Genital Organs. IARC, Lyon, France; 2016.


- OPDIVO package insert.


References
