

PD-L1 IHC 28-8 pharmDx Interpretation Manual - Squamous Cell Carcinoma of the Head and Neck (SCCHN)

FDA-Approved for in vitro diagnostic use

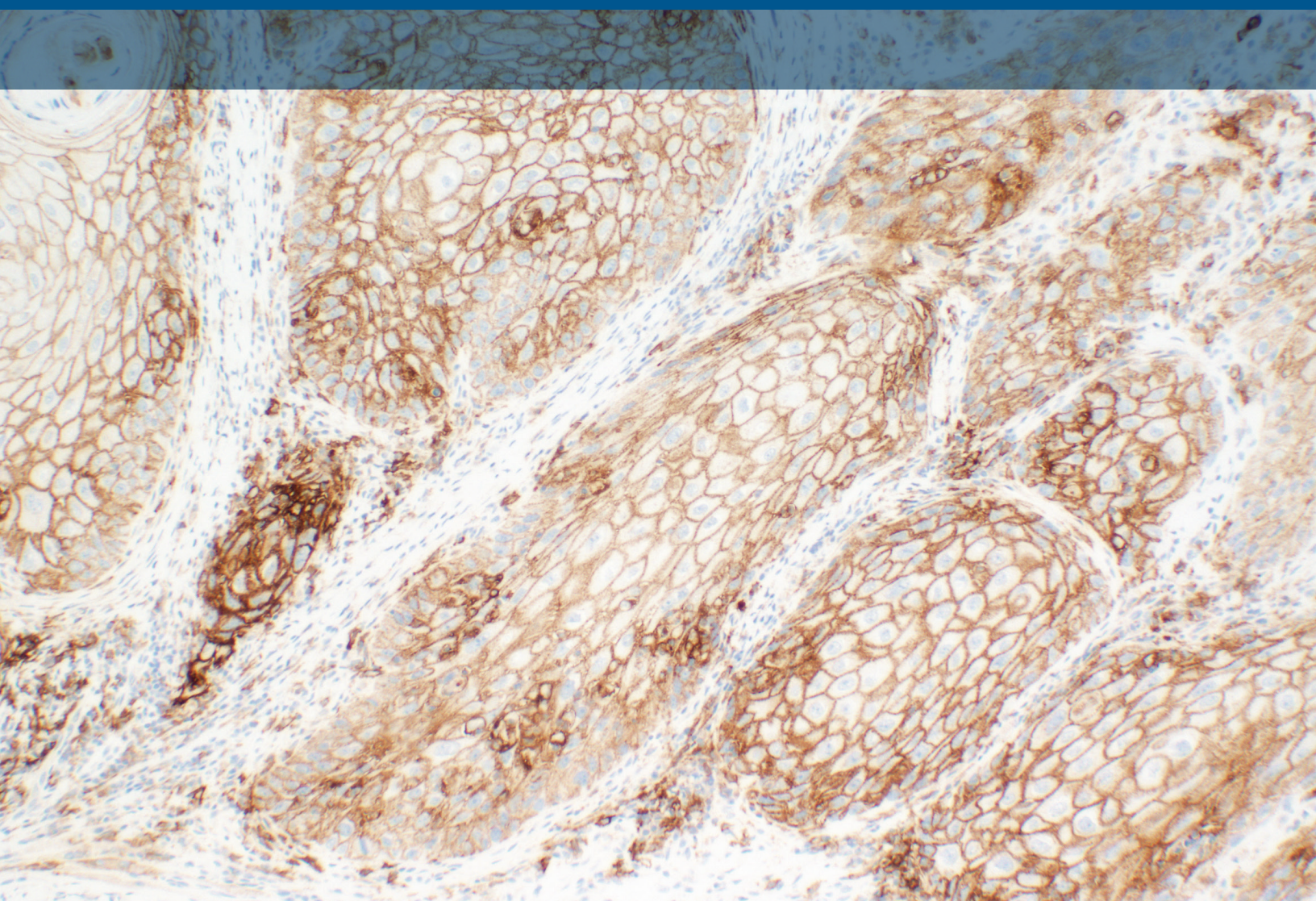


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Introduction

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) |



PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in SCCHN may be associated with enhanced survival from OPDIVO® (nivolumab).

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 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 SCCHN specimens. The goal of this manual is to familiarize you with the requirements for scoring SCCHN 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 SCCHN 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

Pictomicrographs

Note: Pictomicrographs included in this interpretation manual include specimens provided by the following suppliers:

- Tissue samples supplied by BioIVT (Hicksville, NY, USA).
- The data and SCCHN specimens used in this project were provided by Centre Hospitalier Universitaire (CHU) de Nice (Nice, France) with appropriate ethics approval and through Trans-Hit Biomarkers Inc.
- The data and SCCHN specimens used in this project were provided by US Biolab (Gaithersburg, MD, USA) with appropriate ethics approval and through Trans-Hit Biomarkers Inc.

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 26.

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 SCCHN

PD-L1 upregulation in SCCHN 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 trials (CheckMate-141) 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.

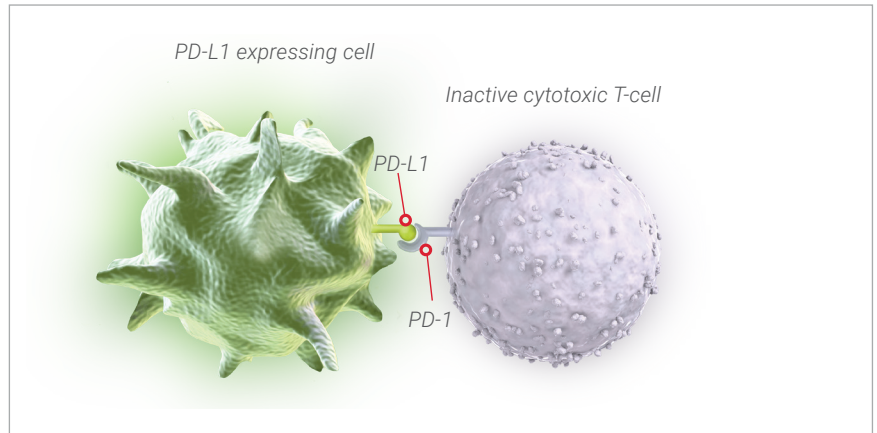


Figure a.

The tumor escapes detection

Inactivation of T-cells reduces tumor cell killing.

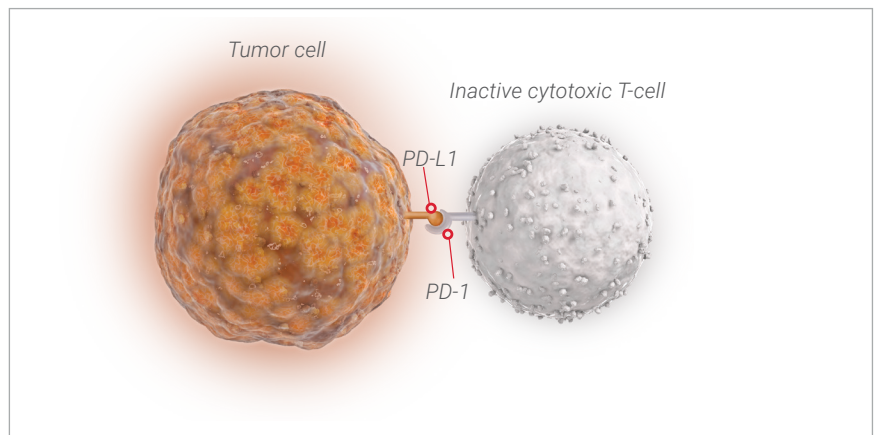


Figure b.

Immuno-oncology therapies harness the immune response to fight tumors

Blocking PD-1/PD-L1 enables cytotoxic T-cells to actively remove tumor cells.

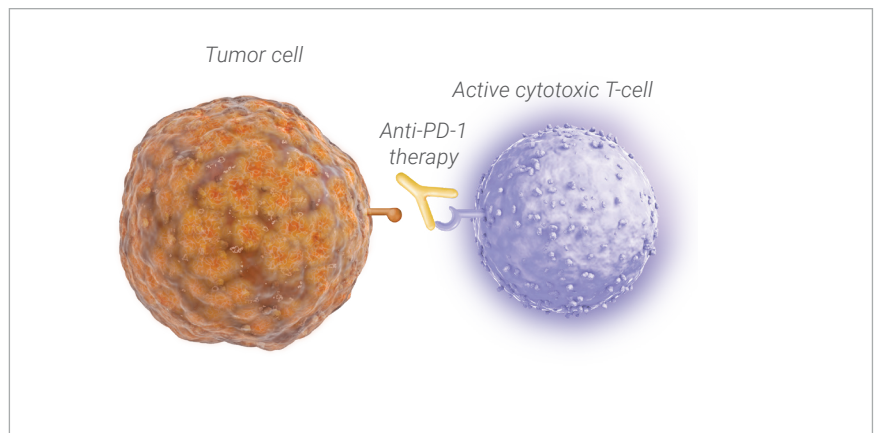


Figure c.

The Clinical Value of PD-L1 IHC 28-8 pharmDx Expression in Head and Neck Cancer

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

- The clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in CheckMate-141 to assess PD-L1 expression in SCCHN patients treated with OPDIVO (nivolumab) versus placebo.
- The study was a phase 3 trial demonstrated a statistically significant improvement in OS for subjects randomized to nivolumab as compared with investigator's choice at a pre-specified interim analysis (78% of the planned number of events for final analysis).
- A pre-specified exploratory sub-group analysis using PD-L1 IHC 28-8 pharmDx was performed. Results from the analysis are below.

The median OS was seven and a half months for nivolumab subjects compared to about five months for investigator's choice subjects with a hazard ratio of 0.70 (95% CI: 0.53, 0.92)*

Table 1. Summary of OS by PD-L1 IHC 28-8 pharmDx expression level and treatment group. Data from a pre-specified exploratory analysis (N=260) of CheckMate-141 (N=361).

| Tumor PD-L1 Expression | < 1% | | ≥ 1% | |
|------------------------|---------------------------|-----------------------|---------------------------|-----------------------|
| | Nivolumab | Investigator's Choice | Nivolumab | Investigator's Choice |
| Median OS | 5.7 months | 5.8 months | 8.7 months | 4.6 months |
| Hazard Ratios | 0.89 (95% CI: 0.54, 1.45) | | 0.55 (95% CI: 0.36, 0.83) | |

Abbreviations: CI = confidence interval

* PD-L1 IHC 28-8 pharmDx Instructions for Use.

Study Data for PD-L1 IHC 28-8 pharmDx in SCCHN Patients CheckMate-141

Detection of PD-L1 expressing tumor cells in SCCHN 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 CheckMate-141 to assess PD-L1 expression in SCCHN patients treated with OPDIVO (nivolumab).
- The study was a phase 3 clinical trial of OPDIVO (nivolumab) in subjects with metastatic or unresectable SCCHN who have progressed or recurred following treatment with a platinum agent.

A phase 3 single arm clinical trial with OPDIVO (nivolumab) was assessed in subjects with metastatic or unresectable SCCHN who have progressed or recurred following treatment with a platinum agent.

Primary study objective

To compare OS of nivolumab to investigator's choice in patients with metastatic or recurrent SCCHN who had experienced disease progression during or within six months of receiving platinum-based therapy administered in either the adjuvant, neo-adjuvant, primary (unresectable locally advanced) or metastatic setting.

Secondary study objectives

- To compare progression free survival (PFS) of nivolumab to investigator's choice.
- To compare objective response rate (ORR) of nivolumab to investigator's choice

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

- Head and neck carcinomas are sixth most common cancer globally, accounting for approximately 550,000 new cases and approximately 300,000 deaths each year. While only a small minority of patients present with metastatic disease initially (approximately 10%; Stage IV-C), approximately 50% of the population initially treated in a locally advanced setting will eventually develop recurrent or refractory disease.
- Recurrent or metastatic SCCHN remains an area of high unmet medical need, since patients who progress after platinum-refractory or resistant disease) have poor prognosis, with a median OS of approximately four to six months. There is no effective standard of care to provide survival benefits in platinum-refractory recurrent or metastatic SCCHN.
- Clinical utility of PD-L1 IHC 28-8 pharmDx in SCCHN was evaluated using specimens from patients enrolled in clinical trial CheckMate-141, a randomized Phase 3 clinical trial of OPDIVO (nivolumab) vs. therapy of investigator's choice in recurrent or metastatic platinum-refractory SCCHN.

The Clinical Value of PD-L1 IHC 28-8 pharmDx Expression in SCCHN Patients CheckMate-141

Table 2. Frequency of Tumor PD-L1 Expression in Quantifiable* Samples from SCCHN CheckMate-141.

| Tumor PD-L1 Expression | Nivolumab (N = 161) | Investigator's Choice (N = 99) | Total (N = 260) |
|--------------------------------|---------------------|--------------------------------|-----------------|
| ≥ 1% PD-L1 Expression Subjects | 88 (54.7%) | 61 (61.6%) | 149 (57.3%) |
| < 1% PD-L1 Expression Subjects | 73 (45.3%) | 38 (38.4%) | 111 (42.7%) |

Clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in CheckMate-141, a phase 3 single arm clinical trial of nivolumab, in which 240 subjects were randomized to receive the drug at 55 sites in 15 countries. Major efficacy outcome measures included confirmed objective response rate (ORR) and progression free survival (PFS).

Tumor specimens were collected from SCCHN 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 SCCHN Specimen Origin - CheckMate-141

| | | |
|------------------------------------|--------------------------------------|-----------------------------------|
| Primary Tumor 29.7% (97/327) | Metastatic Tumor 52% (170/270) | Not reported 18.3% (60/327) |
|------------------------------------|--------------------------------------|-----------------------------------|

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 validated staining runs.

PD-L1 IHC 28-8 pharmDx staining procedure

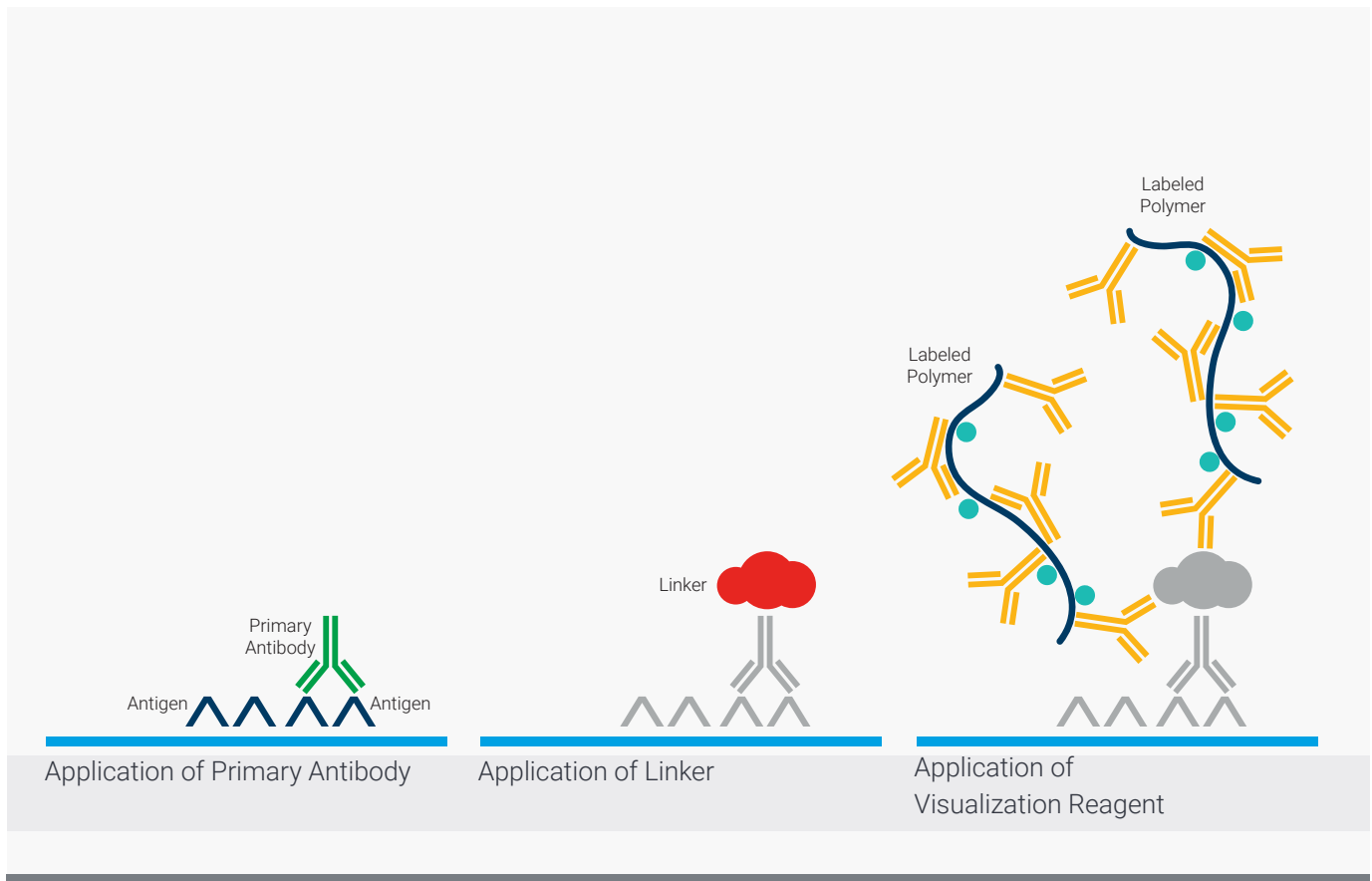


Figure 1a. PD-L1 IHC 28-8 pharmDx staining procedure.

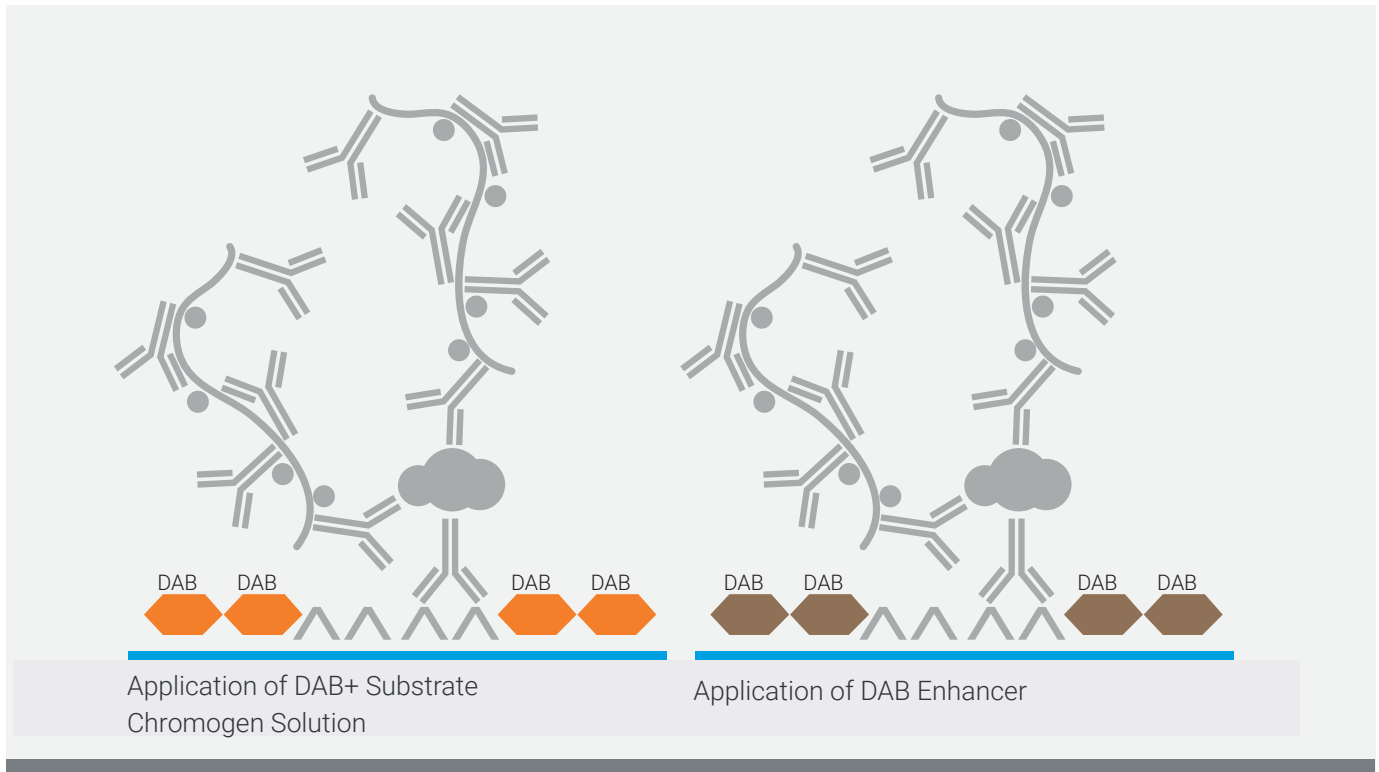


Figure 1b. PD-L1 IHC 28-8 pharmDx staining procedure.



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

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
- LINKER, Anti-Rabbit
- 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 generally are related to procedural deviations and can be controlled and minimized through training and 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.

Tissue Processing

FFPE tissues are suitable for use. Recommended handling and processing conditions are: < 30 minutes ischemia time prior to immersion in fixative, and 24–48 hours fixation time in 10% neutral buffered formalin. Alternative fixatives have not been validated and may give erroneous results. Specimens should be blocked into a thickness of 3 or 4 mm, fixed in 10% neutral buffered formalin, 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. The use of PD-L1 IHC 28-8 pharmDx on decalcified tissues 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 then 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.

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 must represent one of the approved tumor indications for PD-L1 IHC 28-8 pharmDx as listed in the Intended Use of the IFU. 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 gives weak to moderate positive staining. The variety of different cell types present in most tissue sections offers internal negative control sites; this should be verified by the user.

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 1x EnVision FLEX Target Retrieval Solution, Low pH by diluting EnVision FLEX Target Retrieval Solution, Low pH (50x) 1:50 using distilled or deionized water; the pH of 1x EnVision FLEX Target Retrieval Solution must be 6.1 ± 0.2 . Do not modify the pH of 1x EnVision FLEX Target Retrieval Solution after preparation under any circumstance. If a problem is suspected with the EnVision FLEX Target Retrieval Solution pH, please refer to the troubleshooting section for more information. One 30 mL bottle of EnVision FLEX Target Retrieval Solution, Low pH (50x), diluted 1:50 will provide 1.5 L of 1x reagent, sufficient to fill one PT Link tank, which will treat up to 24 slides per use. Discard 1x EnVision FLEX Target Retrieval Solution after three uses and do not use after 5 days following 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 EnVision FLEX Wash Buffer for the wash steps by diluting Wash Buffer (20x) 1:20 using distilled or deionized water and mix thoroughly. 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 1 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 in this section. 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, 1x working solution to cover the tissue sections.
- Pre-heat the EnVision FLEX Target Retrieval Solution, Low pH to 65 °C.
- Immerse Autostainer racks containing mounted, FFPE tissue sections into the pre-heated EnVision FLEX Target Retrieval Solution, Low pH (1x working solution) in PT Link tank. Incubate for 20 minutes at 97 °C.
- As soon as Target Retrieval incubation has been completed and the temperature has cooled to 65 °C, remove each Autostainer slide rack with slides from the PT Link tank and immediately place the slides into a tank (e.g., PT Link Rinse Station, Code PT109) containing room temperature EnVision FLEX Wash Buffer working solution.
- Immerse 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 nonspecific 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? | <input type="checkbox"/> | <input type="checkbox"/> |
| 2. PD-L1 IHC 28-8 pharmDx is used before the expiration date printed on the outside of the box? | <input type="checkbox"/> | <input type="checkbox"/> |
| 3. All PD-L1 IHC 28-8 pharmDx components, including Control Slides, are stored in the dark at 2–8 °C? | <input type="checkbox"/> | <input type="checkbox"/> |
| 4. All PD-L1 IHC 28-8 pharmDx components, including Control Slides, are equilibrated to room temperature (20–25 °C) prior to immunostaining? | <input type="checkbox"/> | <input type="checkbox"/> |
| 5. Appropriate positive and negative control tissues are identified? | <input type="checkbox"/> | <input type="checkbox"/> |
| 6. Tissues are fixed in neutral buffered formalin? | <input type="checkbox"/> | <input type="checkbox"/> |
| 7. Tissues are infiltrated with melted paraffin, at or below 60 °C? | <input type="checkbox"/> | <input type="checkbox"/> |
| 8. Tissue sections of 4–5 µm are mounted on FLEX IHC Microscope Slides, or Superfrost Plus charged slides? | <input type="checkbox"/> | <input type="checkbox"/> |
| 9. SCCHN 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? | <input type="checkbox"/> | <input type="checkbox"/> |
| 10. EnVision FLEX Target Retrieval Solution, Low pH is prepared properly (working solution pH 6.1±0.2)? | <input type="checkbox"/> | <input type="checkbox"/> |
| 11. EnVision FLEX Wash Buffer is prepared properly? | <input type="checkbox"/> | <input type="checkbox"/> |
| 12. DAB+ Substrate-Chromogen Solution is prepared properly? | <input type="checkbox"/> | <input type="checkbox"/> |
| 13. The Deparaffinization, Rehydration, and Target Retrieval 3-in-1 procedure is followed, using PT Link? | <input type="checkbox"/> | <input type="checkbox"/> |
| 14. Slides remain wet with buffer while loading and prior to initiating run on Autostainer Link 48? | <input type="checkbox"/> | <input type="checkbox"/> |
| 15. The PD-L1 IHC 28-8 pharmDx protocol is selected on Autostainer Link 48? | <input type="checkbox"/> | <input type="checkbox"/> |
| 16. Slides are counterstained with EnVision FLEX Hematoxylin? | <input type="checkbox"/> | <input type="checkbox"/> |
| 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. | <input type="checkbox"/> | <input type="checkbox"/> |

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 SCCHN

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 SCCHN 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 26 for an example of a reporting results form for PD-L1 IHC 28-8 pharmDx.

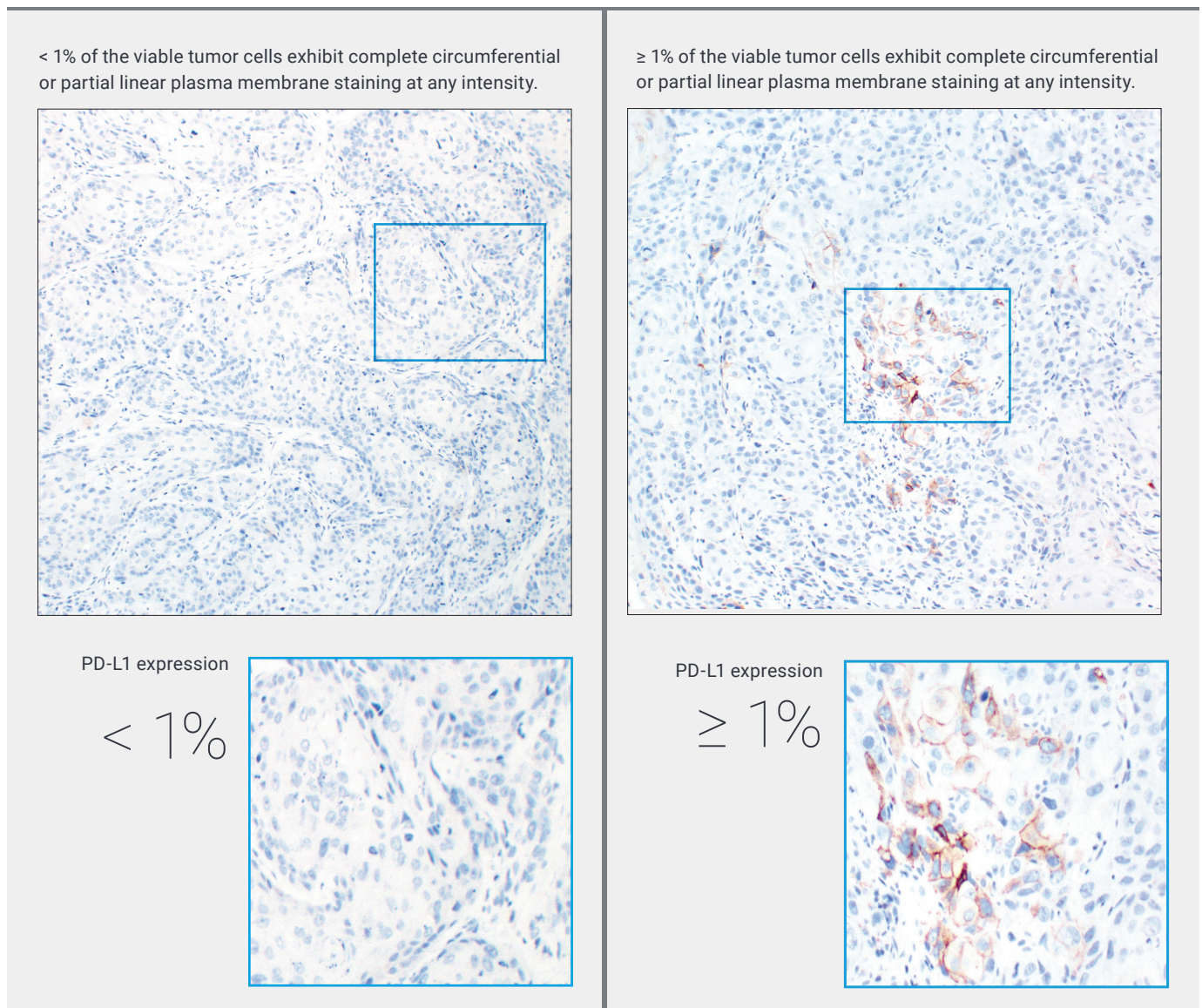


Figure 3. Guidelines for scoring and reporting PD-L1 IHC pharmDx results.

Slide Evaluation Flowchart

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 20–25.

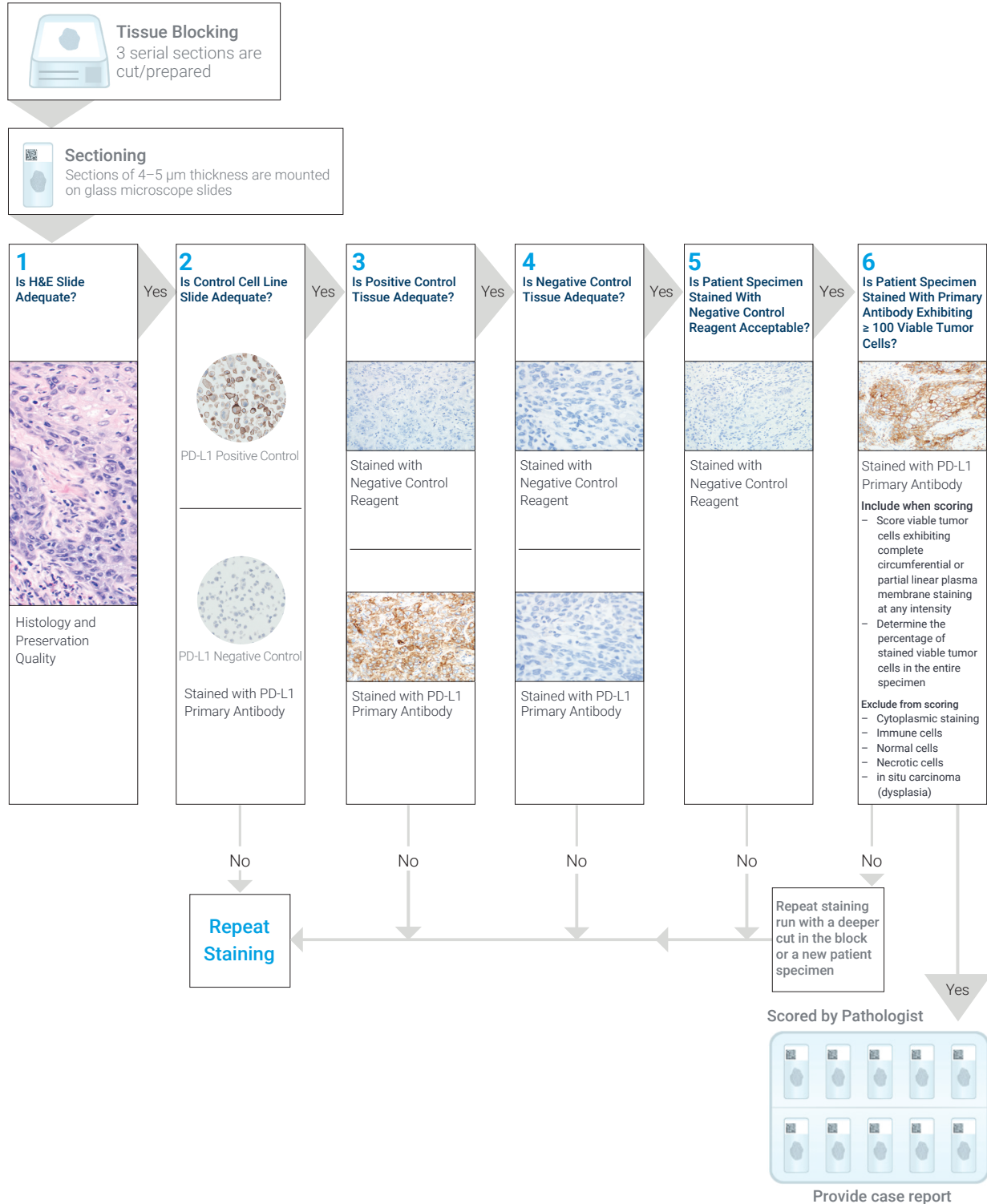


Figure 4. Slide evaluation procedure steps.

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

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 % tumor cell expression.

Positive control tissue slides and negative control tissue slides should be supplied by the laboratory. Only the PD-L1 IHC 28-8 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 5. 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.



PD-L1 IHC

Assess the percentage of cells with plasma membrane staining and the staining intensity. Evaluate the overall staining intensity using the following guide:

| | |
|----|-------------------|
| 0 | No staining |
| 1+ | Weak staining |
| 2+ | Moderate staining |
| 3+ | Strong staining |



PD-L1 positive

PD-L1 negative

Figure 5. 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 6:

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

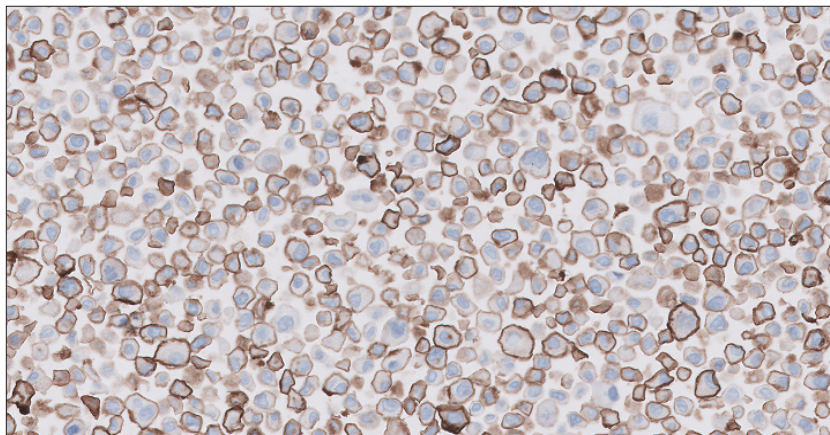


Figure 6. Acceptable Positive PD-L1 Control.

For the PD-L1 negative cell pellet on the Control Slide, the following staining is acceptable, see Figure 7:

- No plasma membrane staining
- Any background staining is of less than 1+ staining intensity

Staining of a few cells in the negative pellet may occasionally be observed. The presence of 10 or less cells with distinct plasma membrane staining, or cytoplasmic staining with $\geq 1+$ intensity within the boundaries of the cell pellet are acceptable.

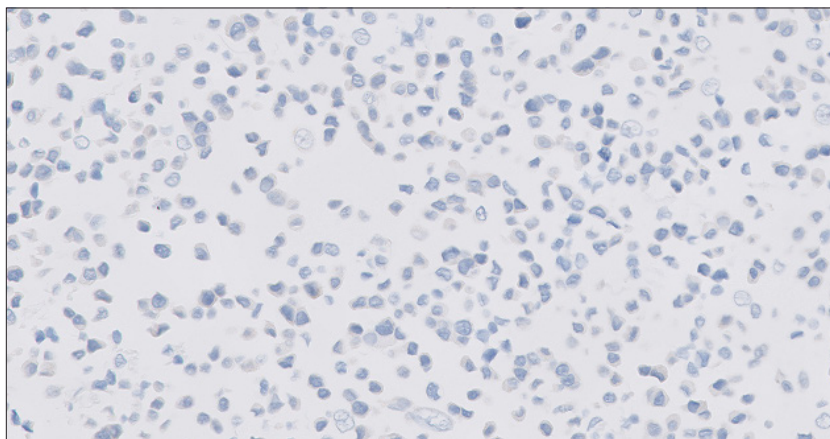


Figure 7. Acceptable Negative PD-L1 Control.

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 $\leq 1+$ 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 $\leq 1+$ 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 $\leq 1+$ 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 % 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 % 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

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 % 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 8). 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 (blue circle in Figure 8).
- 50 out of 1000 viable tumor cells are staining PD-L1 positive in the entire tumor specimen (Method 1) which may also be described as 50% PD-L1 positive in a single region representing 10% of the tumor area (Method 2).

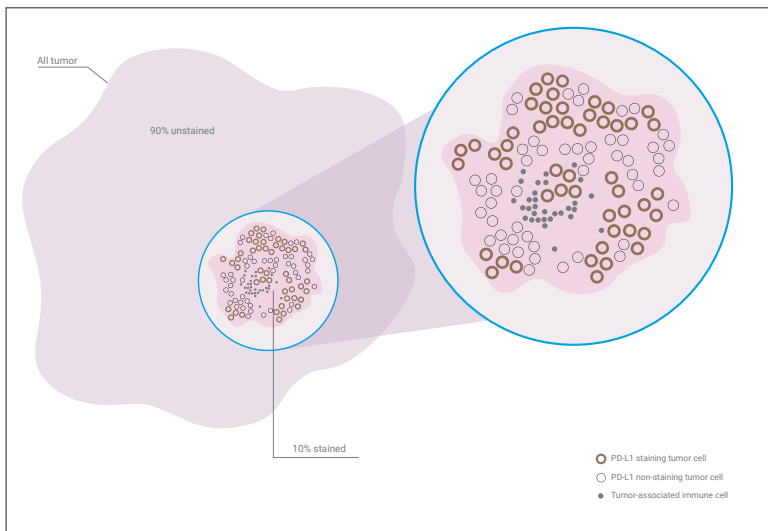


Figure 8. Example of a tumor with a small PD-L1 staining area.

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

Method 1

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

Method 2

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

Example 2: Calculating PD-L1 % 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 9.

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%.

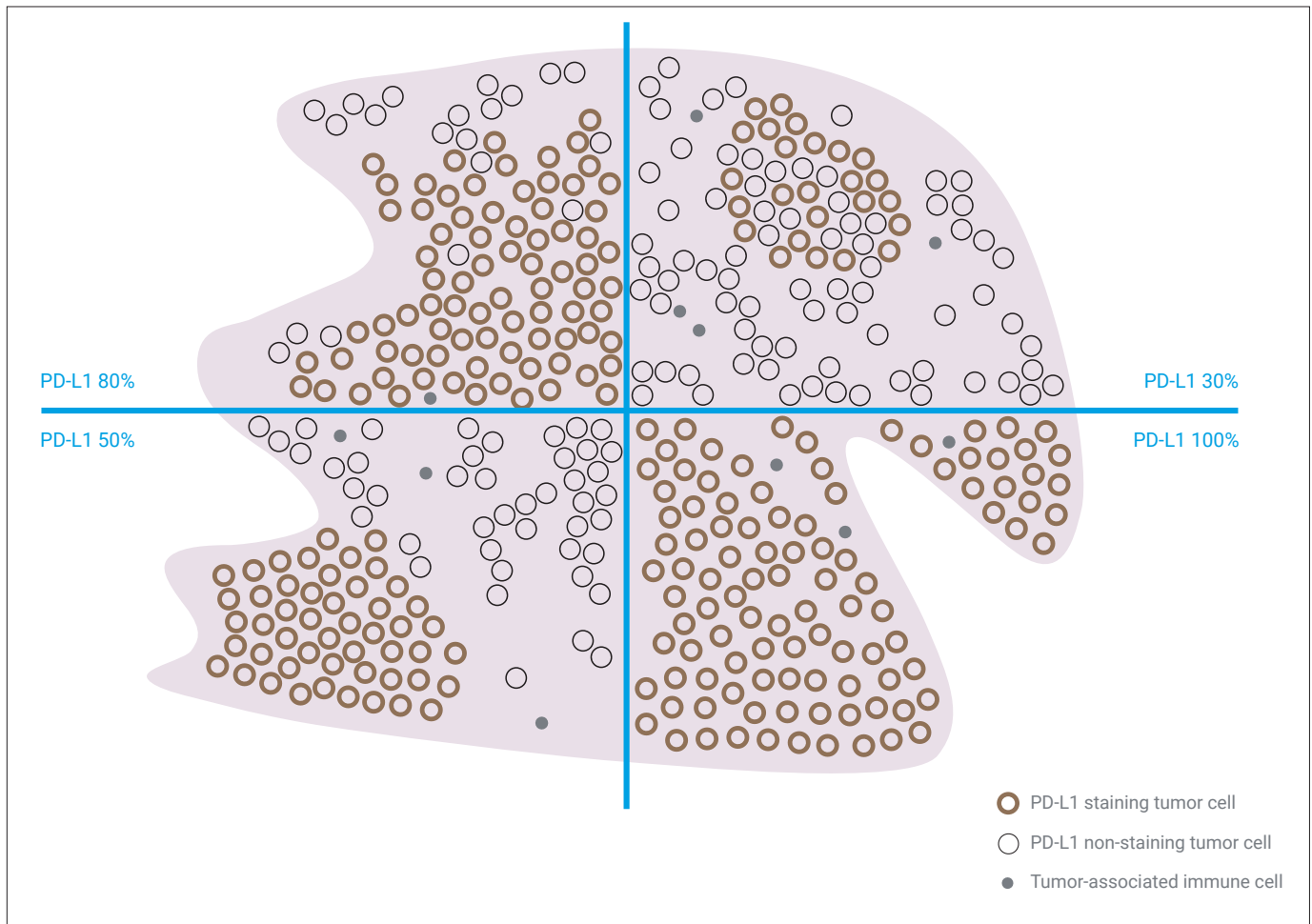


Figure 9. Example with heterogenous PD-L1 staining area.

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 SCCHN

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 pharmDx 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 SCCHN patient specimens may indicate an enhanced survival rate benefit to OPDIVO (nivolumab) treatment for the patient.

Viable Tumor Cells Present: ≥ 100 cells Not evaluable

PD-L1 % tumor cell expression is $< 1\%$

Percent of SCCHN cells with complete circumferential and/or partial linear membrane PD-L1 staining is $< 1\%$.

PD-L1 % tumor cell expression is $\geq 1\%$

Percent of SCCHN cells with complete circumferential and/or partial linear membrane PD-L1 staining is $\geq 1\%$.

Other Comments to Treating Physician: _____

Note: PD-L1 IHC 28-8 pharmDx was validated for invasive SCCHN 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.

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PD-L1 IHC 28-8 pharmDx

Immunostaining Examples in SCCHN

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

An example of squamous cell carcinoma of the tonsil stained with PD-L1 IHC 28-8 pharmDx assay. The staining shows a range of PD-L1 expression. This specimen would be appropriate to use 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.

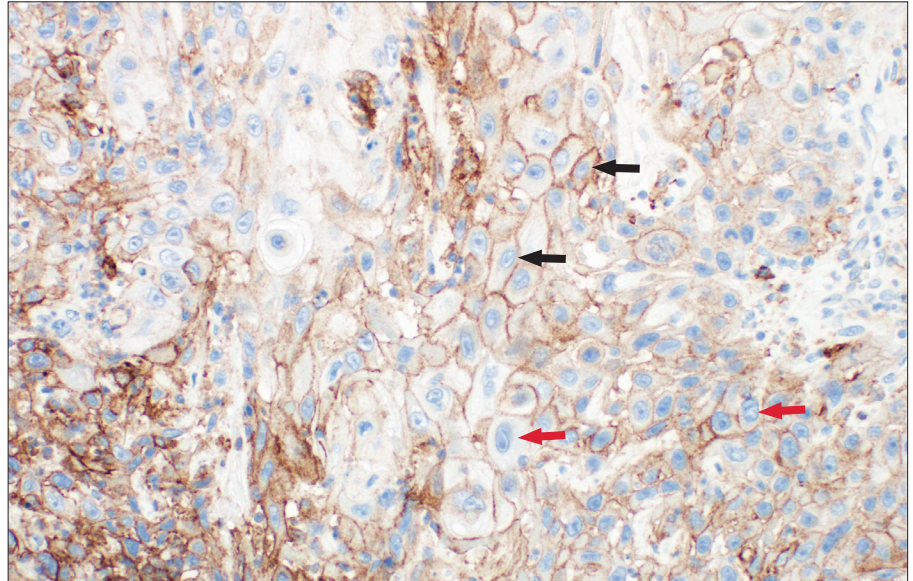


Figure 10. 20x magnification.

Squamous cell carcinoma of the larynx
PD-L1 % tumor cell expression < 1%.

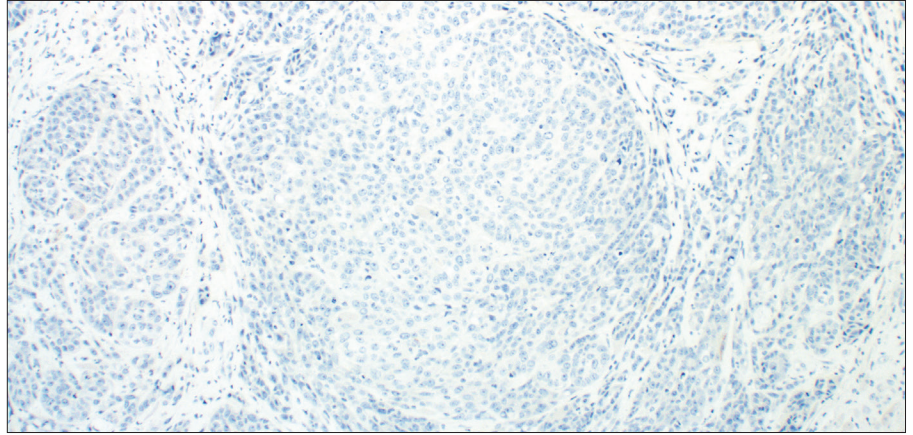


Figure 11. 10x magnification.

Squamous cell carcinoma of the larynx
PD-L1 % tumor cell expression \geq 1%.

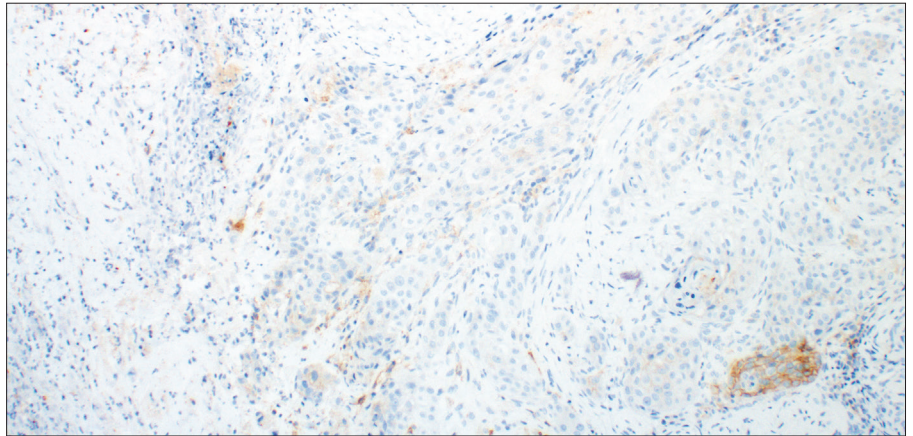


Figure 12. 10x magnification.

Squamous cell carcinoma of the tonsil demonstrating $\geq 1\%$, moderate PD-L1 % tumor cell expression.

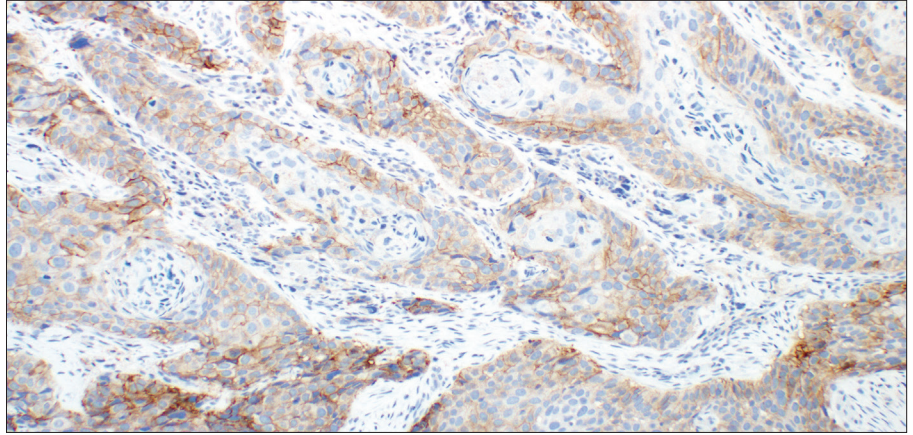


Figure 13. 10x magnification.

Squamous cell carcinoma of the tonsil demonstrating $\geq 1\%$, high PD-L1 % tumor cell expression.

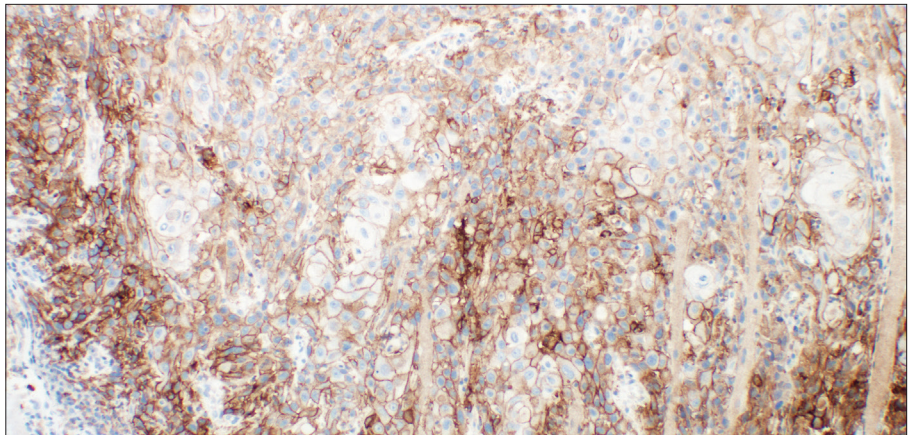


Figure 14. 10x magnification.

Squamous cell carcinoma of the pharynx showing strong staining of intra-tumoral immune cells (**black arrow**), while the tumor cells are negative (**red arrow**) for PD-L1.

Note the staining of intra-tumoral mononuclear inflammatory cells (histiocytes and lymphocytes) are not included in determining the PD-L1 % tumor cell expression.

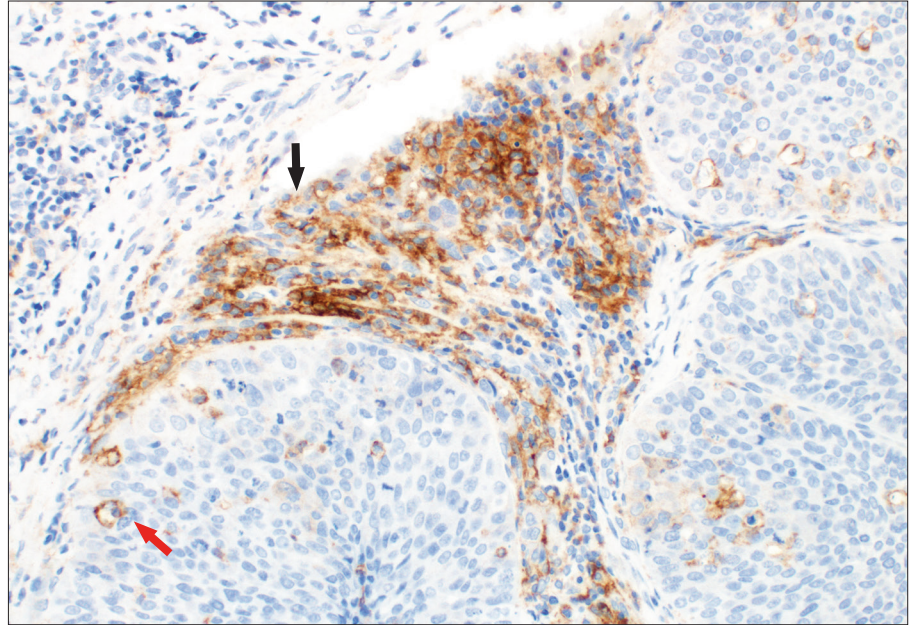


Figure 15. 20x magnification.

Squamous cell carcinoma of the tongue showing PD-L1 staining of immune cells and positive staining within tumor.

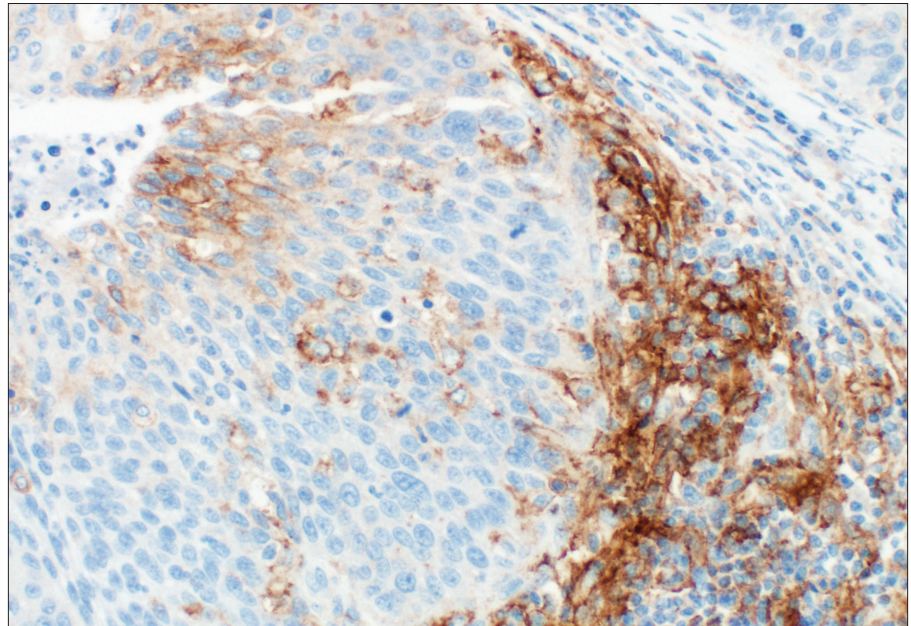


Figure 16. 20x magnification.

H&E stain case of SCCHN of the tongue showing in situ component.

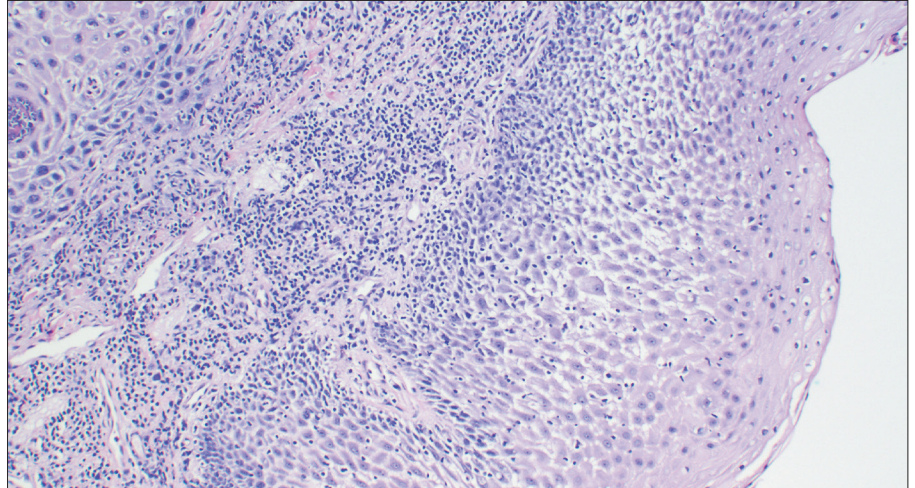


Figure 17a. 20x magnification.

Squamous cell carcinoma of the tongue showing positive staining for PD-L1 in the invasive squamous cell carcinoma (**black arrow**). Note that in this case, there is staining in the normal squamous epithelium (**red arrow**). The staining in the normal squamous epithelium is not included in calculating percent positive staining of the tumor for PD-L1.

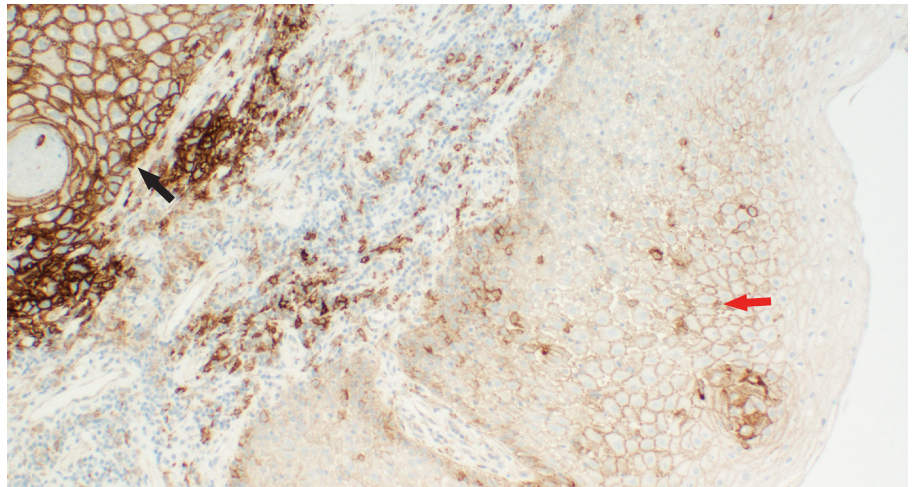


Figure 17b. 20x magnification.

H&E stain of SCCHN of the larynx showing normal squamous cell carcinoma.

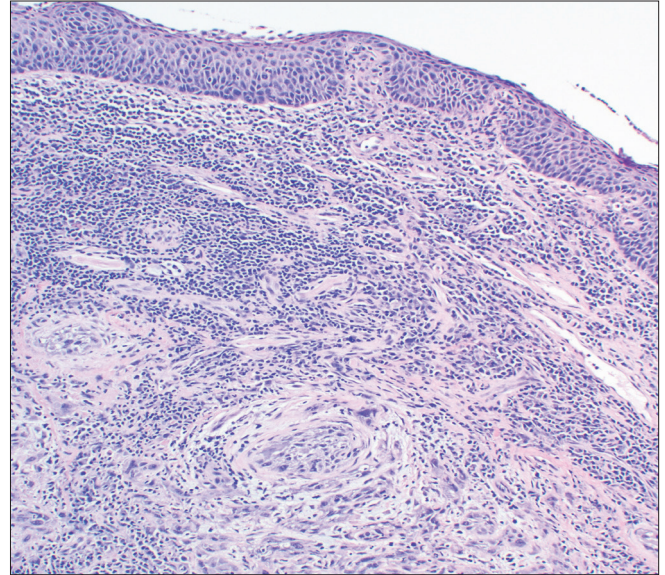


Figure 18a. 20x magnification.

Squamous cell carcinoma of the larynx showing normal plasma membrane (**black arrow**) staining for PD-L1. When determining the PD-L1 % tumor cell expression for the specimen stained normal urothelium, dysplasia, and in situ carcinoma components (**red arrow**) are not included in the denominator. Only the invasive component is evaluated when determining % tumor cell expression.



Figure 18b. 20x magnification.

Artifacts

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 patient specimen stained with NCR is useful in determining the level of non-specific staining in the same patient tissue specimen stained with PD-L1. All specimens must have $\leq 1+$ non-specific staining.

Immune cells

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

Necrosis

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

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

Squamous cell carcinoma of the tongue.
Tumor cells with granular staining in the cytoplasm and no linear membrane staining should not be included in scoring.

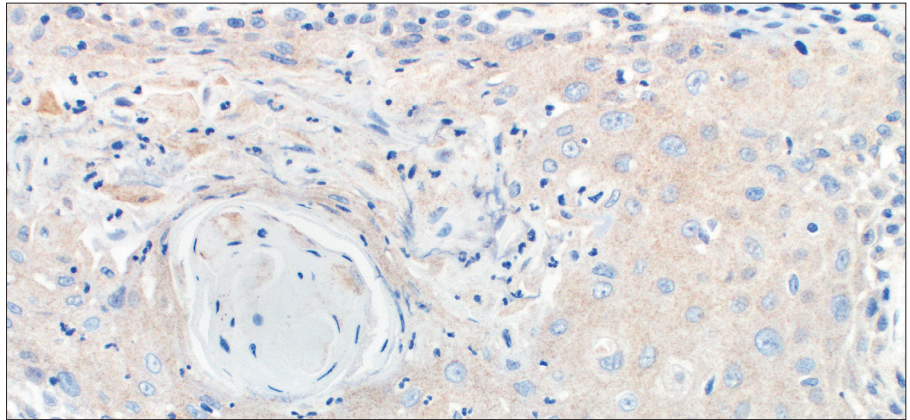


Figure 19. 20x magnification.

Squamous cell carcinoma of the tongue.
Cells with linear membrane staining that is distinguishable from cytoplasmic staining are included in scoring.

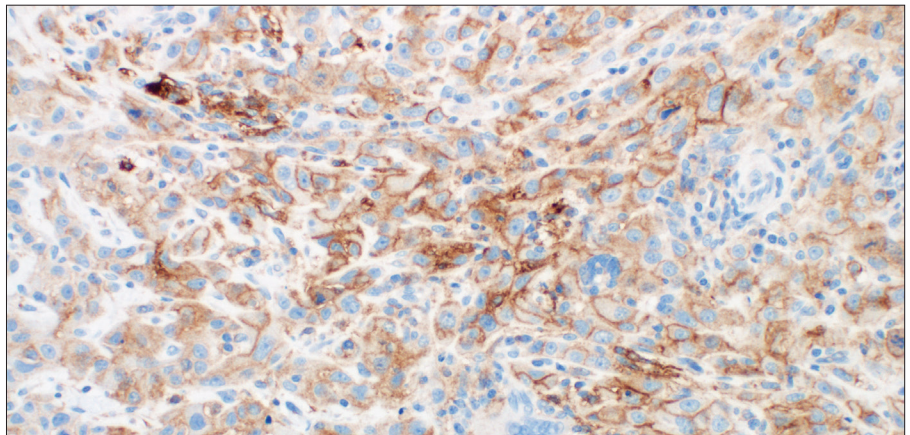


Figure 20. 20x magnification.

Squamous cell carcinoma of the tongue.
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**).

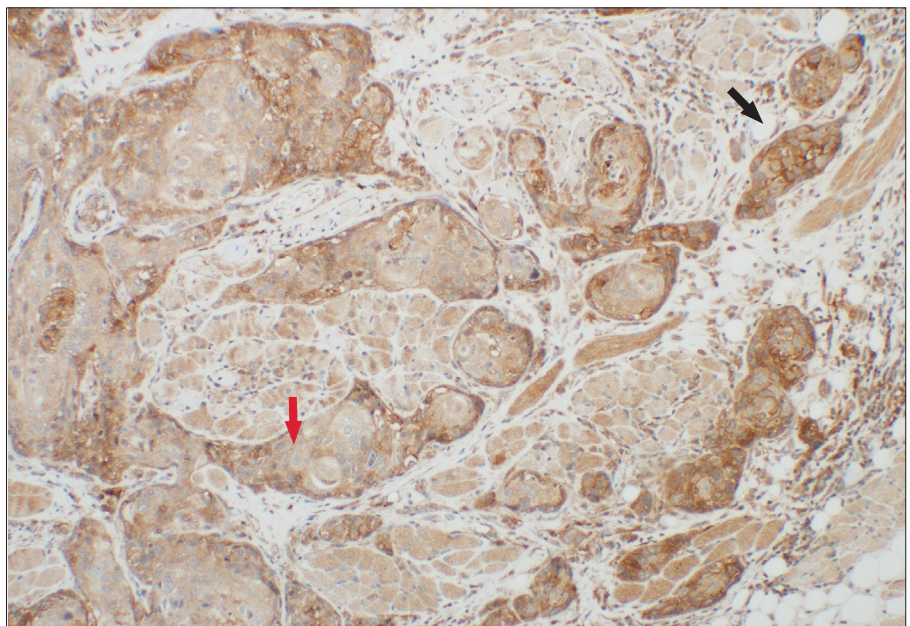


Figure 21. 40x magnification.

Squamous cell carcinoma of the tongue.
 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.

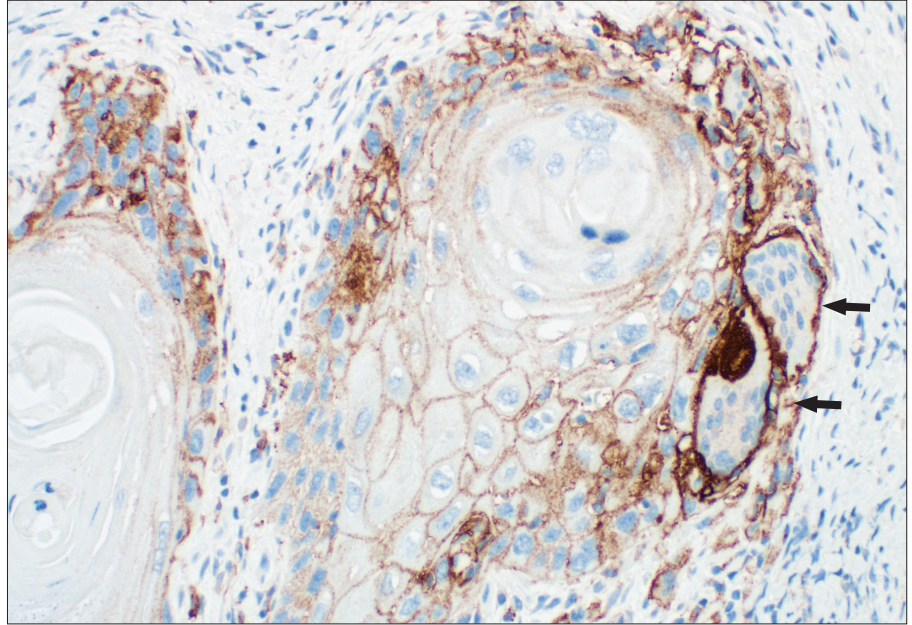


Figure 22. 20x magnification.

Squamous cell carcinoma of the larynx.
 Necrotic tissue may show non-specific staining and should not be included in scoring % 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).

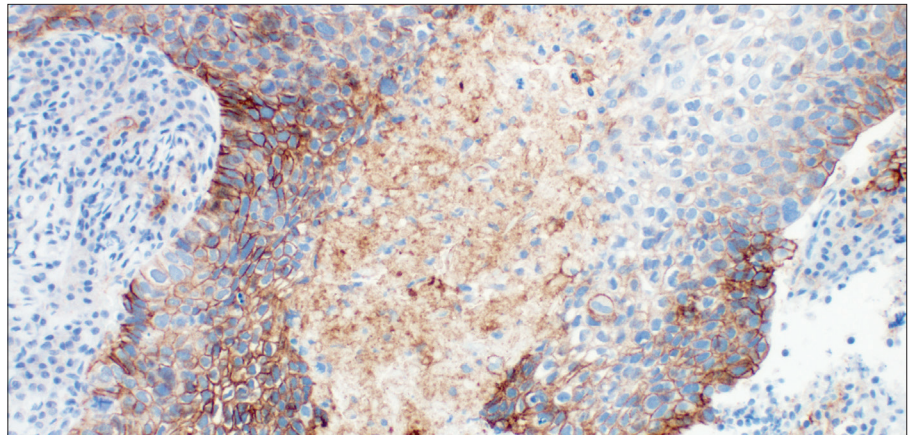


Figure 23. 20x magnification.

Squamous cell carcinoma of the larynx. When scoring for percent positivity, percentage is determined by the number of positively stained cells and not area. Note in this example, the absence of staining in tightly packaged basaloid cells which take up less area than an equal number of well differentiated positively-stained cells. A majority of the cells looks positive by surface area, however when enumerating cells, only 30% of the tumor cells are positive.

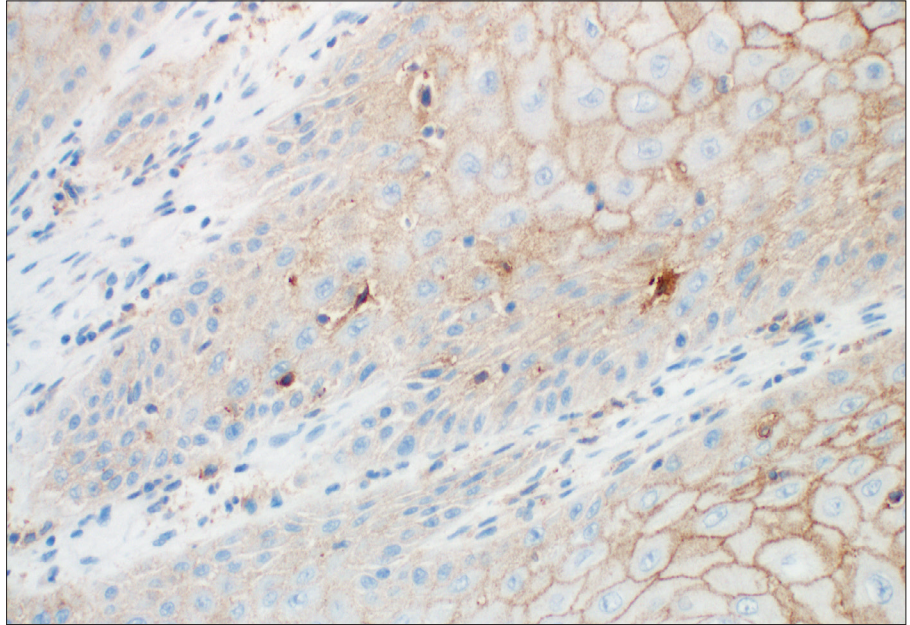


Figure 24. 20x magnification.

Squamous cell carcinoma of the hypopharynx. In this example, the tightly packed basaloid cells are staining positive for PD-L1, while the well-differentiated squamous cells are not staining for PD-L1. If determining the percent positive tumor staining based on the surface area that is staining then the score would be recorded incorrectly as 20% in this example. However, correct implementation of the scoring algorithm, by quantifying the number of positively staining tumor cells out of the total number of viable tumor cells, will result in a more accurate score of 60% positive PD-L1 staining.

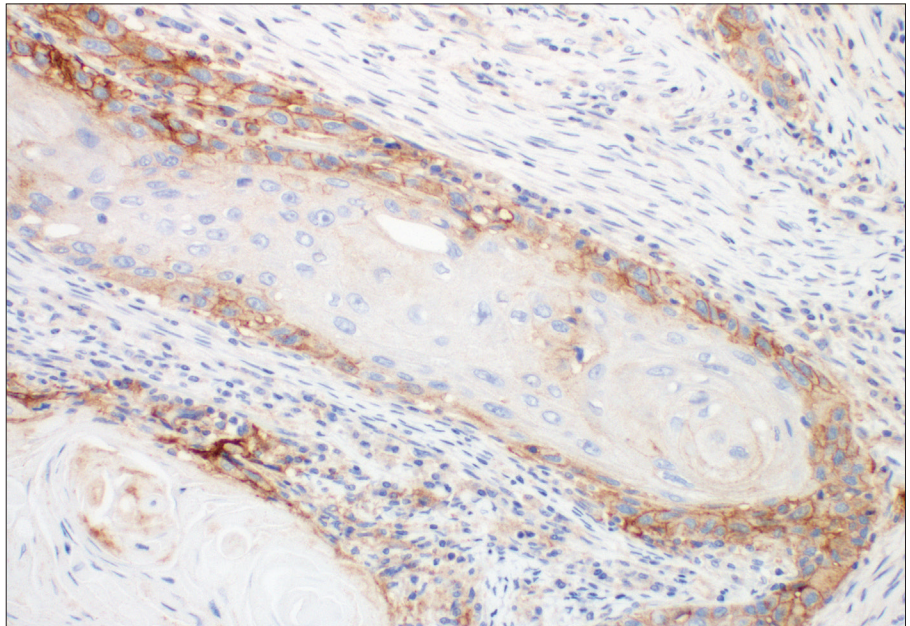


Figure 25. 20x magnification.

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 |

Note: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please 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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