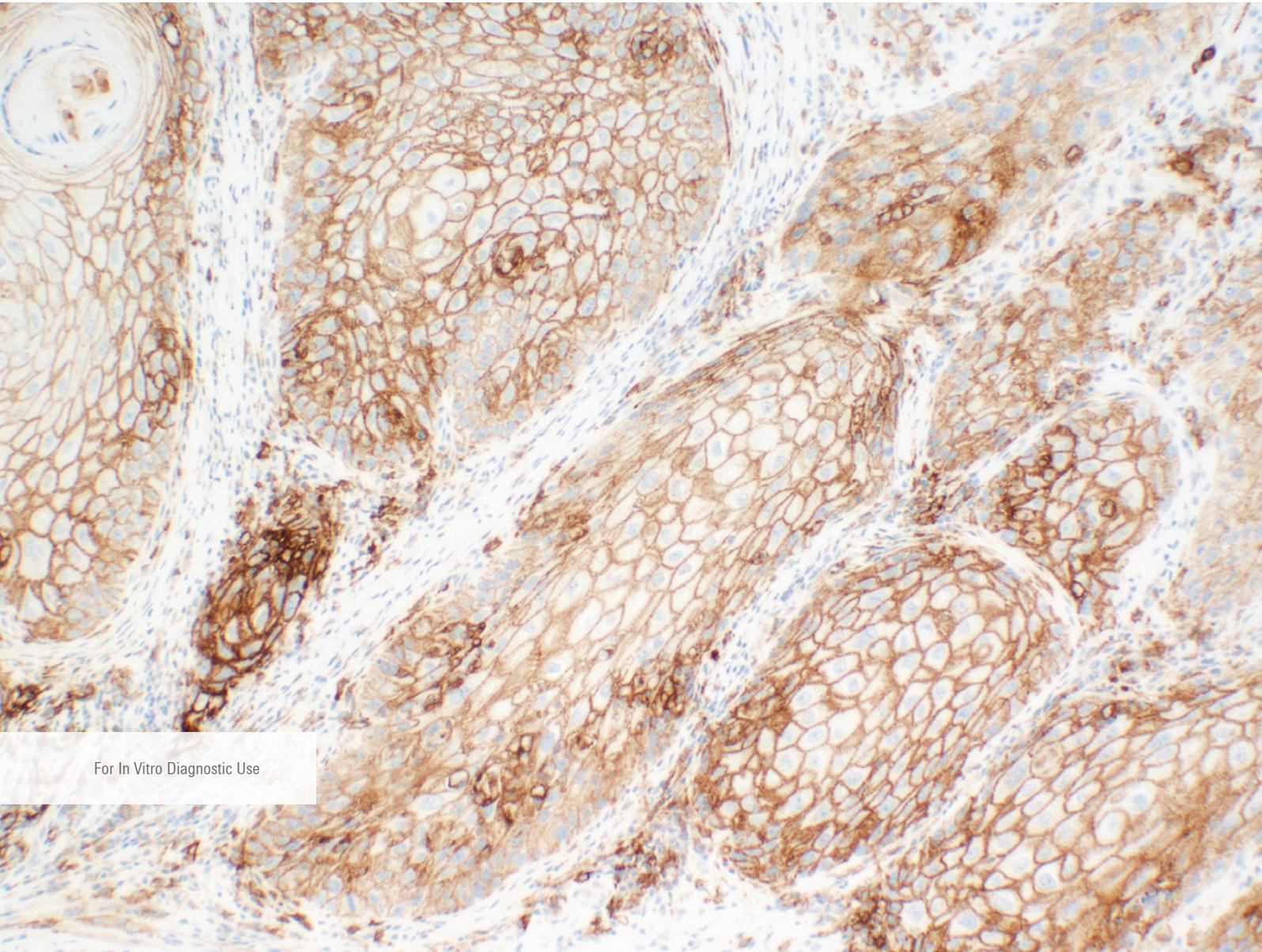


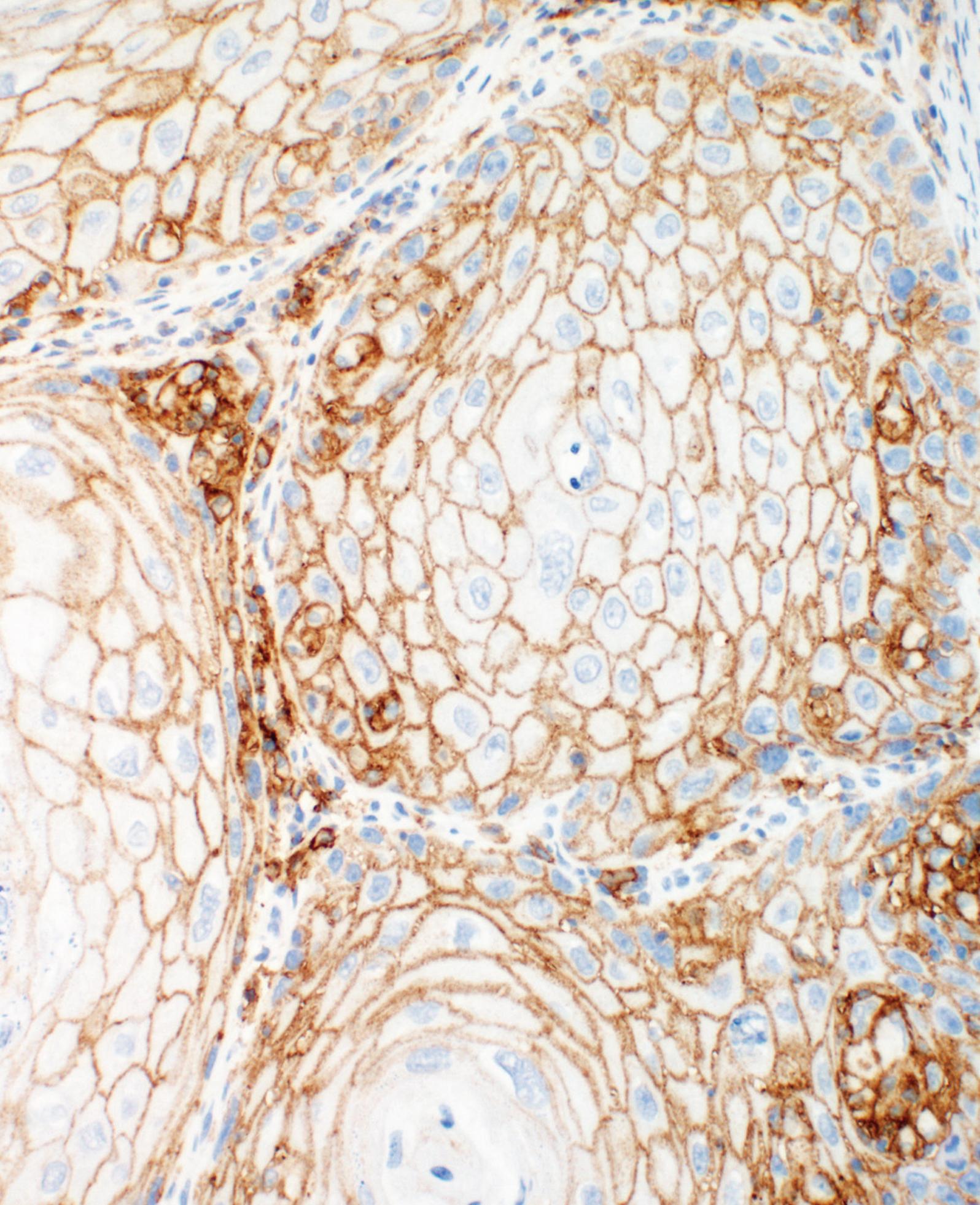
Squamous Cell Carcinoma of the Head and Neck (SCCHN)



For In Vitro Diagnostic Use

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Introduction

Intended Use in Squamous Cell Carcinoma of the Head and Neck

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), 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).

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 melanoma may be used as an aid in the assessment of patients for whom OPDIVO (nivolumab) and YERVOY® (ipilimumab) combination treatment is being considered.

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 SCCHN specimens stained with PD-L1 IHC 28-8 pharmDx. Photomicrographs of example cases are provided for reference.

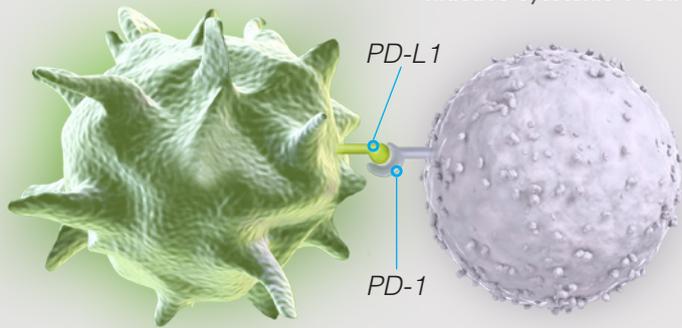
PD-L1 IHC 28-8 pharmDx IFU contains guidelines and technical tips for ensuring high-quality staining in your laboratory.

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 provided or visit www.agilent.com.

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

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

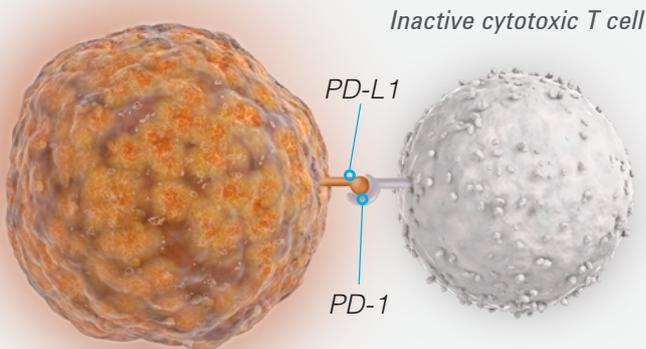
PD-L1 expressing cell



Limiting damage to healthy tissue

Inactivation of T cells limits damage to healthy tissue.

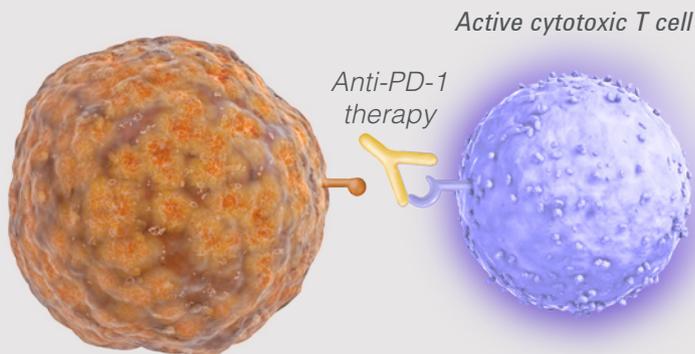
Tumor cell



The tumor escapes detection

Inactivation of T cells reduces tumor cell killing.

Tumor cell



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 Head and Neck Cancer

Detection of PD-L1 expressing tumor cells in squamous cell carcinoma of head and neck (SCCHN) patient specimens may indicate an enhanced survival benefit to OPDIVO (nivolumab) treatment for the patient.

- Clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in CA209141, an open label, randomized Phase 3 clinical trial of nivolumab vs. therapy of investigator's choice in recurrent or metastatic platinum-refractory squamous cell carcinoma of the head and neck (SCCHN).
- The study achieved its primary objective, demonstrating superior Overall Survival (OS) with OPDIVO (nivolumab) versus investigator's choice therapy (cetuximab, methotrexate, or docetaxel).

A pre-specified exploratory sub-group analysis using PD-L1 IHC 28-8 pharmDx was performed. Results from the analysis are below.

The Phase 3 CA209141 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). The median OS was 7.5 months for nivolumab subjects compared to 5.1 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 CA209141 (N=361).

PD-L1 Expression	<1%		≥1%	
	Nivolumab	Investigator's Choice	Nivolumab	Investigator's Choice
Median OS (95% CI)	5.7 mos. (4.4-12.7)	5.8 mos. (4.0-9.8)	8.7 mos. (5.7-9.1)	4.6 mos. (3.8-5.8)
Hazard Ratios	0.89 (95% CI: 0.54, 1.45)		0.55 (95% CI: 0.36, 0.83)	

Abbreviations: CI = confidence interval

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

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

Head and neck carcinomas are the 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 (~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-based treatment (platinum-refractory or resistant disease) have poor prognosis, with a median OS of approximately 4 to 6 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 CA209141, a randomized Phase 3 clinical trial of OPDIVO (nivolumab) vs. therapy of investigator's choice in recurrent or metastatic platinum-refractory squamous cell carcinoma of the head and neck (SCCHN).

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 6 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.

Baseline (pre-study) tumor tissue specimens were collected prior to randomization.

Frequencies of PD-L1 expression at each of the predefined baseline expression levels in all randomized subjects in CA209141 are presented in Table 2.

Table 2: Frequency of PD-L1 Expression in Quantifiable* Samples from SCCHN - CA209141

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%)

*327 of 361 samples were PD-L1 quantifiable from study CA209141.

361 patients were randomized at 55 sites in 15 countries to one of two treatment arms (240 nivolumab vs. 121 to investigator's choice) and stratified according to prior cetuximab treatment (yes/no). The major efficacy outcome measure for CA209141 was Overall Survival (OS). Additional efficacy outcome measures included Progression Free Survival (PFS) and Objective Response Rate (ORR).

Table 3: Baseline SCCHN Specimen Origin - Study CA209141^[1]

Tumor specimens were collected from SCCHN tumors from either a primary or metastatic site, consistent with the inclusion requirements for the study. 327 subjects (out of 361 total subjects) had tumor tissue collected at baseline with the following site proportion:

Primary Tumor	Metastatic Tumor	Not reported
29.7% (97/327)	52.0% (170/327)	18.3% (60/327)

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.

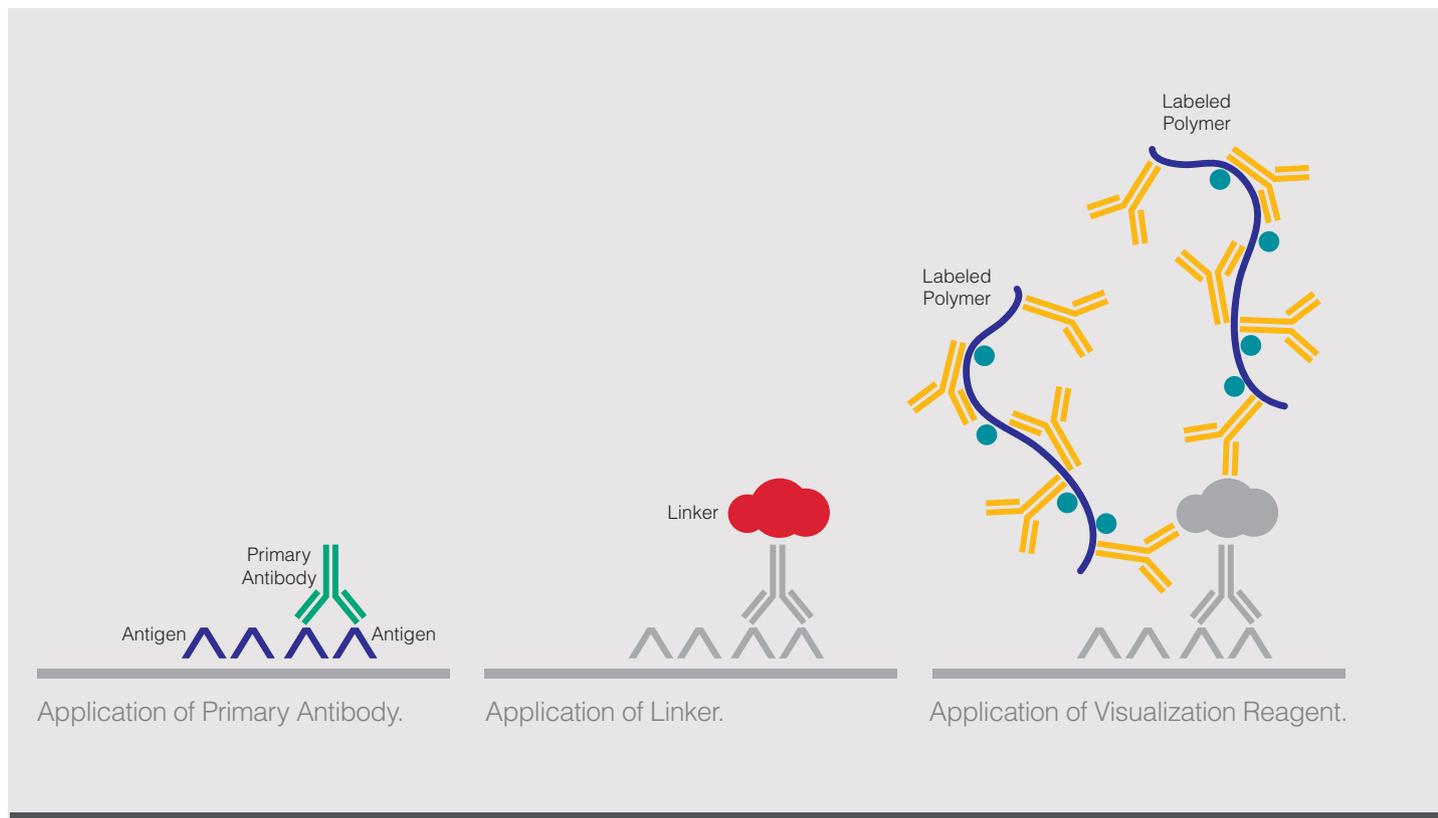


Figure 1: PD-L1 IHC 28-8 pharmDx staining procedure.

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

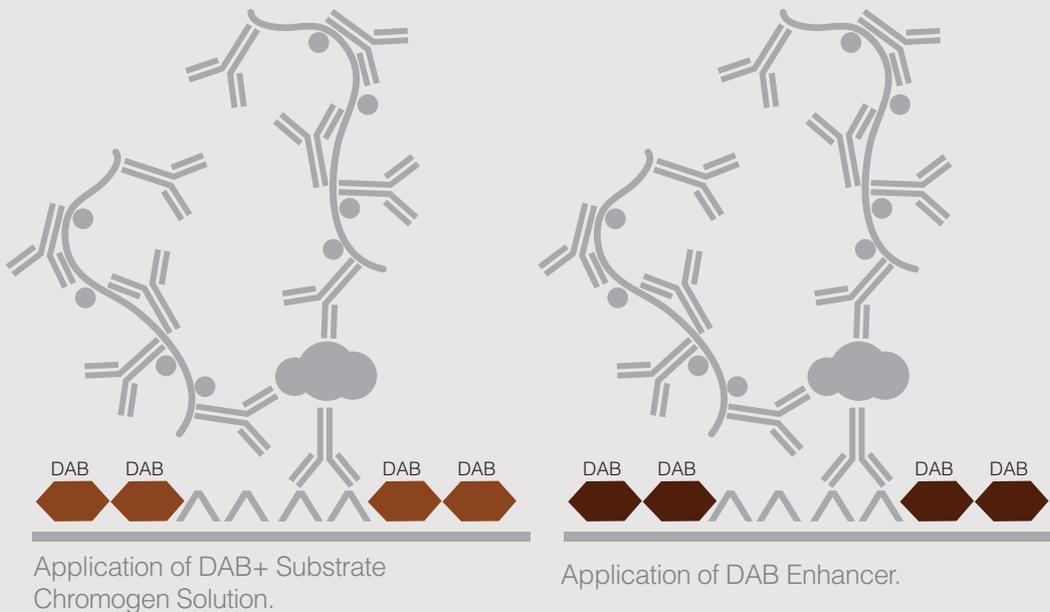
- EnVision FLEX Target Retrieval Solution, Low pH, 50x
- Peroxidase-Blocking Reagent
- Primary Antibody: Monoclonal Rabbit Anti-PD-L1, Clone 28-8
- Negative Control Reagent
- PD-L1 IHC 28-8 pharmDx Rabbit LINKER
- Visualization Reagent-HRP
- DAB+ Substrate Buffer
- DAB+ Chromogen
- DAB Enhancer
- PD-L1 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 the Autostainer Link 48. All reagents must be used as indicated in the IFU in order for the test to perform as specified.



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. 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 SCCHN 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 SCCHN-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 and select 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 the Autostainer Link 48. Ensure slides remain wet with buffer while loading and prior to initiating the run. Dried tissue sections may display increased non-specific staining.

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

95%	EtOH	(1 min)
95%	EtOH	(1 min)
100%	EtOH	(1 min)
100%	EtOH	(1 min)
Xylene		(3 min)
Xylene		(3 min)

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 the Autostainer Link 48 and PT Link? 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 SCCHN 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: _____

Guidelines for Scoring PD-L1 IHC 28-8 pharmDx

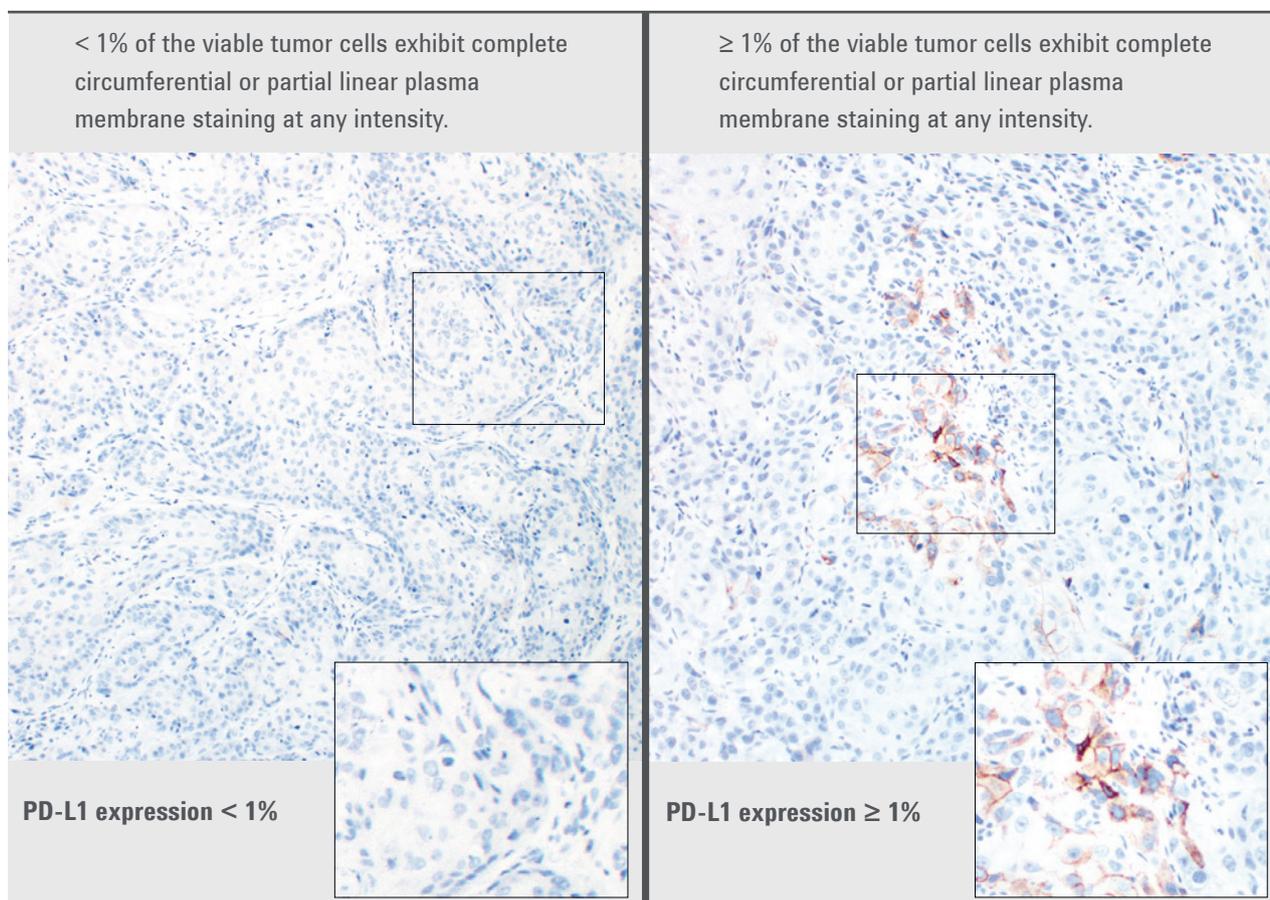
Agilent emphasizes that scoring of PD-L1 IHC 28-8 pharmDx must be performed in accordance with the guidelines established in the IFU, within the context of best practices and the pathologist's experience.

This assay was validated for invasive SCCHN tissue samples and not for lesions with foci of dysplasia or carcinoma *in situ*. 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.

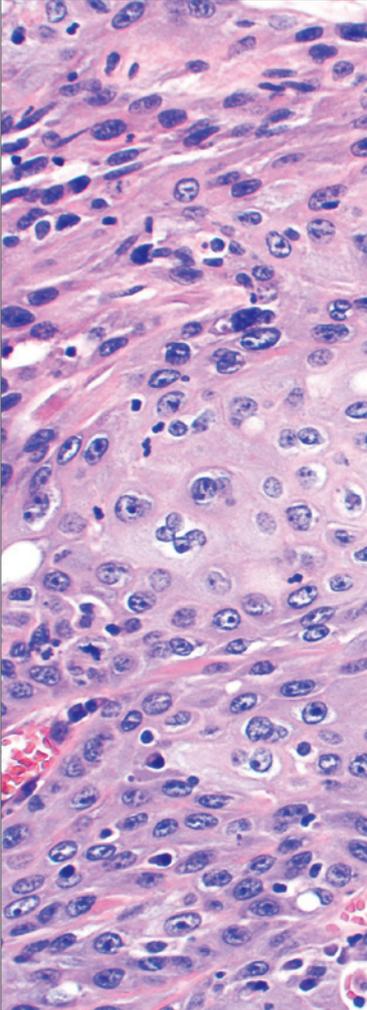
Figure 3: Guidelines for scoring and reporting PD-L1 IHC 28-8 pharmDx results



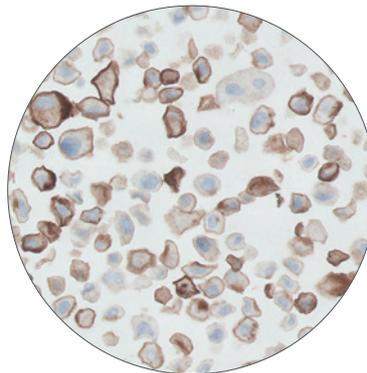
Recommended Slide Order for Interpretation of PD-L1 IHC 28-8 pharmDx

The following flow of slide review is recommended when conducting interpretation of PD-L1 IHC 28-8 pharmDx. Refer to detailed description on pages 18-19.

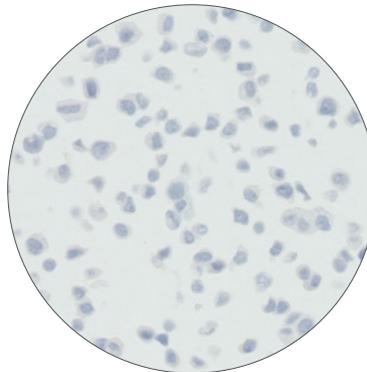
1
Patient Specimen stained with H&E
Histology and preservation quality
ACCEPTABLE



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

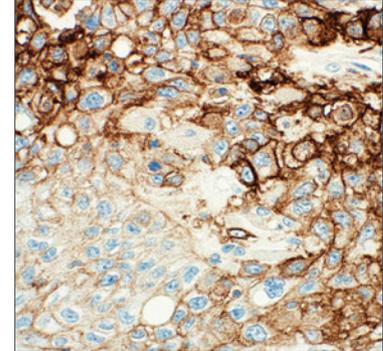


PD-L1 Positive Control

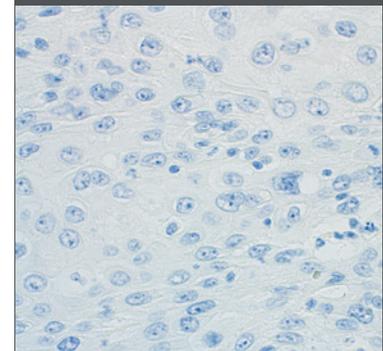


PD-L1 Negative Control

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



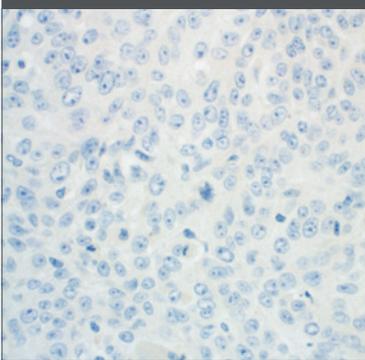
3B
Positive Control Tissue
Stained with Negative Control Reagent
ACCEPTABLE



4A

Negative Control Tissue
Stained with *PD-L1 Primary Antibody*

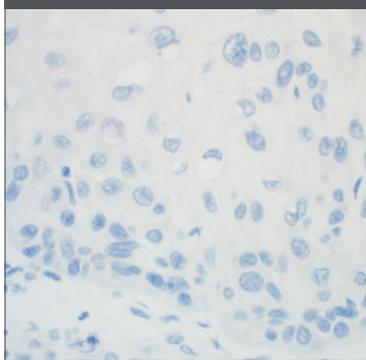
ACCEPTABLE



5

Patient Specimen
Stained with *Negative Control Reagent*

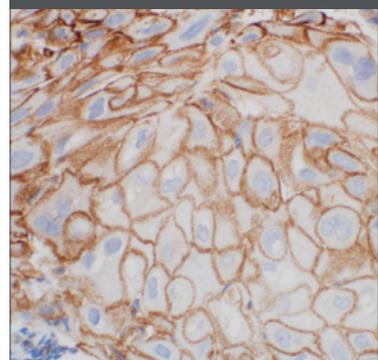
ACCEPTABLE



6

Patient Specimen
Stained with *PD-L1 Primary Antibody*

ACCEPTABLE



≥100 viable tumor cells should be present for scoring.

Include when scoring:

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

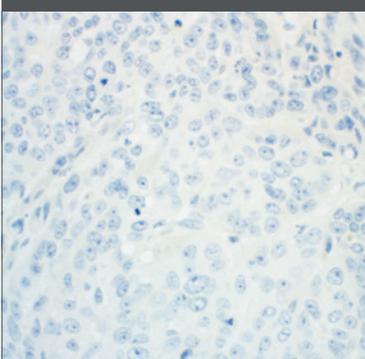
Exclude from scoring:

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

4B

Negative Control Tissue
Stained with *Negative Control Reagent*

ACCEPTABLE



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 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.

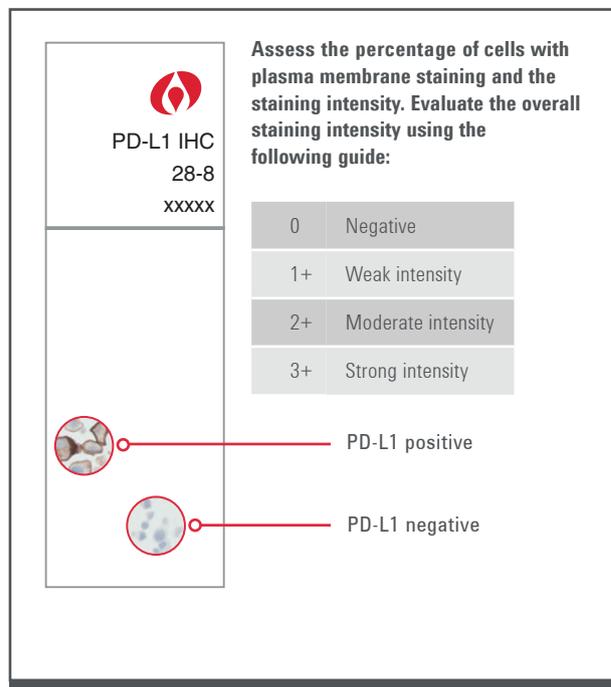


Figure 4: Each Control Slide contains sections of cell pellets with positive and negative PD-L1 expression.

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

- 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

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

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

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 $\geq 1+$ intensity within the boundaries of the negative cell pellet are acceptable.

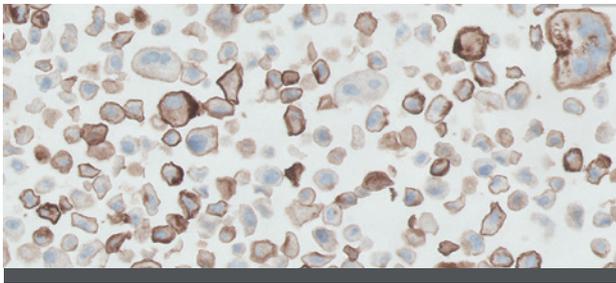


Figure 5: Acceptable Positive PD-L1 Control.

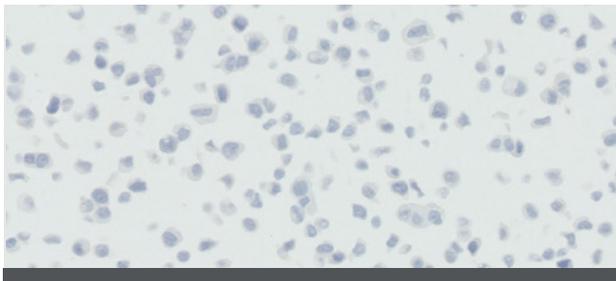


Figure 6: Acceptable Negative PD-L1 Control.

3. Positive Control Tissue Slides

Examine the positive SCCHN 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 $\leq 1+$ 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 SCCHN 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 $\leq 1+$ 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 $\leq 1+$. 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.

- 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 cytoplasmic staining from scoring. Exclude immune cells, necrotic cells, normal cells, and *in situ* carcinoma from scoring.
- 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.
- 3** Record if the specimen has PD-L1 expression $< 1\%$ or $\geq 1\%$. 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.

$$\% \text{ PD-L1 positive} = \frac{\text{Number of tumor cells expressing PD-L1 positive membrane staining}}{\text{Total number of viable tumor cells present in the section}}$$

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.

Reporting Results

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

Viable Tumor Cells Present ≥ 100 cells Not Evaluable

PD-L1 expression < 1%:

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

PD-L1 expression $\geq 1\%$:

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

% Percent Expression PD-L1 Tumor Cells: _____%

Other Comments to Treating Physician: _____

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.

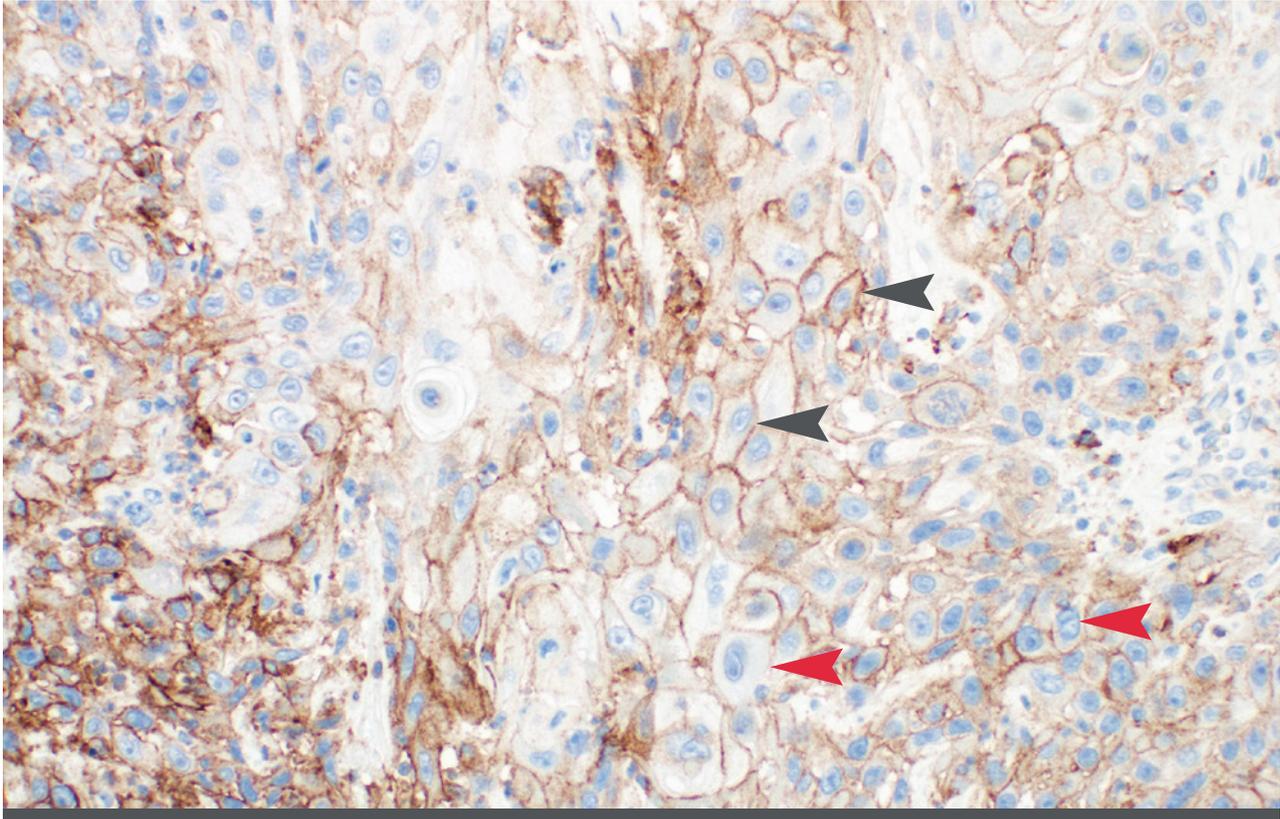


Figure 7: An example of a squamous cell carcinoma of the tonsil stained with PD-L1 IHC 28-8 pharmDx assay. The staining shows a range of PD-L1 expression. This specimen would be appropriate to use as a positive control specimen for detection of subtle changes in assay sensitivity. Note the partial linear (**red arrow**) and complete circumferential (**black arrow**) plasma membrane staining. 20x magnification.

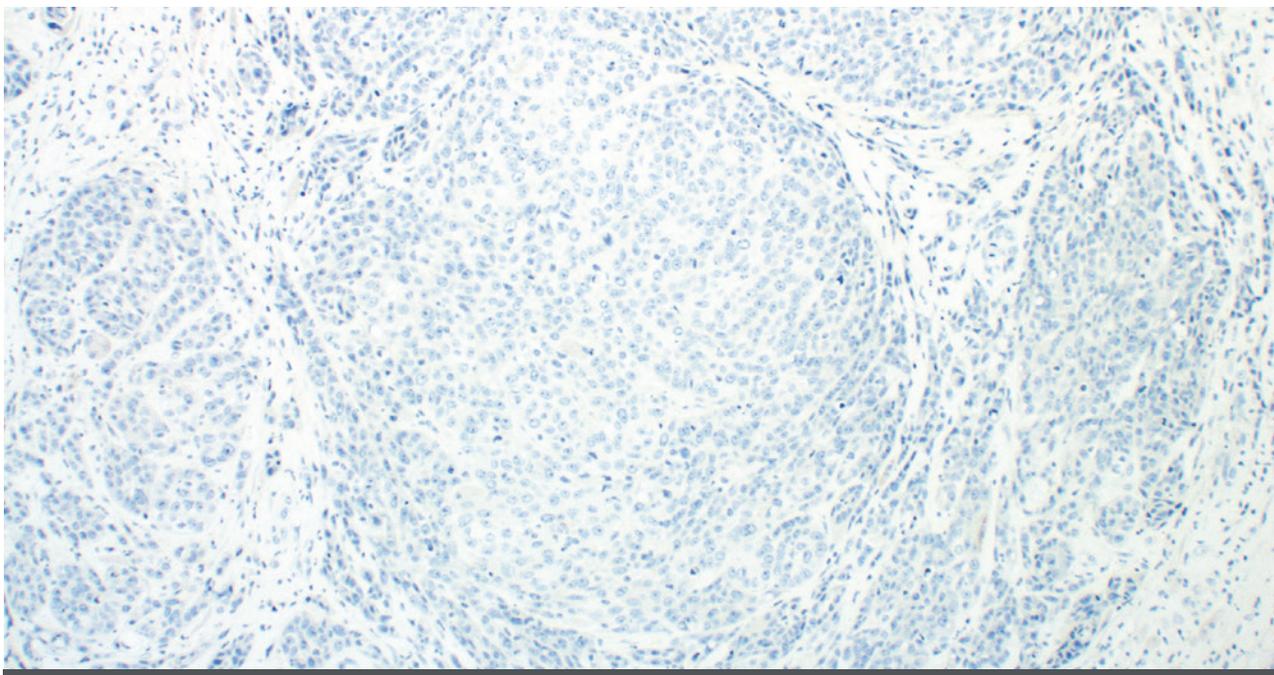


Figure 8: Squamous cell carcinoma of the larynx. PD-L1 expression < 1%. 10x magnification.

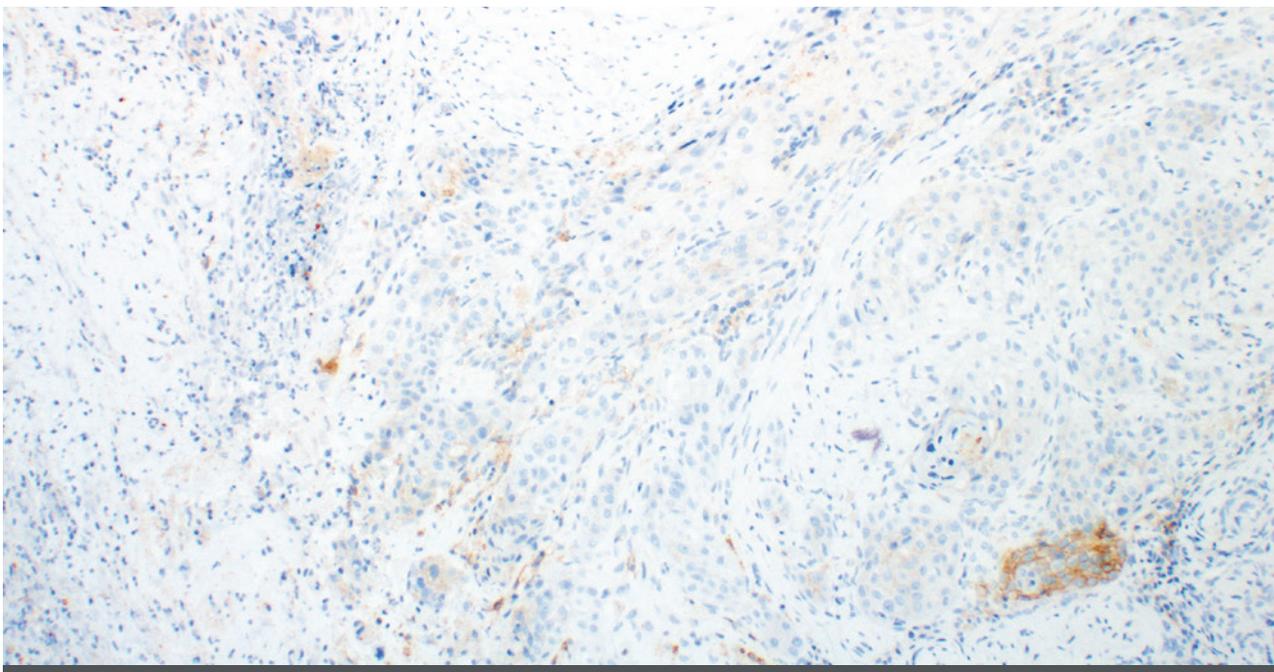


Figure 9: Squamous cell carcinoma of the larynx. PD-L1 expression \geq 1%. 10x magnification.

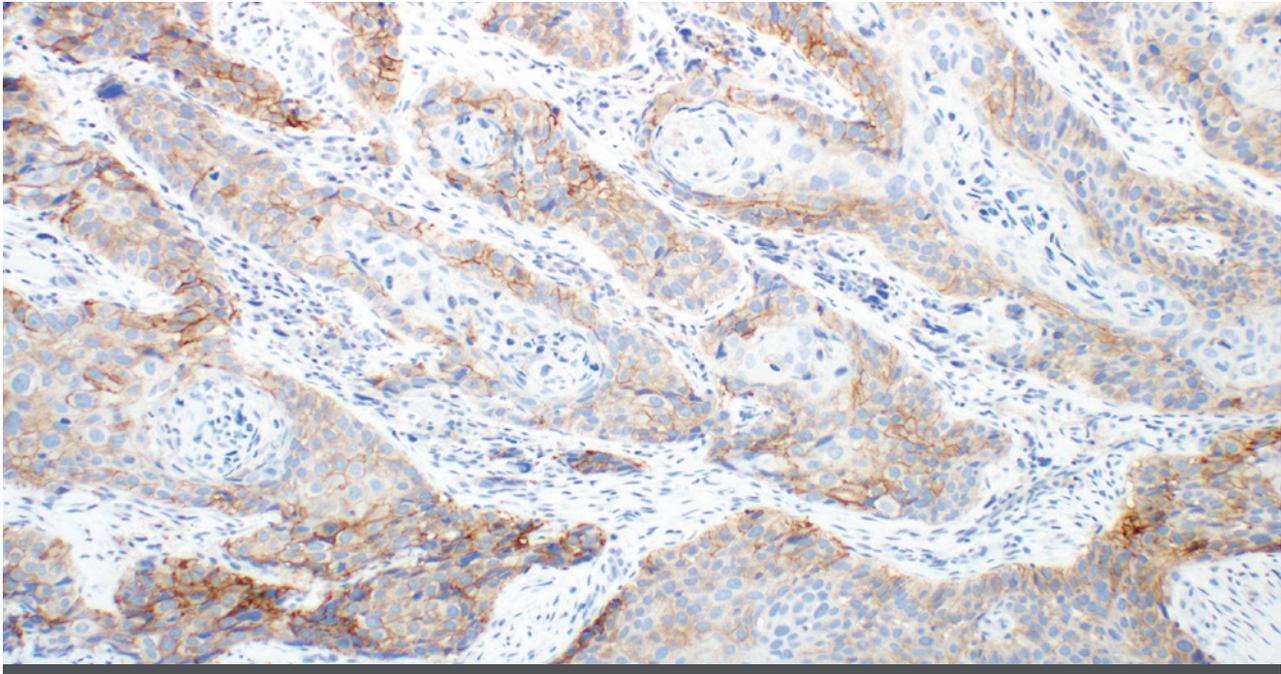


Figure 10: Squamous cell carcinoma of the tonsil demonstrating >1%, moderate PD-L1 expression. 10x magnification.

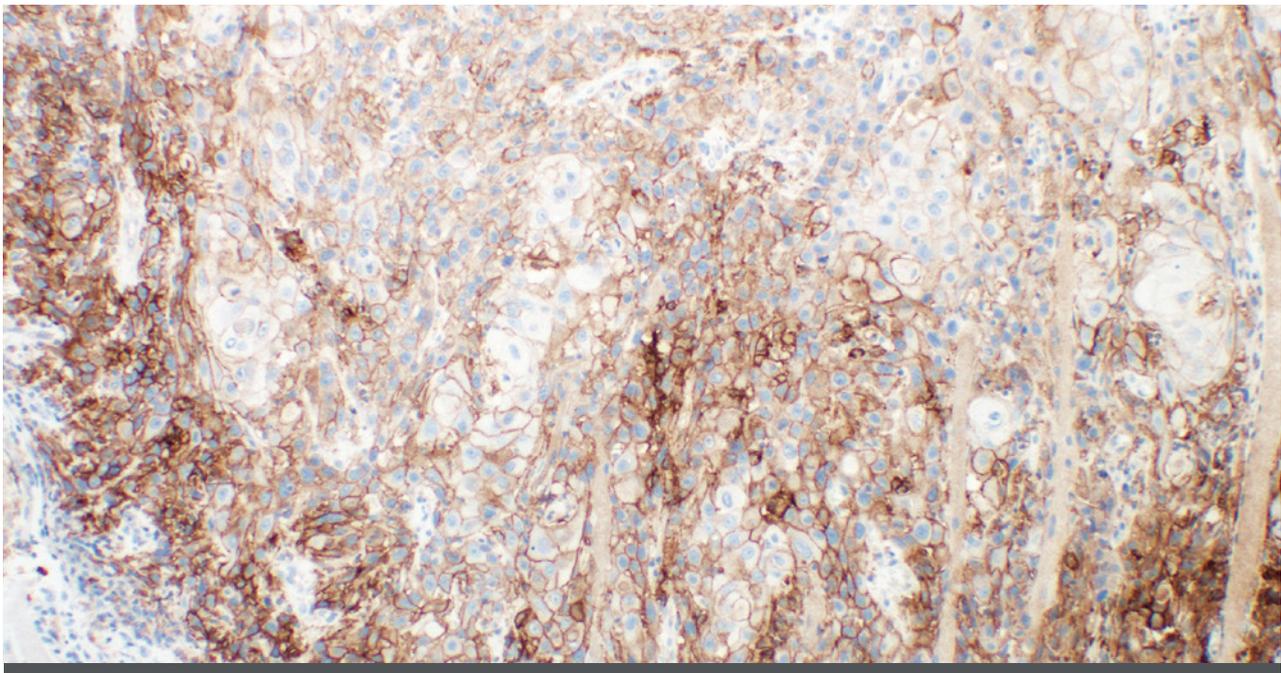


Figure 11: Squamous cell carcinoma of the tonsil demonstrating >1%, high PD-L1 expression. 10x magnification.

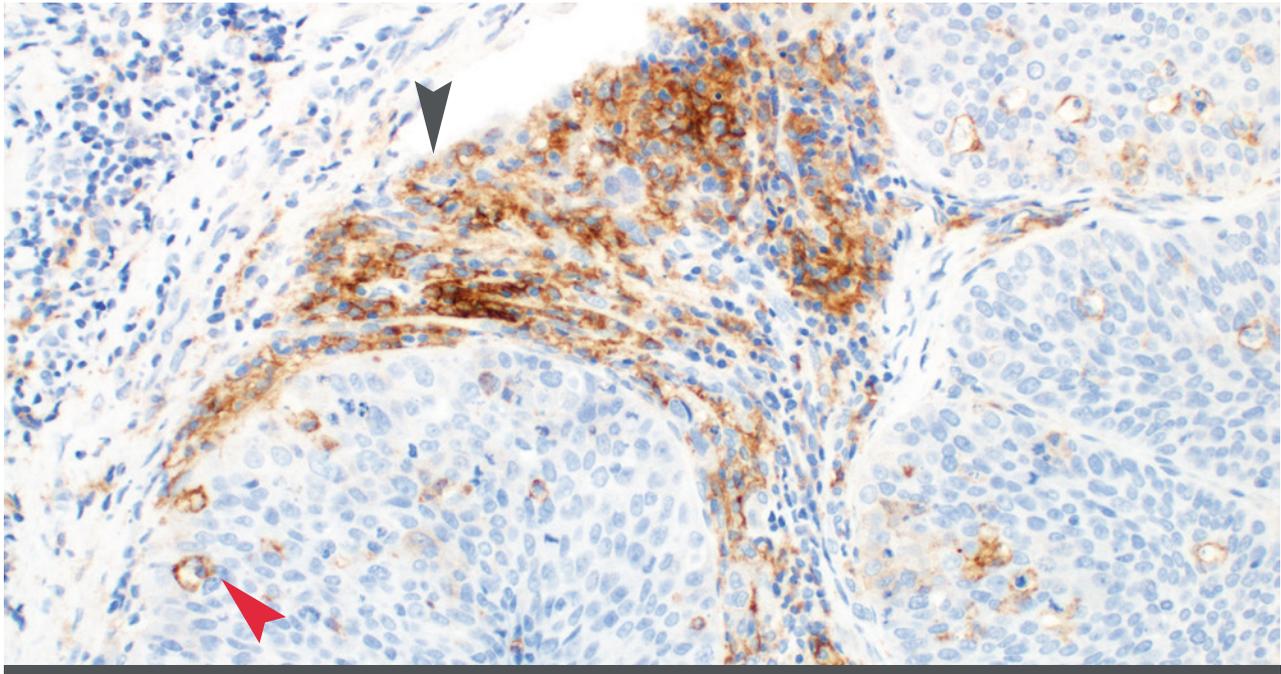


Figure 12: Squamous cell carcinoma of the pharynx, showing strong staining of peri-tumoral associated immune cells (**black arrow**), while the tumor cells are negative for PD-L1 positivity. Note the staining of the intra-tumoral histiocytes (**red arrow**), which are not included in determining the percent PD-L1 positive score. 20x magnification.

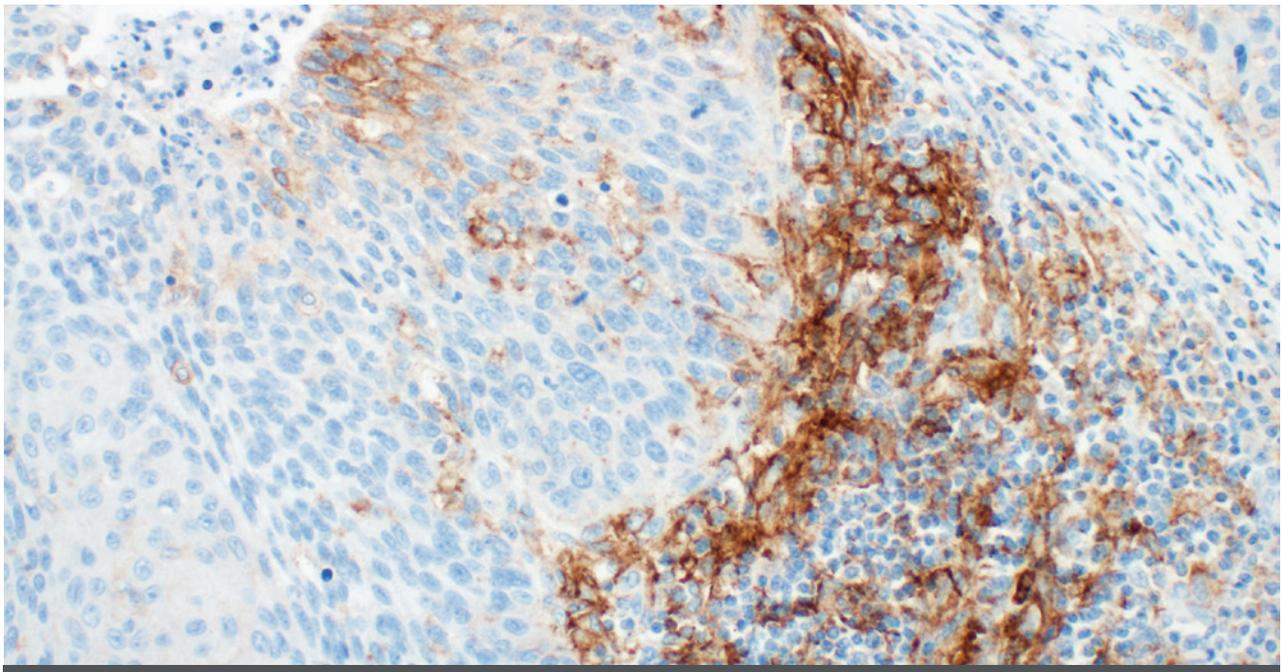


Figure 13: Squamous cell carcinoma of the tongue showing staining of immune cells and positive staining within tumor. 20x magnification.

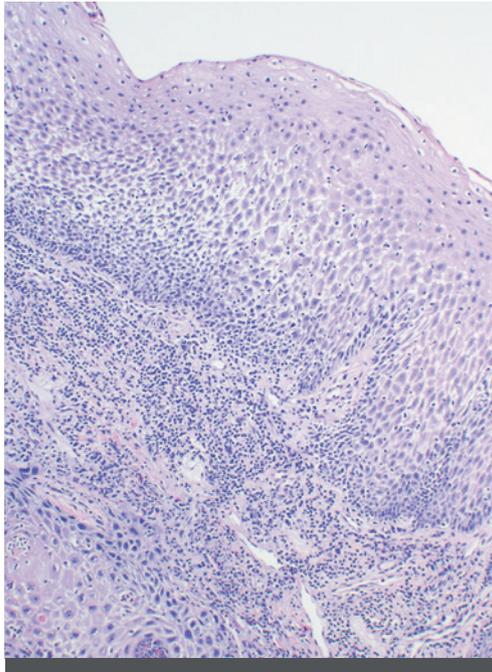


Figure 14A: H&E stain of a case of squamous cell carcinoma of the tongue.

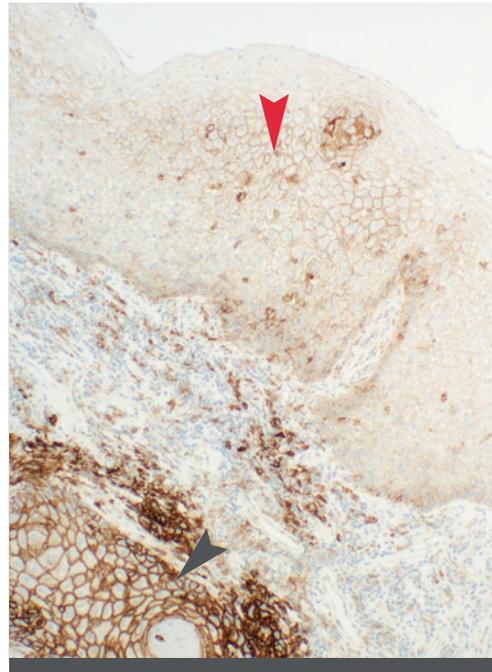


Figure 14B: Strongly positive PD-L1 staining is observed in the invasive squamous cell carcinoma (**black arrow**). Note that in this case, there is staining in the normal squamous epithelium (**red arrow**). The staining in the normal squamous epithelium is not included in calculating % positive staining of the tumor for PD-L1. 10x magnification.

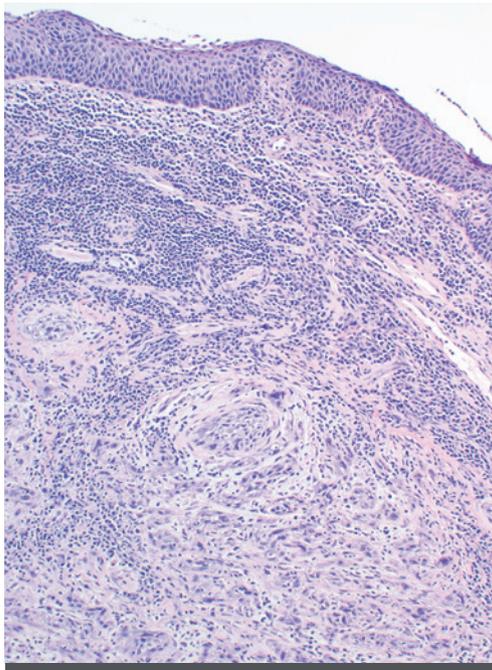


Figure 15A: H&E stain of a case of squamous cell carcinoma of the larynx.

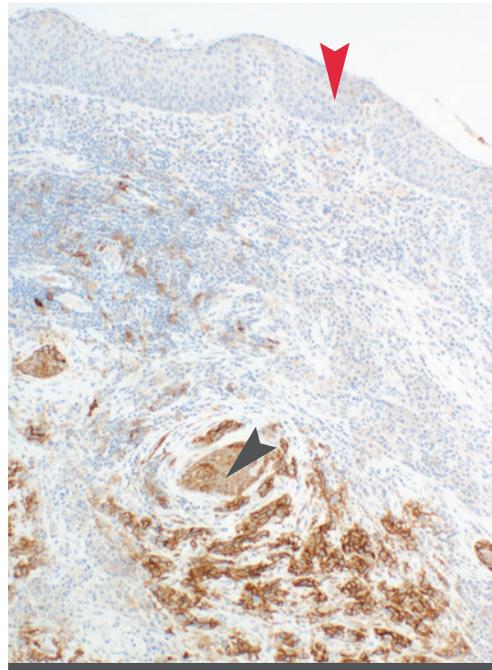


Figure 15B: Strongly positive plasma membrane staining of invasive squamous cell carcinoma (**black arrow**). The *in situ* component (**red arrow**), is not staining for PD-L1 in this case. Regardless of staining status, the *in situ* component is not included in the denominator for determining the PD-L1 percentage score for the specimen. Only the invasive component is evaluated. 10x magnification.

Challenging Cases for SCCHN

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 $\leq 1+$ 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.

Squamous Cell Differentiation

Due to cell differentiation in this tumor type, a wide range of cell sizes may be observed. Approximating percent positive staining by staining area may produce an inaccurate score due the wide range of cell sizes. See page 19 for instructions on scoring the patient specimen stained with primary antibody.

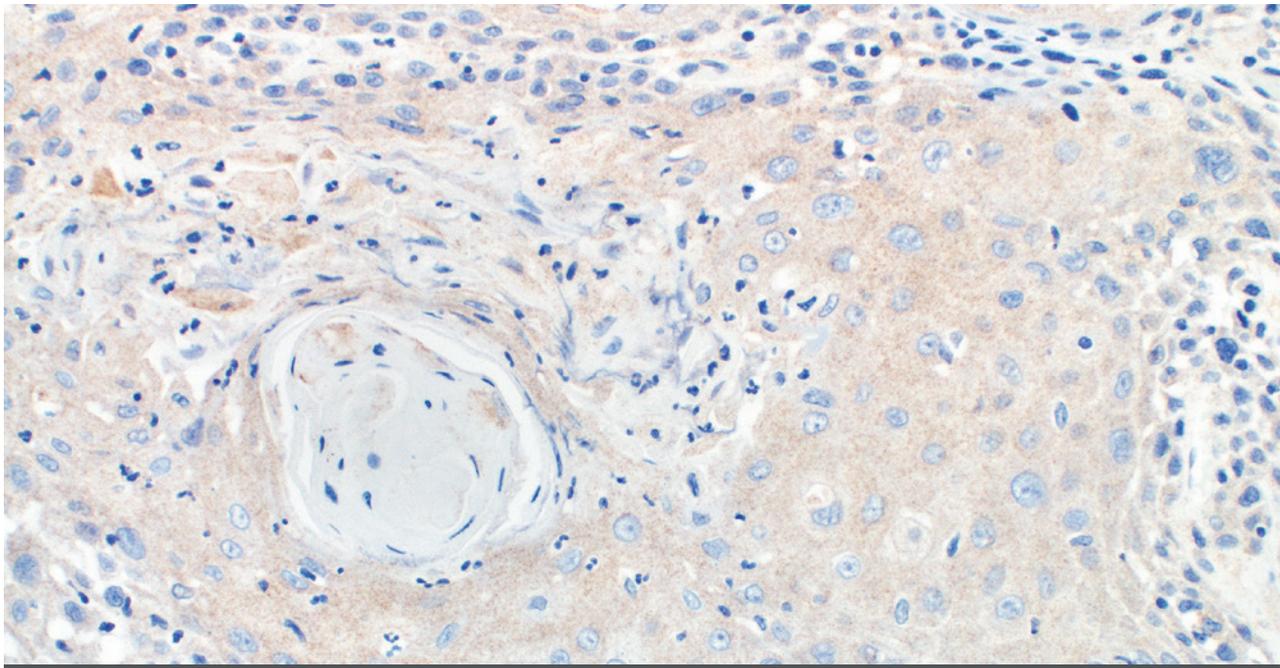


Figure 16: Squamous cell carcinoma of the tongue. Granular staining in the cytoplasm of tumor cells with no positive linear membrane staining is not considered positive. 20x magnification.

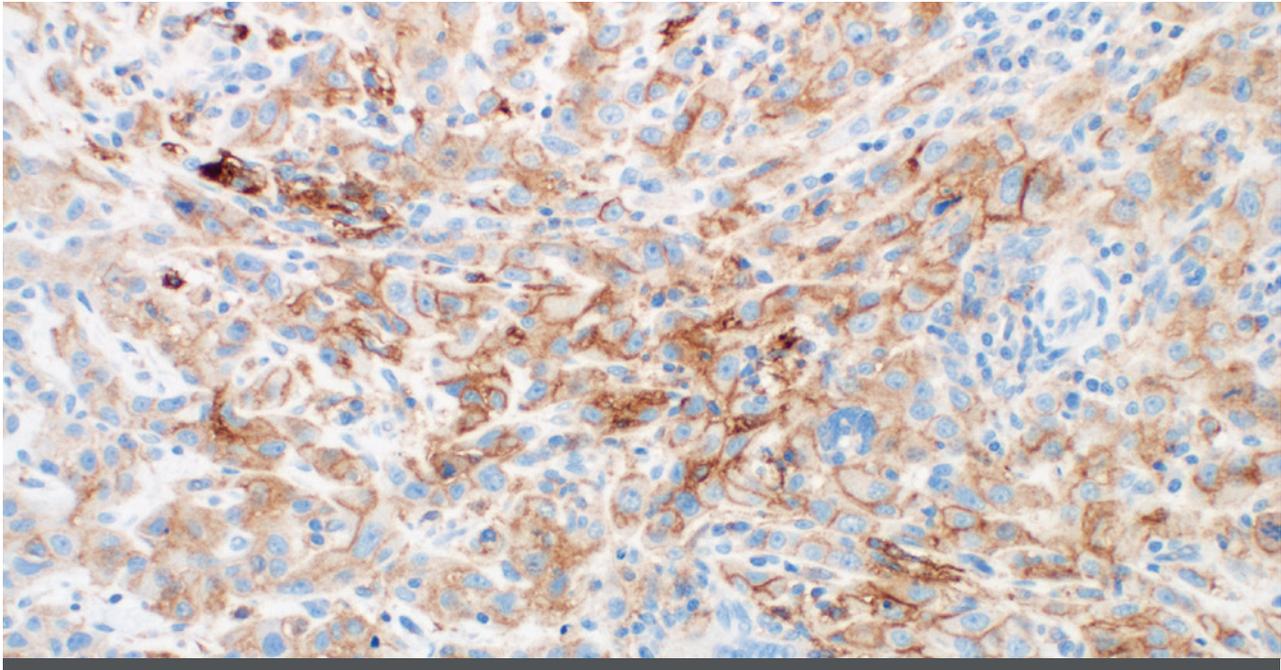


Figure 17: Squamous cell carcinoma of the tongue. Positive linear membrane staining of the tumor is observed and distinguishable from the non-specific cytoplasmic staining. 20x magnification.

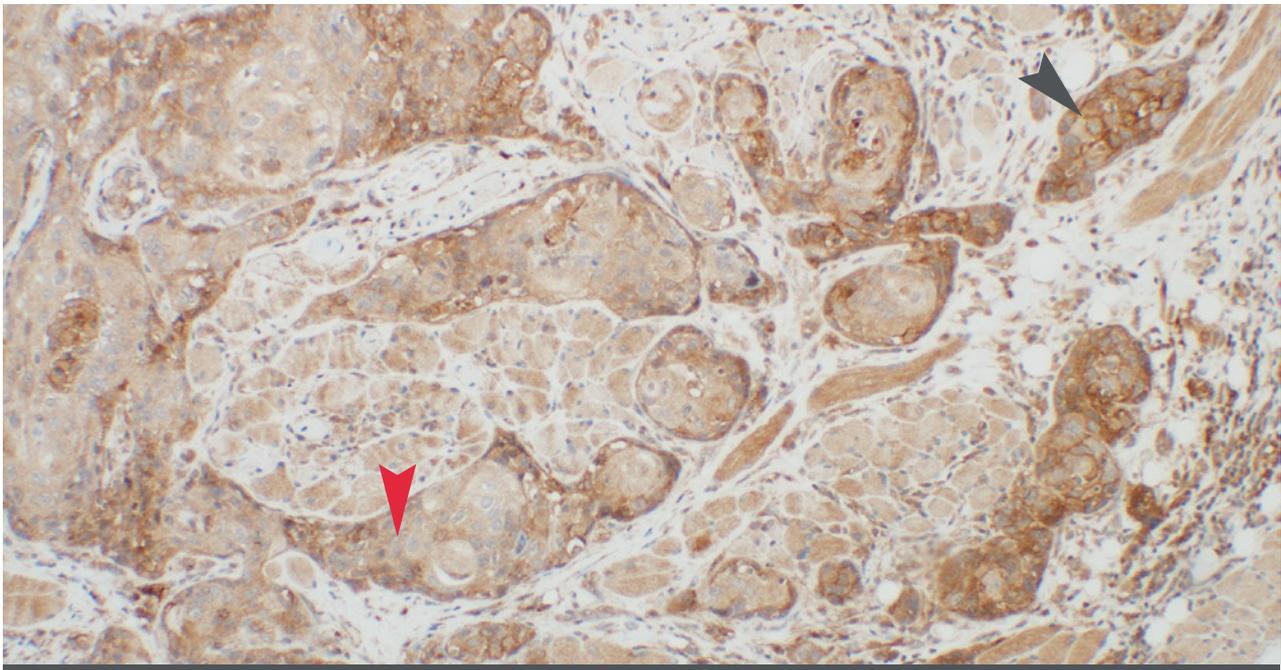


Figure 18: Squamous cell carcinoma of the tongue. This is an example of an indeterminate case due to excess cytoplasmic staining that can hamper scoring. Positive linear membrane staining of the tumor is observed (**black arrow**), however cytoplasmic staining is excessive in much of the specimen (**red arrow**). 10x magnification.

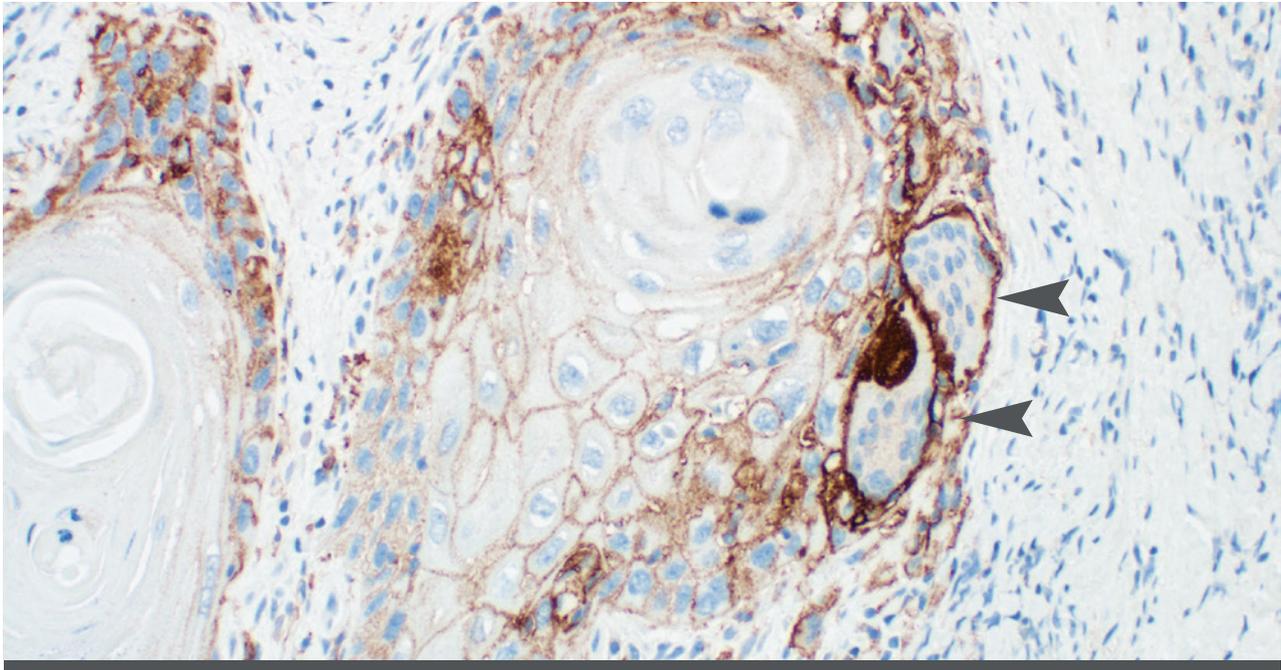


Figure 19: Well to moderately differentiated keratinizing squamous cell carcinoma of the tongue. Note plasma membrane staining of foreign body giant cells (FBGC). Because of the nature of this tumor, presence of FBGCs is a feature that can be observed in well differentiated squamous cell carcinomas and represent a reaction to foreign material, such as keratin clumps, produced by tumor cells that is spilled into the stroma. FBGCs are formed as a result of the fusion of macrophages. Exclude FBGCs from scoring for PD-L1 positive staining evaluation of the tumor. 20x magnification.

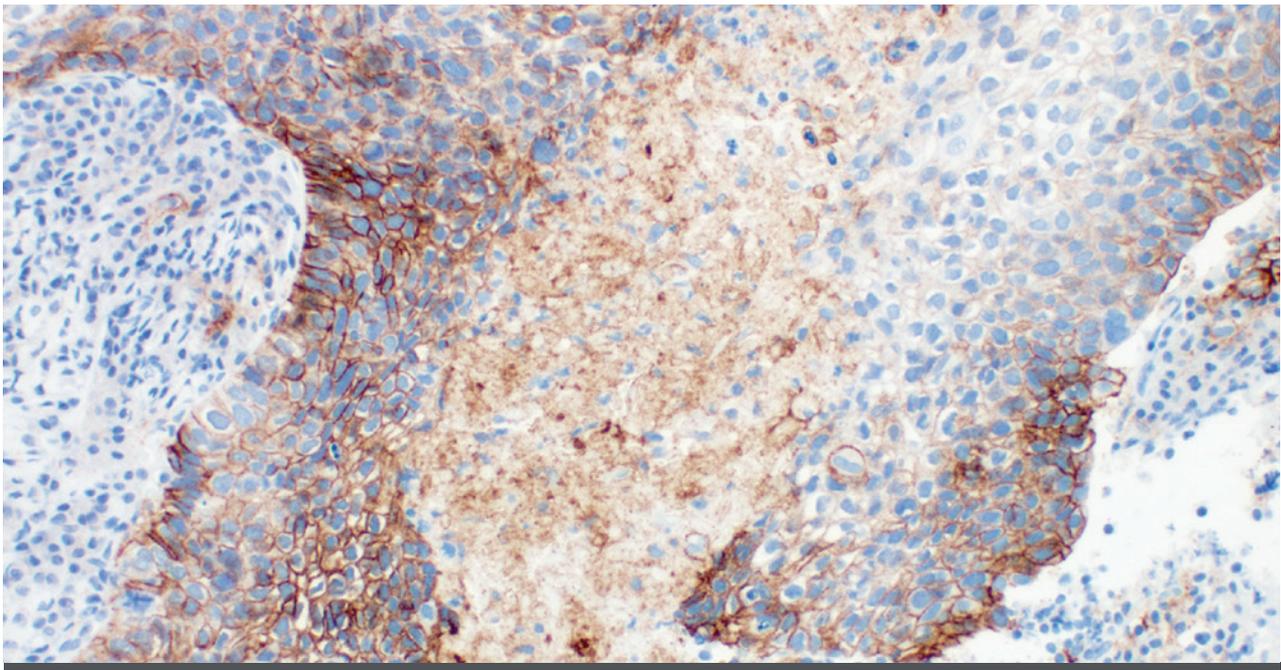


Figure 20: Squamous cell carcinoma of the larynx showing a necrotic region. Adjacent viable tumor cells are evaluable for PD-L1 positive staining. Necrotic tissue may show non-specific staining and should not be included in scoring percent positivity of the tumor. Care should be taken to only include viable tumor cells for scoring and not necrotic regions. If specimen is excessively necrotic, the specimen is considered not evaluable. A minimum of 100 viable tumor cells should be present for evaluating the specimen. 20x magnification.

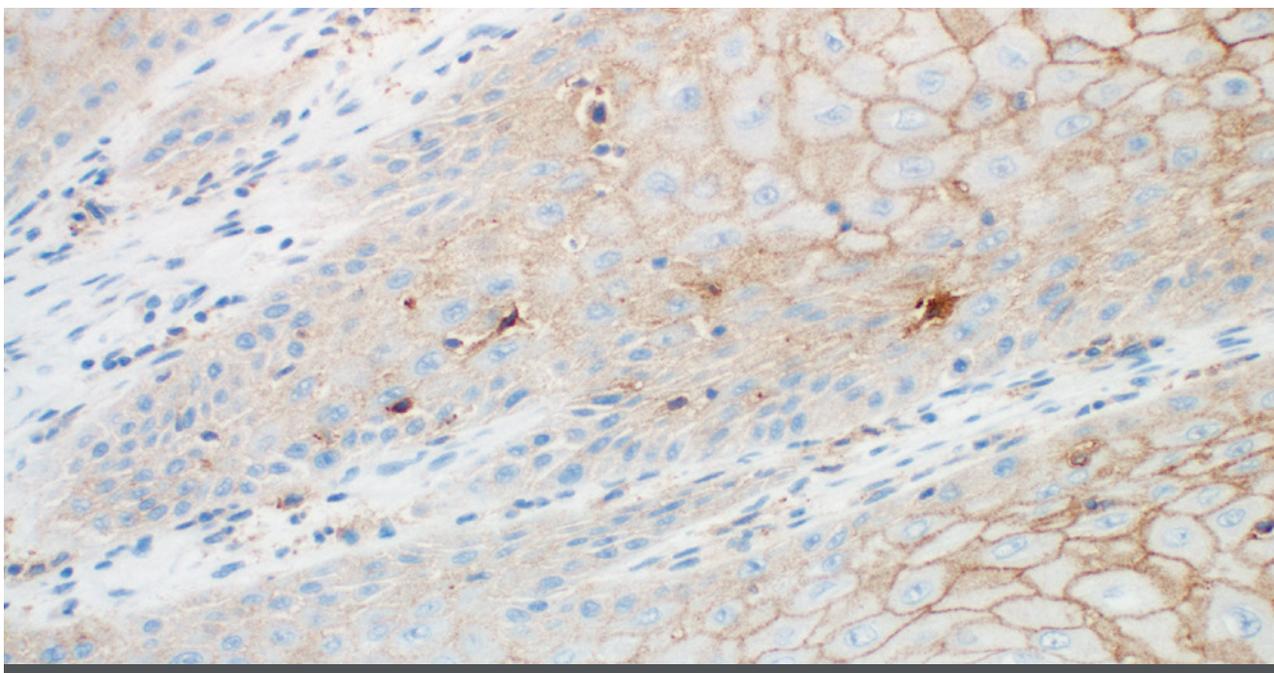


Figure 21: PD-L1 positive staining observed in squamous cell carcinoma of the larynx. When scoring for percent positivity, percentage is determined by the number of positively stained cells and not area. Note in this example, the absence of staining in tightly packed basaloid cells which take up less 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. 20x magnification.

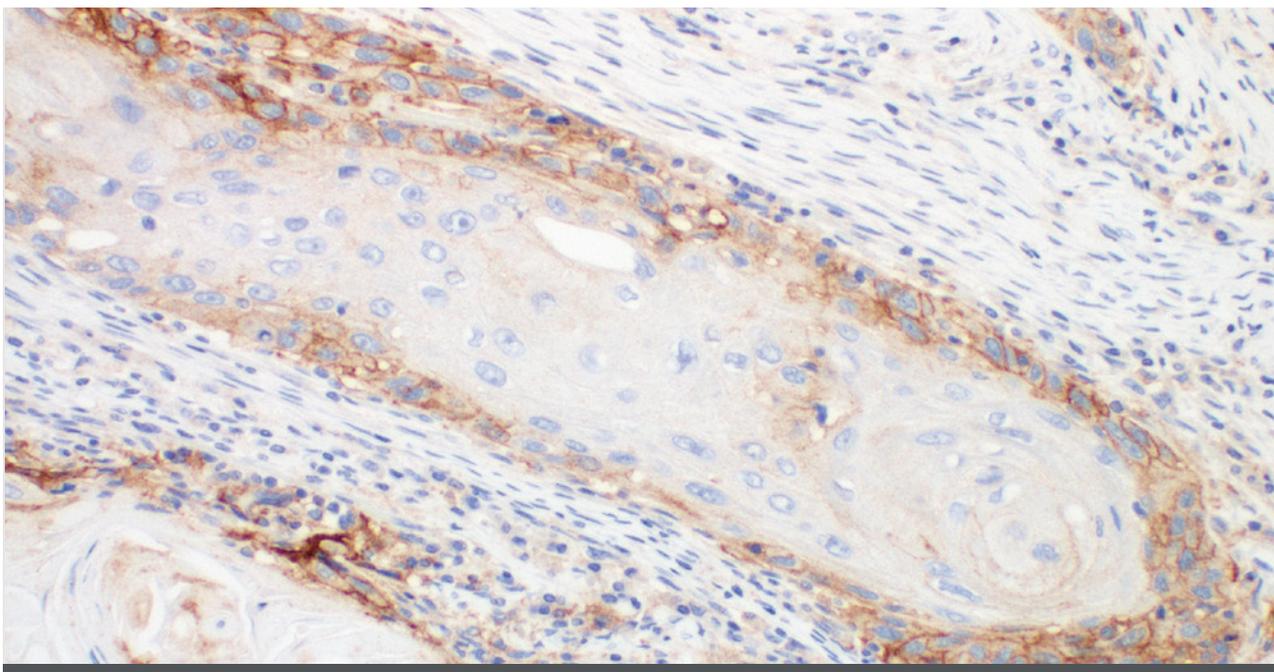


Figure 22: Squamous cell carcinoma of the hypopharynx. In this example, the tightly packed basaloid cells are staining positive for PD-L1, while the well-differentiated squamous cells are not staining for PD-L1. If determining the percent positive tumor staining based on the surface area that is staining, 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. 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	Verify that the SK005 PD-L1 IHC 28-8 pharmDx protocol was selected for programming of slides
	1b Lack of reaction with DAB+ Substrate-Chromogen Solution	Verify that DAB+ Substrate-Chromogen Solution was prepared properly
	1c Sodium azide in wash buffer	Use only Dako Wash Buffer, Code K8007
	1d Degradation of Control Slide	Check kit expiration date and kit storage conditions on outside of package
2 Weak staining of specimen slides	2a Inappropriate fixation method used	Ensure that only approved fixatives and fixation methods are used
	2b Insufficient reagent volume applied	Check size of tissue section and reagent volume applied
	2c Inappropriate wash buffer used	Use only Dako Wash Buffer, Code K8007
	2d Reagents used when cold	Allow reagents to come to room temperature (20-25 °C) prior to staining
3 Weak staining of specimen slides or of the positive cell line on the Dako-provided Control Slide	3a Inadequate target retrieval	Verify that the 3-in-1 pre-treatment procedure was correctly performed
	3b Inappropriate wash buffer used	Use only Dako Wash Buffer, Code K8007
4 Excessive background staining of slides	4a Paraffin incompletely removed	Verify that the 3-in-1 pre-treatment procedure was correctly performed
	4b Slides dried while loading onto the Autostainer Link 48	Ensure slides remain wet with buffer while loading and prior to initiating run
	4c Non-specific binding of reagents to tissue section	Check for proper fixation of the specimen and/or the presence of necrosis
	4d Inappropriate fixation method used	Ensure that only neutral buffered formalin fixative and approved fixation methods are used
5 Tissue detached from slides	5a Use of incorrect microscope slides	Use Dako FLEX IHC Microscope Slides (Code K8020), or Fisherbrand Superfrost Plus slides
	5b Inadequate preparation of specimens	Cut sections should be placed in a 58 ± 2 °C oven for one hour prior to staining
6 Excessively strong specific staining	6a Inappropriate fixation method used	Ensure that only approved fixatives and fixation methods are used
	6b Inappropriate wash buffer used	Use only Dako Wash Buffer, Code K8007
	6c Temperature of staining reagents too high	It is recommended that reagents are equilibrated to 20-25 °C prior to staining
7 Target Retrieval Solution is cloudy in appearance when heated	7 When heated the Target Retrieval Solution turns cloudy in appearance	This is normal and does not influence staining
8 Artifacts in specimen giving a bubbling staining appearance	8 Inadequate slide dehydration procedure prior to slide mounting	Ensure adequate slide dehydration procedure was used. An example of dehydration procedure is given in this manual

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- [1] PD-L1 IHC 28-8 pharmDx Instructions for Use.

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