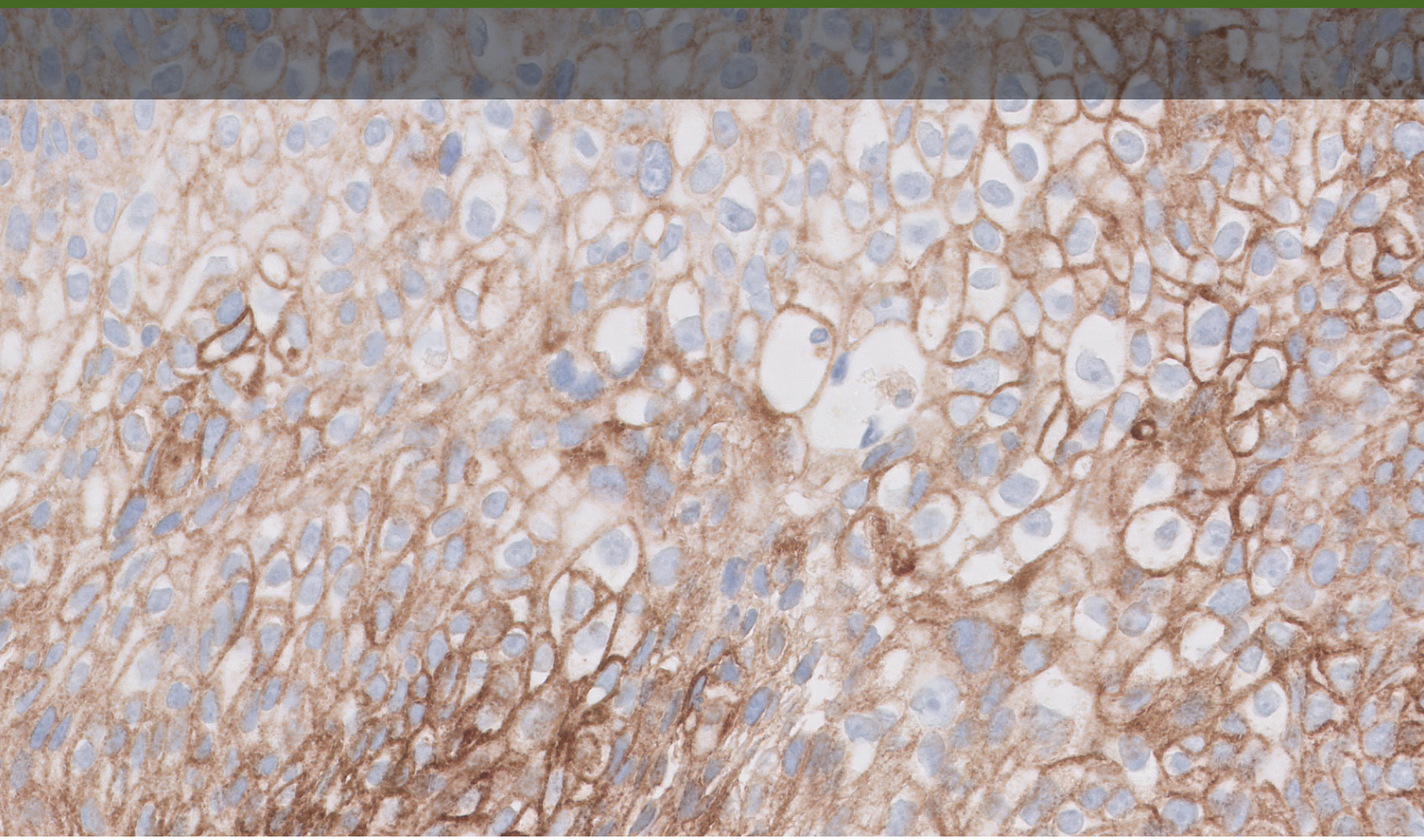
Agilent Dako

PD-L1 IHC 22C3 pharmDx Interpretation Manual – Head and Neck Squamous Cell Carcinoma (HNSCC)

FDA-approved for in vitro diagnostic use

Rx only



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Trusted Answers

For countries outside of the United States, see the local KEYTRUDA product label for approved indications and expression cutoff values to guide therapy.

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Intended Use

For in vitro diagnostic use.

PD-L1 IHC 22C3 pharmDx is a qualitative immunohistochemical assay using monoclonal mouse anti-PD-L1, Clone 22C3 intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) non-small cell lung cancer (NSCLC), gastric or gastroesophageal junction (GEJ) adenocarcinoma, cervical cancer, urothelial carcinoma and head and neck squamous cell carcinoma (HNSCC) tissues using EnVision FLEX visualization system on Autostainer Link 48.

PD-L1 protein expression in NSCLC is determined by using Tumor Proportion Score (TPS), which is the percentage of viable tumor cells showing partial or complete membrane staining at any intensity.

PD-L1 protein expression in gastric or GEJ adenocarcinoma, cervical cancer, urothelial carcinoma and HNSCC is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100.

Companion Diagnostic Indications

Tumor Indication	PD-L1 Expression Level	Intended Use
NSCLC	TPS ≥ 1%	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying NSCLC patients for treatment with KEYTRUDA® (pembrolizumab).**
Gastric or GEJ Adenocarcinoma	CPS ≥ 1	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying gastric or GEJ adenocarcinoma patients for treatment with KEYTRUDA® (pembrolizumab).
Cervical Cancer	CPS ≥ 1	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying cervical cancer patients for treatment with KEYTRUDA® (pembrolizumab).
Urothelial Carcinoma	CPS ≥ 10	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying urothelial carcinoma patients for treatment with KEYTRUDA® (pembrolizumab).**
HNSCC	CPS ≥ 1	PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying HNSCC patients for treatment with KEYTRUDA® (pembrolizumab).**

*** See the KEYTRUDA® product label for specific clinical circumstances guiding PD-L1 testing.*

Introduction

PD-L1 IHC 22C3 pharmDx is the only companion diagnostic FDA-approved as an aid in identifying patients with head and neck squamous cell carcinoma (HNSCC) for treatment with KEYTRUDA® (pembrolizumab). This Interpretation Manual is provided as a tool to help guide pathologists and laboratory personnel in achieving correct and reproducible results in assessing PD-L1 expression in FFPE HNSCC specimens. PD-L1 expression evaluation may be used to identify patients for treatment with KEYTRUDA.

The manual provides detailed scoring guidelines and technical information from the PD-L1 IHC 22C3 pharmDx Instructions for Use (IFU) to ensure high-quality staining and diagnostic assessment. To help familiarize you with the requirements for scoring HNSCC stains with PD-L1 IHC 22C3 pharmDx, example cases of various PD-L1 expression levels are provided as references. These example cases and in-depth recommendations for interpretation of HNSCC specimens stained with PD-L1 IHC 22C3 pharmDx can help individual labs achieve reproducible and reliable results.

PD-L1 IHC 22C3 pharmDx is considered a qualitative immunohistochemical assay. PD-L1 expression in HNSCC is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100.

HNSCC tissue specimens that are tested for PD-L1 expression are scored and divided into PD-L1 expression levels based on a Combined Positive Score (CPS):

- CPS < 1
- CPS ≥ 1
- CPS ≥ 20

Note: PD-L1 expression level CPS ≥ 20 may be of interest to treating physician but does not determine eligibility for first-line treatment with KEYTRUDA as a single agent.

PD-L1 expression (CPS ≥ 1) is used to inform patient eligibility for first-line treatment with KEYTRUDA. For more details on staining and interpretation, please refer to the current version of the IFU provided with PD-L1 IHC 22C3 pharmDx, Code SK006 or visit www.agilent.com.

Assay Interpretation

The clinical interpretation of any staining, or the absence of staining, must be complemented by the evaluation of proper controls. 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 33.

Photomicrographs

The included photomicrographs are of HNSCC, except for Figure 35 which is squamous cell carcinoma from the cervix.

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

Tissue samples were provided by the Cooperative Human Tissue Network which is funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects.

The data and biospecimens used in this project were provided by US Biolab, Rockville, MD, with appropriate ethics approval and through Trans-Hit Biomarkers Inc.

Tissue samples supplied by Asterand Bioscience.

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 1). 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 2). 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

PD-1/PD-L1 interaction between tumor cells and activated T-cells (Figure 3) is a mechanistic pathway used by immunotherapeutic agents. When the tumor cell is unable to interact with the activated T-cell, the immune system remains active, helping to prevent immunosuppression.

PD-L1 IHC 22C3 pharmDx Detects PD-L1 in HNSCC Specimens

PD-L1 upregulation in HNSCC is a biomarker for response to anti-PD-1 therapy. PD-L1 IHC 22C3 pharmDx was the only PD-L1 assay used in the KEYTRUDA® (pembrolizumab) clinical trial (KEYNOTE-048) to evaluate the relationship between PD-L1 expression and clinical efficacy. KEYTRUDA is a humanized monoclonal PD-1-blocking antibody.

The PD-1/PD-L1 Pathway

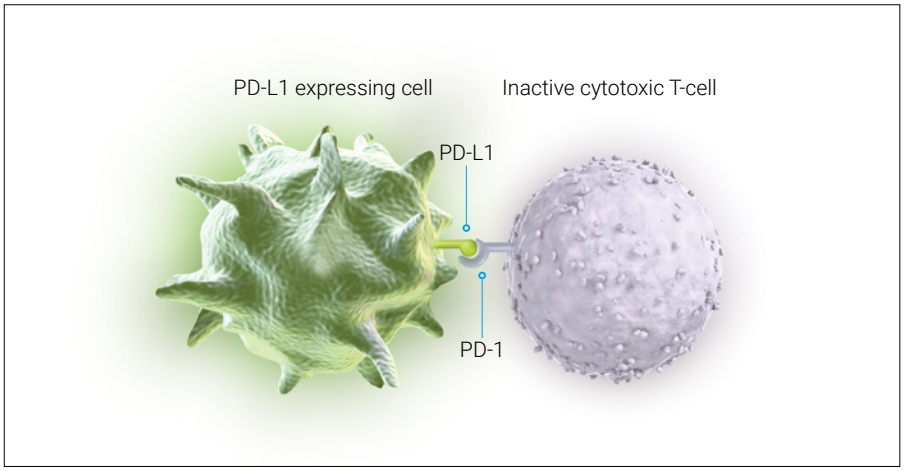


Figure 1: Inactivation of T-cells limits damage to normal tissue.

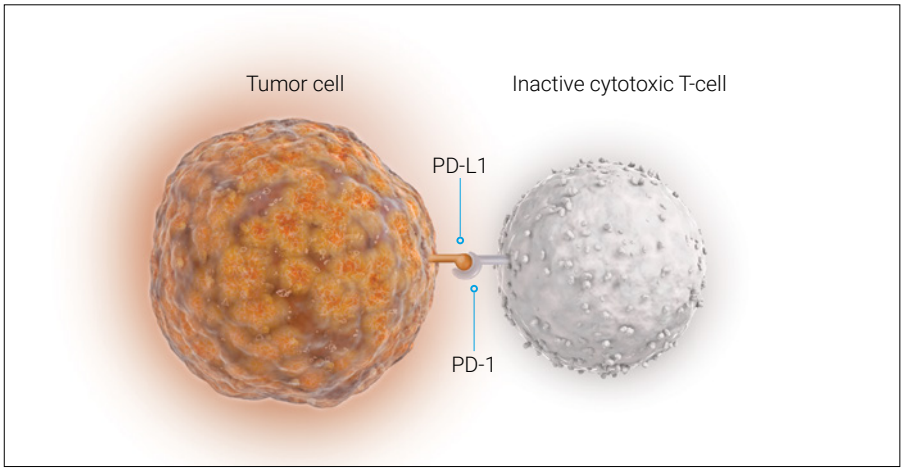


Figure 2: Inactivation of T-cells reduces tumor cell death and elimination.

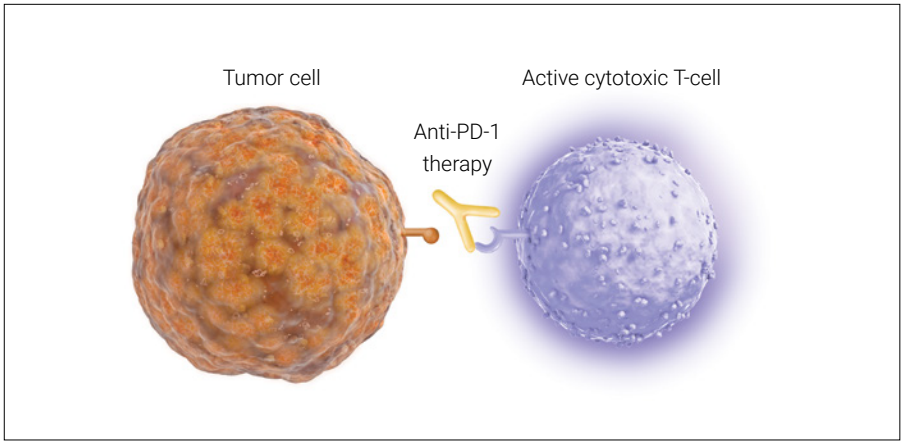


Figure 3: Blocking the PD-1/PD-L1 interaction helps to enable active T-cells and tumor cell death and elimination.

PD-L1 IHC 22C3 pharmDx Overview

What is PD-L1 IHC 22C3 pharmDx?

PD-L1 IHC 22C3 pharmDx is the only companion diagnostic indicated as an aid in identifying patients with HNSCC for treatment with KEYTRUDA® (pembrolizumab). PD-L1 IHC 22C3 pharmDx is a qualitative immunohistochemical (IHC) assay intended for use in the detection of PD-L1 protein in FFPE HNSCC tissue samples using EnVision FLEX visualization system on Autostainer Link 48.

Components of PD-L1 IHC 22C3 pharmDx

PD-L1 IHC 22C3 pharmDx contains optimized reagents to perform an IHC staining procedure using a linker and a chromogen enhancement reagent (Figure 4). Deparaffinization, rehydration, and target retrieval is performed using a 3-in-1 procedure on PT Link. Following peroxidase block, specimens are incubated with the monoclonal mouse primary antibody to PD-L1 or the Negative Control Reagent. Specimens are then incubated with a Mouse LINKER, followed by incubation 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 may then be counterstained and coverslipped. Results are interpreted using a light microscope.

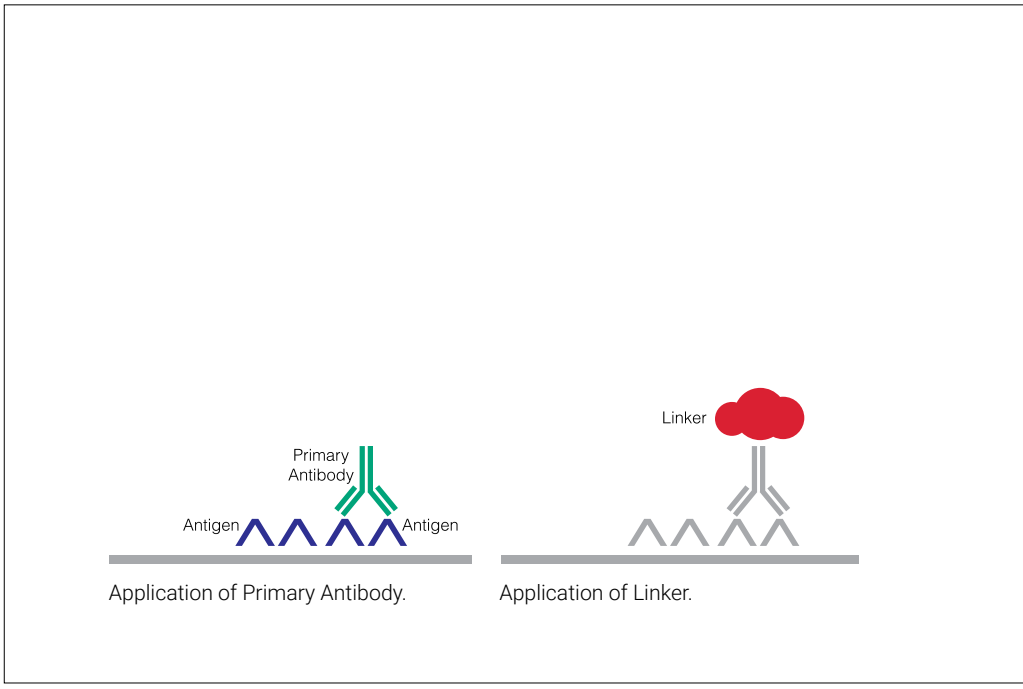


Figure 4: PD-L1 IHC 22C3 pharmDx staining procedure.

Kit Configuration (SK006)

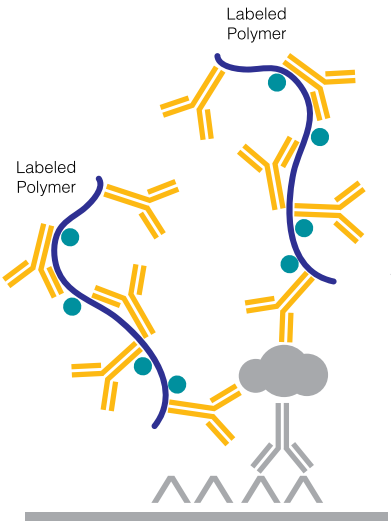
Figure 5: PD-L1 IHC 22C3 pharmDx components.

PD-L1 IHC 22C3 pharmDx (Code SK006) contains reagents to perform 50 tests in up to 15 individual runs (Figure 5):

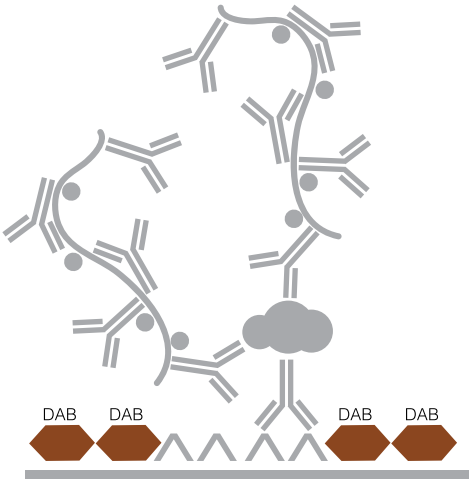
1. EnVision FLEX Target Retrieval Solution, Low pH, (50×)
2. Peroxidase-blocking Reagent
3. Primary Antibody: Monoclonal Mouse Anti-PD-L1, Clone 22C3
4. Negative Control Reagent
5. Mouse LINKER
6. Visualization Reagent-HRP
7. DAB+ Substrate Buffer
8. DAB+ Chromogen
9. DAB Enhancer
10. PD-L1 IHC 22C3 pharmDx Control Cell Line Slides*

EnVision FLEX Wash Buffer, (20×) (Code K8007) and EnVision FLEX Hematoxylin (Code K8008) are required but not included in the kit.

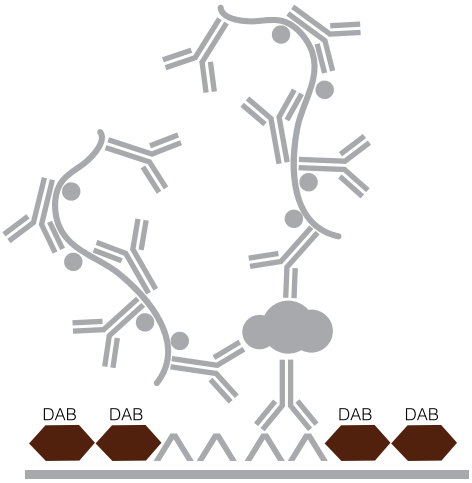
** Dr. AF Gazdar and Dr. JD Minna at NIH are acknowledged for their contribution in developing NCI-H226 (ATCC Number: CRL-5826™)*



Application of Visualization Reagent.



Application of DAB+ Substrate Chromogen Solution.



Application of DAB Enhancer.

Technical Considerations

Technical problems related to PD-L1 IHC 22C3 pharmDx may arise and can be attributed to two factors: specimen collection and preparation prior to performing the test, and the actual performance of the test itself. Technical problems are generally related to procedural deviations and can be controlled and minimized through training and, where necessary, clarification of the product instructions.

Specimen Preparation

Specimens must be handled to preserve the tissue for immunohistochemical staining. Determine intact tumor morphology and the presence of sufficient tumor cells for evaluation. Use standard methods of tissue processing for all specimens.

In-house Control Tissue

Differences in processing and embedding in the user's laboratory may produce significant variability in results. Include positive and negative in-house control tissue in each staining run, in addition to the PD-L1 IHC 22C3 pharmDx Control Cell Line Slide.

Select positive and negative control tissue from fresh specimens of the same tumor indication as the patient specimen. Fix, process, and embed the control tissue in the same manner. Control tissues processed differently from the patient specimen validate reagent performance only and do not verify tissue preparation.

The ideal positive control tissue provides a complete dynamic representation of weak-to-moderate staining of tumor cells and tumor-associated mononuclear inflammatory cells (MICs: lymphocytes and macrophages). The ideal negative control tissue should demonstrate no staining on tumor cells and immune cells. However, because prevalence of PD-L1 expression on immune cells is high, a few staining immune cells are acceptable.

Optional Additional In-house Control: Tonsil Tissue

Tonsil stained with PD-L1 should be pre-screened to exhibit strong staining in portions of the crypt epithelium and weak-to-moderate staining of the follicular macrophages in the germinal centers. PD-L1 expression of the endothelium, fibroblasts, as well as the surface epithelium should be negative.

Tissue Processing

FFPE tissues have been validated for use. Block specimens into a thickness of 3 mm or 4 mm, fix in formalin and dehydrate and clear in a series of alcohols and xylene, followed by infiltration with melted paraffin. The paraffin temperature should not exceed 60 °C. Feasibility studies on NSCLC tissue samples were performed with fixation in 10% neutral buffered formalin for 12–72 hours. Fixation times of 3 hours or less should not be used for PD-L1 assessment. The use of PD-L1 IHC 22C3 pharmDx on decalcified tissues or tissues processed with other fixatives has not been validated and is not recommended.

Cut tissue specimens into sections of 4–5 µm. After sectioning, tissues should be mounted on Dako FLEX IHC Microscope Slides (Code K8020) or Superfrost Plus slides, and then placed in a 58 ± 2 °C oven for 1 hour. To preserve antigenicity, store tissue sections in the dark at 2–8 °C and stain within 6 months of sectioning (preferred), or at room temperature up to 25 °C and stain within 4 months of sectioning.

PD-L1 IHC 22C3 pharmDx Staining Procedure

The PD-L1 IHC 22C3 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. All of the required steps and incubation times for staining are pre-programmed in the DakoLink software.

Reagent Storage

Store all components of PD-L1 IHC 22C3 pharmDx, including Control Cell Line Slides, in the dark at 2–8 °C when not in use.

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 of the package.

EnVision FLEX Target Retrieval Solution, Low pH

Dilute EnVision FLEX Target Retrieval Solution, Low pH, (50×) 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 tank. Discard 1× EnVision FLEX Target Retrieval Solution, Low pH after 3 uses or 5 days after dilution.

EnVision FLEX Wash Buffer

Dilute EnVision FLEX Wash Buffer (20×) 1:20 using distilled or deionized water (reagent-quality water). Store unused 1× buffer at 2–8 °C for no more than 1 month. 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 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 will 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 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

The following quality controls should be included in each staining run:

- One PD-L1 IHC 22C3 pharmDx Control Cell Line Slide stained with the primary antibody
- Positive and negative in-house control tissues stained with the primary antibody
- Subsequent sections of each patient specimen stained with the Negative Control Reagent

Deparaffinization, Rehydration, and Target Retrieval

Use PT Link 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 1× 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 preheated Target Retrieval Solution, Low pH in PT Link tank. 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 slides into a tank (e.g., PT Link Rinse Station, Code PT109) containing room temperature 1× EnVision FLEX Wash Buffer working solution
- Leave Autostainer rack with slides in room temperature 1× 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
- Select the PD-L1 IHC 22C3 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, Code K8008

Mounting

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

Technical Checklist

Use the checklist below to ensure correct usage of PD-L1 IHC 22C3 pharmDx:

Customer Name/Institution _____

Name and Title _____

Autostainer Link 48 Serial Number _____ Software Version _____

	Yes	No
Regular preventive maintenance is performed on the Autostainer Link 48 and PT Link?	☐	☐
PD-L1 IHC 22C3 pharmDx is used before the expiration date printed on the outside of the box?	☐	☐
All PD-L1 IHC 22C3 pharmDx components, including Control Cell Line Slides, are stored in the dark at 2–8 °C?	☐	☐
All PD-L1 IHC 22C3 pharmDx components, including Control Cell Line Slides, are equilibrated to room temperature (20–25 °C) prior to immunostaining?	☐	☐
Appropriate positive and negative control tissues from HNSCC are identified?	☐	☐
Tissues are fixed in neutral buffered formalin?	☐	☐
Tissues are infiltrated with melted paraffin, at or below 60 °C?	☐	☐
Tissue sections of 4–5 µm are mounted on Dako FLEX IHC Microscope Slides or Superfrost Plus slides?	☐	☐
Specimens are oven-dried at 58 ± 2 °C for 1 hour?	☐	☐
Specimens are stained within 6 months of sectioning when stored in the dark at 2–8 °C (preferred) or within 4 months when stored in the dark at room temperature up to 25 °C?	☐	☐
EnVision FLEX Target Retrieval Solution, Low pH is prepared properly? pH of 1× Target Retrieval Solution must be 6.1 ± 0.2.	☐	☐
EnVision FLEX Wash Buffer is prepared properly?	☐	☐
DAB+ Substrate-Chromogen Solution is prepared properly?	☐	☐
Slides are counterstained with EnVision FLEX Hematoxylin?	☐	☐
The Deparaffinization, Rehydration, and Target Retrieval 3-in-1 procedure is followed using PT Link?	☐	☐
Slides remain wet with buffer while loading and prior to initiating run on Autostainer Link 48?	☐	☐
The PD-L1 IHC 22C3 pharmDx protocol is selected on Autostainer Link 48?	☐	☐
Do you have all the necessary equipment to perform the PD-L1 IHC 22C3 pharmDx according to protocol? If not, specify what is missing in comments below.	☐	☐

Additional observations or comments:

_____

_____

Slide Evaluation

General Considerations

PD-L1 IHC 22C3 pharmDx evaluation should be performed by a qualified pathologist using a light microscope. Details of the PD-L1 IHC 22C3 pharmDx interpretation guidelines are reviewed on page 30. Before examining the patient specimen for PD-L1 staining, it is important to examine the controls to assess staining quality.

PD-L1 interpretation is best assessed by requesting 3 serial tissue sections (H&E, PD-L1 stain, and NCR stain) so that if the H&E is first assessed and is acceptable, IHC staining of the remaining 2 serial sections is likely to be acceptable.

Each PD-L1 IHC 22C3 pharmDx is configured with Control Cell Line Slides that should be included in each IHC run. Guidelines on interpreting the Control Cell Line Slide are reviewed to the right. In-house control tissue slides should also be assessed with every IHC run.

Specimen Adequacy

Confirm the Presence of at Least 100 Viable Tumor Cells

A hematoxylin and eosin (H&E) stain of the tissue specimen is evaluated first to assess tissue histology and preservation quality. PD-L1 IHC 22C3 pharmDx and the H&E staining should be performed on serial sections from the same paraffin block of the specimen. Tissue specimens should be intact, well preserved, and should confirm tumor indication.

A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide for the specimen to be considered adequate for PD-L1 evaluation.

Instructions for Patient Specimens With Less Than 100 Viable Tumor Cells

Tissue from a deeper level of the block, or potentially another block, could have a sufficient number of viable tumor cells for PD-L1 IHC 22C3 pharmDx testing.

Evaluating Controls

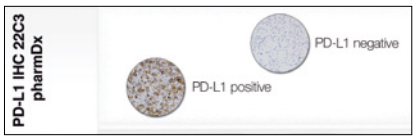


Figure 6: Each Control Cell Line Slide contains sections of cell pellets with positive and negative PD-L1 expression.

PD-L1 IHC 22C3 pharmDx Control Cell Line Slide

Examine the PD-L1 IHC 22C3 pharmDx Control Cell Line Slide to determine that reagents are functioning properly. Each slide contains sections of cell pellets with positive and negative PD-L1 expression (Figure 6). Assess the percentage of positive cells, staining intensity, and non-specific staining in both cell pellets. If any staining of the Control Cell Line Slide is not satisfactory, all results with the patient specimens should be considered invalid. Do not use the Control Cell Line Slide as an aid in interpretation of patient results.

Evaluate the overall staining intensity using the following guide:

0	Negative
1+	Weak intensity
2+	Moderate intensity
3+	Strong intensity

Positive Control Cell Pellet

The following staining is acceptable for the PD-L1 positive cell pellet (Figure 7):

- Cell membrane staining of ≥ 70% of cells
- ≥ 2+ average staining intensity
- Non-specific staining < 1+ intensity

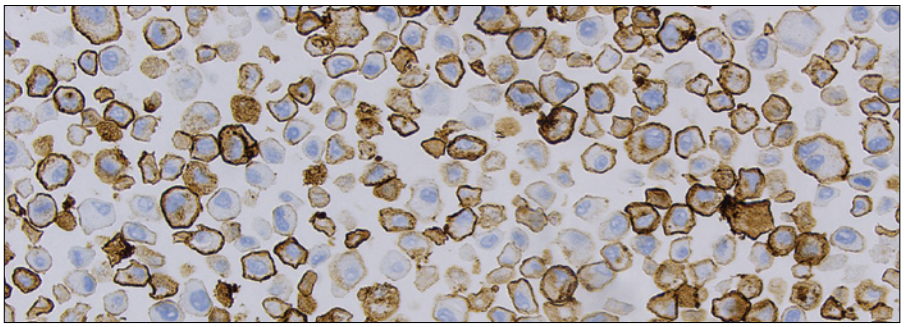


Figure 7: Positive cell pellet with acceptable staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20× magnification).

Negative Control Cell Pellet

For the PD-L1 negative cell pellet, the following staining is acceptable (Figure 8):

- The majority of cells should demonstrate no staining. **Note:** The presence of 10 or fewer cells with distinct cell membrane staining is acceptable
- Non-specific staining < 1+ intensity

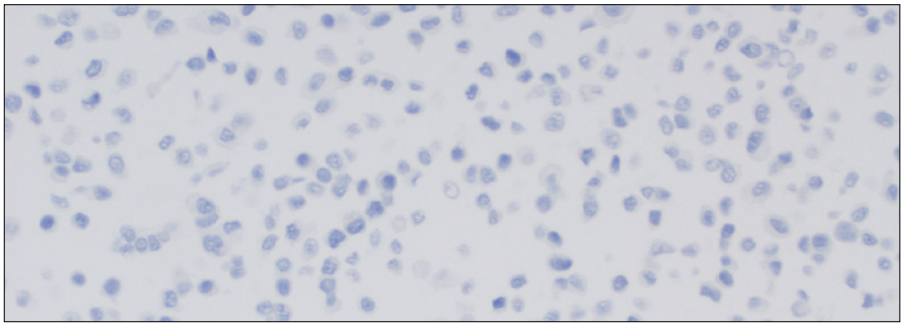


Figure 8: Negative cell pellet with no staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20× magnification).

Positive and Negative In-house Control Tissue (HNSCC)

Examine the positive in-house HNSCC control tissue to determine that the tissues are correctly prepared and reagents are functioning properly. The ideal positive control tissue provides a complete dynamic representation of weak-to-moderate staining of tumor cells and tumor-associated mononuclear inflammatory cells (MICs) (Figure 9). If staining of positive in-house control tissue is not satisfactory, all results with the patient specimen should be considered invalid.

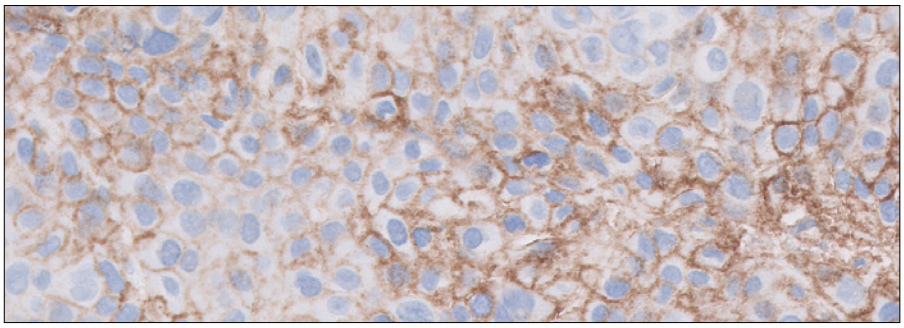


Figure 9: Positive in-house control tissue (20× magnification).

The ideal HNSCC negative control tissue should demonstrate no staining of tumor cells and immune cells (Figure 10). However, because prevalence of PD-L1 expression on immune cells is high, a few staining immune cells are acceptable. Examine the negative in-house control tissue to determine the expected staining. The variety of different cell types present in most tissue sections offers internal negative control sites; this should be verified by the user.

If unwanted staining occurs in the in-house control tissues, results with the patient specimen should be considered invalid.

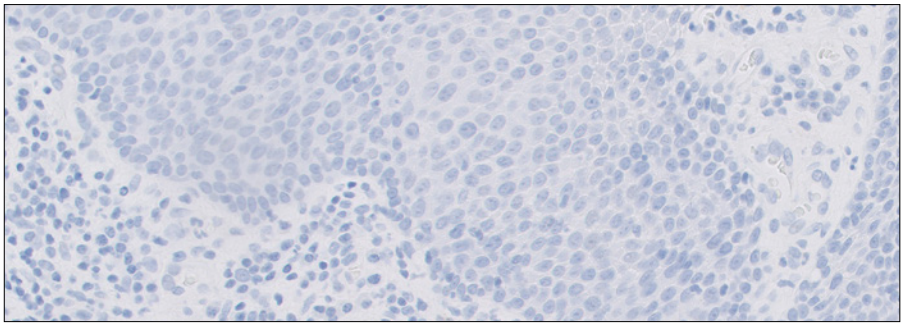


Figure 10: Negative in-house control tissue demonstrating lack of staining of tumor cells and MICs (20× magnification).

Optional Control Tissue

In addition to the Control Cell Line Slide and in-house control tissues, FFPE tonsil may also be used as an optional control specimen. Tonsil stained with PD-L1 should exhibit strong membrane staining in portions of the crypt epithelium and weak-to-moderate membrane staining of the follicular macrophages in the germinal centers (Figure 11).

PD-L1 expression of the endothelium, fibroblasts, and the surface epithelium should be absent.

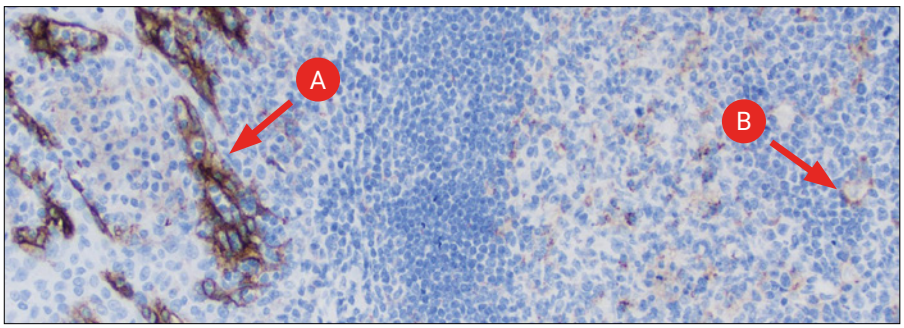


Figure 11: Tonsil stained with PD-L1 primary antibody exhibiting strong membrane staining in portions of the crypt epithelium (A) and weak-to-moderate membrane staining of follicular macrophages in the germinal centers (B) (10× magnification).

Do not use in-house control tissue as an aid in interpretation of patient results.

Negative Control Reagent (NCR)

Examine the slides stained with the NCR to identify non-specific background staining that may interfere with PD-L1 staining interpretation, making the specimen non-evaluable. Satisfactory performance is indicated by the absence of staining (Figure 12).

Examine the patient specimens stained with the NCR to determine if there is any non-specific staining that may interfere with interpreting the PD-L1 stained slide.

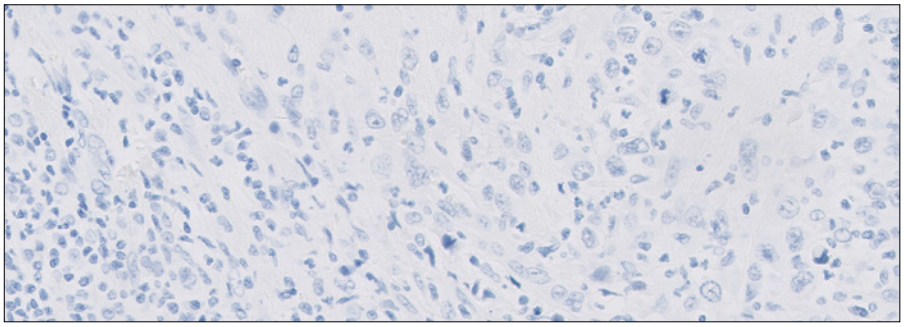


Figure 12: HNSCC tissue specimen stained with NCR (20× magnification).

NCR-stained slides indicate non-specific background staining and allow for better interpretation of patient specimens stained with the primary antibody.

Slide Evaluation Flowchart

Figure 13: Recommended order of slide evaluation.

Combined Positive Score

Definition of Combined Positive Score (CPS)

PD-L1 expression in HNSCC is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages*) divided by the total number of viable tumor cells, multiplied by 100. Although the result of the calculation can exceed 100, the maximum score is defined as CPS 100.

CPS is defined accordingly:

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells (tumor cells, lymphocytes, macrophages)}}{\text{Total \# viable tumor cells}} \times 100$$

** Macrophages and histiocytes are considered the same cells*

CPS Numerator Inclusion and Exclusion Criteria

Any perceptible and convincing partial or complete linear membrane staining (≥ 1+) of viable tumor cells that is perceived as distinct from cytoplasmic staining is considered PD-L1 staining and should be included in the scoring.

Any membrane and/or cytoplasmic staining (≥ 1+) of lymphocytes and macrophages (mononuclear inflammatory cells, MICs) within tumor nests and/or adjacent supporting stroma is considered PD-L1 staining and should be included in the CPS numerator. Only MICs directly associated with the response against the tumor are scored.

See Tables 1 and 2 on page 26 for additional CPS inclusion/exclusion criteria.

Determining Combined Positive Score

- At lower magnifications, examine all well-preserved tumor areas. Evaluate overall areas of PD-L1 staining and non-staining tumor cells, keeping in mind that partial membrane staining or 1+ membrane staining may be difficult to see at low magnifications. Ensure there are at least 100 viable tumor cells in the sample
  - A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide (biopsy and resection) for the specimen to be considered adequate for evaluation
- For specimens with less than 100 viable tumor cells, tissue from a deeper level of the block or potentially another block could have a sufficient number of tumor cells for evaluation of PD-L1 expression
- At higher magnification (20×), evaluate PD-L1 expression and calculate CPS:
  - Determine the total number of viable tumor cells, both PD-L1 staining and non-staining (CPS denominator)
  - Determine the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) (CPS numerator; see Tables 1 and 2 on page 26 for additional CPS inclusion/exclusion criteria)
  - Calculate CPS
- Evaluation of membrane staining should be performed at no higher than 20× magnification. Slide reviewer should not perform the CPS calculation at 40× magnification

Table 1: CPS Numerator Inclusion/Exclusion Criteria

Tissue Elements	Included in the Numerator	Excluded from the Numerator
Tumor Cells	Convincing partial or complete linear membrane staining (at any intensity) of viable invasive tumor cells	– Non-staining tumor cells – Tumor cells with only cytoplasmic staining
Immune Cells	Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma†: – Lymphocytes (including lymphocyte aggregates) – Macrophages‡ Only MICs directly associated with the response to the tumor are scored	– Non-staining MICs – MICs (including lymphoid aggregates) associated with ulcers or other inflammatory processes – MICs associated with carcinoma in situ – MICs associated with benign structures – Neutrophils, eosinophils, and plasma cells
Other Cells	Not included	– Carcinoma in situ – Benign cells – Stromal cells (including fibroblasts) – Necrotic cells and/or cellular debris

** In MICs, membrane and cytoplasmic staining are often indistinguishable due to high nuclear to cytoplasmic ratio. Therefore, membrane and/or cytoplasmic staining of MICs are included in the score*

† Adjacent MICs are defined as being within the same 20× field as the tumor. However, MICs that are NOT directly associated with the response to the tumor should be excluded

‡ Macrophages and histiocytes are considered the same cells

Table 2: CPS Denominator Inclusion/Exclusion Criteria

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	All viable invasive tumor cells	Any necrotic or non-viable tumor cells
Immune Cells	Not included	All immune cells of any type
Other Cells	Not included	– Carcinoma in situ – Benign cells – Stromal cells (including fibroblasts) – Necrotic cells and/or cellular debris

Suggested Methods

Agilent recommends that scoring be performed within the context of the pathologist's past experience and best judgment in interpreting IHC stains. We offer three different examples of techniques that may be used when determining the respective Combined Positive Scores (CPS) of various staining patterns.

The entire IHC slide should be reviewed to determine which of the following example techniques may be used.

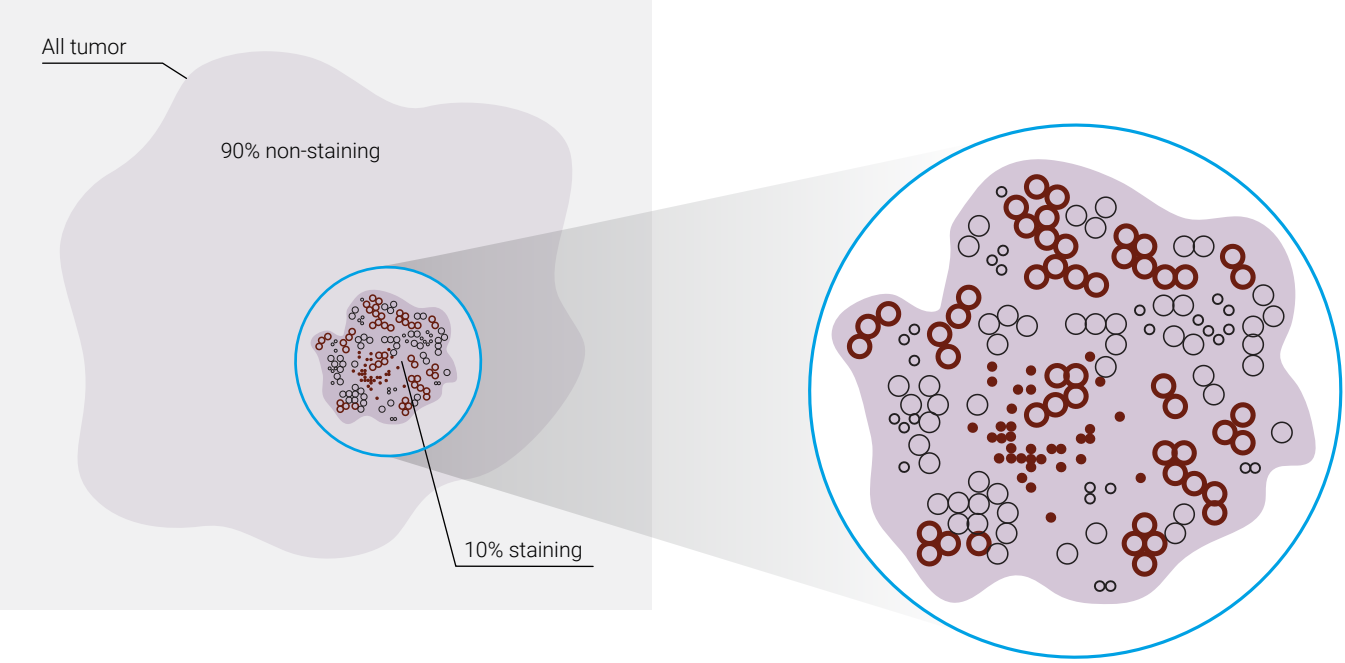
Example 1: Calculation of Combined Positive Score Based on a Small PD-L1 Staining Area

First: Evaluate the tumor area for perceptible and convincing staining as described in "Determining Combined Positive Score" on page 25.

Assessment: *10% of area shows staining, 90% of area shows no staining*

Second: Evaluate the area of staining to estimate the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages).

Assessment: *There are approximately 100 viable tumor cells and about 80 PD-L1 staining cells (per the CPS numerator)*



Calculate the Combined Positive Score of the entire tumor area:

Assessment:

CPS of area with staining:

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells}^{\S}}{\text{Total \# viable tumor cells}} \times 100 = \frac{\sim 80 \text{ PD-L1 staining cells}}{100 \text{ tumor cells}} \times 100 = 80$$

CPS of entire tumor area: 10% × 80 ≈ CPS 8

Clinical Interpretation: CPS ≥ 1

§ *Including tumor cells, lymphocytes, macrophages*

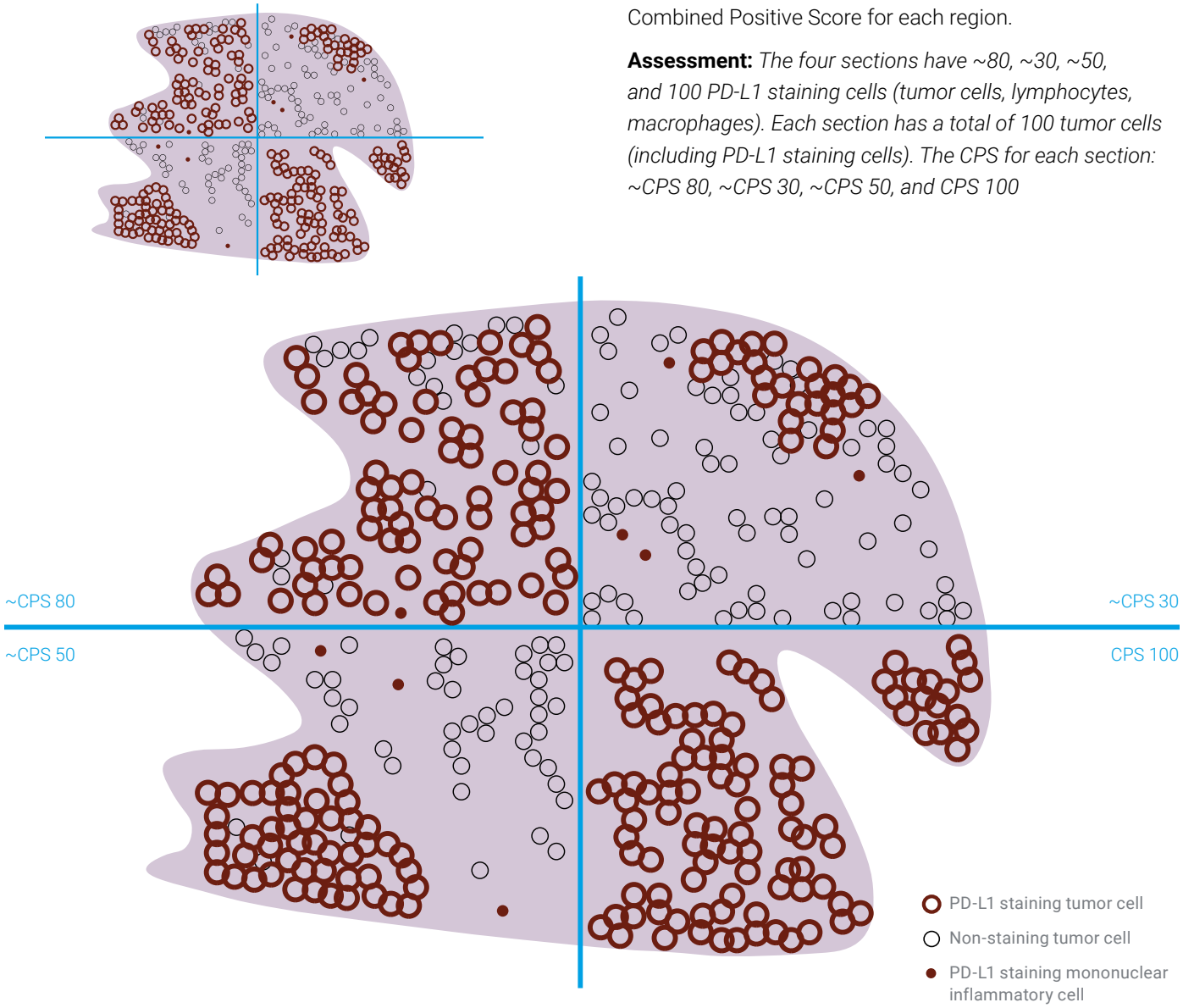
Figure 14: Example of tumor with small PD-L1 staining area.

Example 2: Calculation of Combined Positive Score Based on a Heterogeneous PD-L1 Staining Area

First: Visually divide the tumor area into regions with equal numbers of tumor cells.

Second: Observe each region and estimate the total number of viable tumor cells and PD-L1 staining cells (tumor cells, lymphocytes, macrophages). Calculate the Combined Positive Score for each region.

Assessment: *The four sections have ~80, ~30, ~50, and 100 PD-L1 staining cells (tumor cells, lymphocytes, macrophages). Each section has a total of 100 tumor cells (including PD-L1 staining cells). The CPS for each section: ~CPS 80, ~CPS 30, ~CPS 50, and CPS 100*



Calculate the Combined Positive Score of the entire tumor area:

Assessment:

Combined Positive Score:
(80 + 30 + 50 + 100) / 4 ≈ CPS 65

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells (tumor cells, lymphocytes, macrophages)}}{\text{Total \# viable tumor cells}} \times 100$$

Clinical Interpretation: CPS ≥ 20

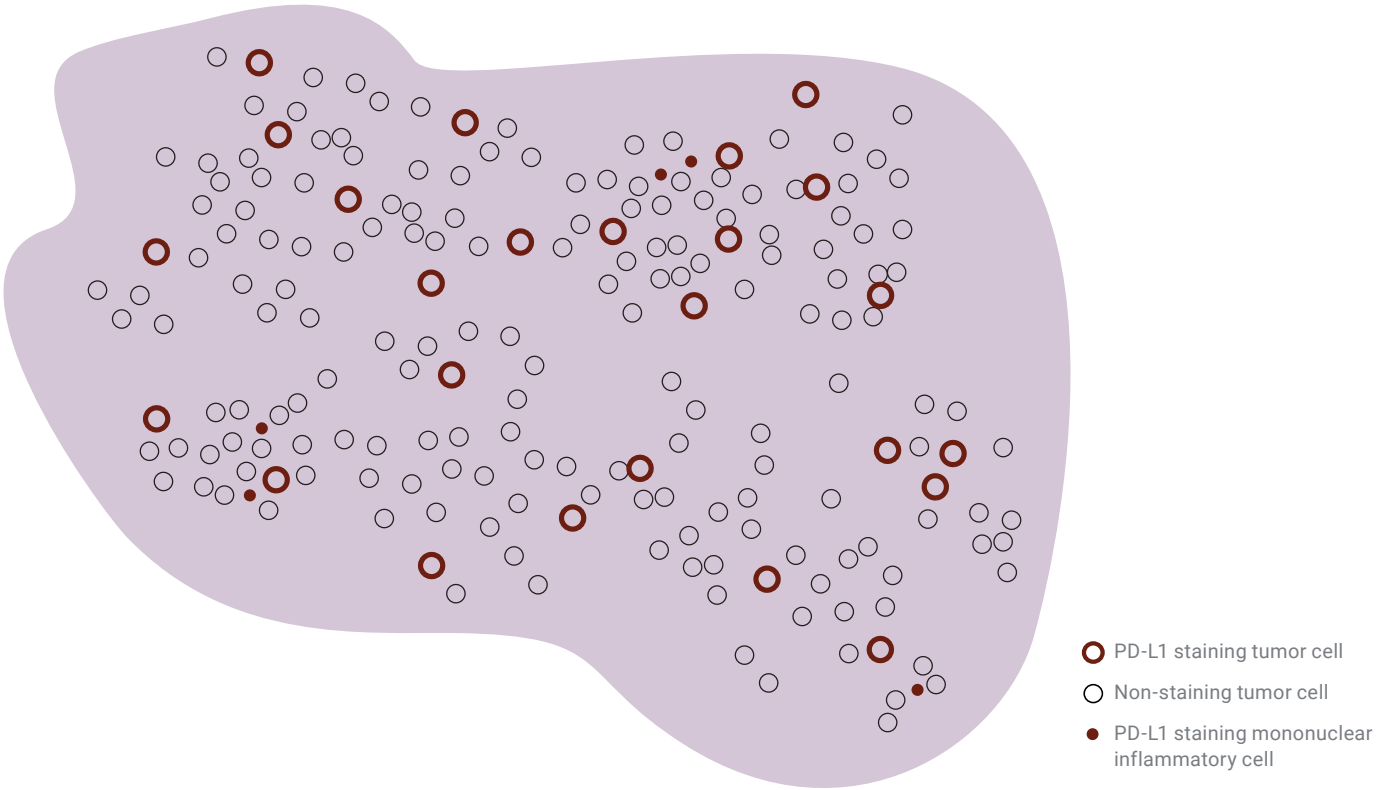
Figure 15: Example with heterogeneous PD-L1 staining area.

Example 3: Calculation of Combined Positive Score for a Near Cut-off Specimen

First: Evaluate the specimen for perceptible and convincing staining as described in "Determining Combined Positive Score" on page 25.

Second: Confirm that there is no staining in areas that appeared void of staining at lower magnifications. Evaluate all staining areas and estimate the total number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages). Then re-evaluate the entire specimen (staining and non-staining areas) and estimate the total number of viable tumor cells (PD-L1 staining and non-staining tumor cells). Calculate the Combined Positive Score.

Assessment: *Tumor specimen has perceptible and convincing staining. There are 30 PD-L1 staining cells (tumor cells, lymphocytes, macrophages). There are approximately 200 viable tumor cells present in the entire specimen*



Calculate the Combined Positive Score of the entire tumor area:

Assessment:

Combined Positive Score:

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells*}}{\text{Total \# viable tumor cells}} \times 100 = \frac{\text{30 PD-L1 staining cells}}{\text{200 tumor cells}} \times 100 = \text{CPS 15}$$

Clinical Interpretation: CPS ≥ 1

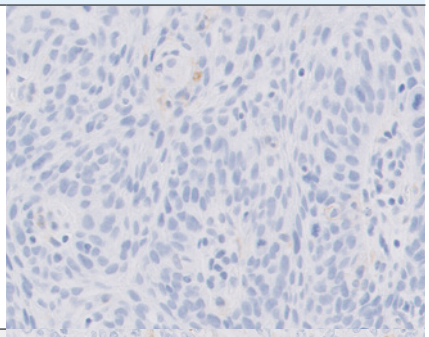
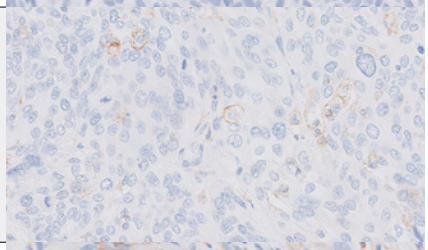
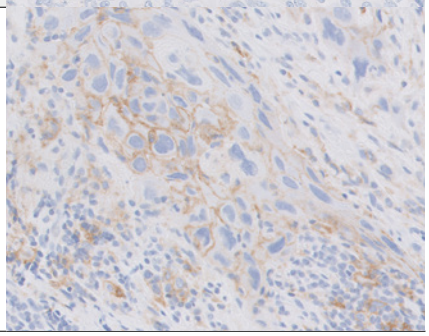
** Including tumor cells, lymphocytes, macrophages*

Figure 16: Example of near cut-off specimen.

Interpretation of CPS

The Combined Positive Score (CPS) determines the PD-L1 expression levels of the specimen. See the table below for scoring interpretation examples.

Table 3: CPS and Corresponding PD-L1 Expression Levels

CPS	PD-L1 Expression Level	Image (20× magnification)
< 1	CPS is less than 1	
≥ 1	CPS is greater than or equal to 1	
≥ 20	CPS is greater than or equal to 20	

Note: PD-L1 expression level CPS ≥ 20 may be of interest to treating physician but does not determine eligibility for first-line treatment with KEYTRUDA® (pembrolizumab) as a single agent.

Clinicians should use caution when interpreting test results at the CPS ≥ 20 cut-off because PD-L1 IHC 22C3 pharmDx failed to meet pre-specified acceptance criteria for positive percent agreement in two independent inter-site reproducibility studies and overall percent agreement in one inter-site reproducibility study conducted on HNSCC specimens at the CPS ≥ 20 cut-off. All pre-specified acceptance criteria were met in the independent inter-site reproducibility study conducted on HNSCC specimens at the CPS ≥ 1 cut-off.

Identifying Patients with HNSCC for Treatment

PD-L1 IHC 22C3 pharmDx is the only companion diagnostic indicated as an aid in identifying patients with HNSCC for treatment with KEYTRUDA.

Clinical Validation of PD-L1 IHC 22C3 pharmDx in Previously Untreated Patients With HNSCC

The clinical validity of PD-L1 IHC 22C3 pharmDx in evaluating PD-L1 expression in previously untreated patients with HNSCC is based on the KEYTRUDA KEYNOTE-048 study sponsored by Merck & Co. Specimens from previously untreated patients with metastatic or with unresectable, recurrent HNSCC were tested for PD-L1 expression using PD-L1 IHC 22C3 pharmDx. In the KEYTRUDA single agent and control* arms, 85% of enrolled HNSCC patients had tumors that expressed PD-L1 with a Combined Positive Score (CPS) of greater than or equal to 1 (Table 4). Clinical efficacy of KEYTRUDA treatment is presented in the Clinical Performance Evaluation section on pages 65–68.

Table 4: PD-L1 Prevalence in Patients with Metastatic or with Unresectable, Recurrent HNSCC Enrolled in KEYNOTE-048

PD-L1 Expression	CPS < 1	CPS ≥ 1	CPS ≥ 20†
Prevalence % (n)	15% (89)	85% (512)	43% (255)

** Cetuximab, platinum, and FU*
† Prevalence calculation for CPS ≥ 20 based on patients with known PD-L1 expression; 4 patients had unknown PD-L1 expression status

PD-L1 IHC 22C3 pharmDx Testing Scheme

Use the following flowchart to help you understand which patients are indicated for treatment with KEYTRUDA® (pembrolizumab) based on their CPS.

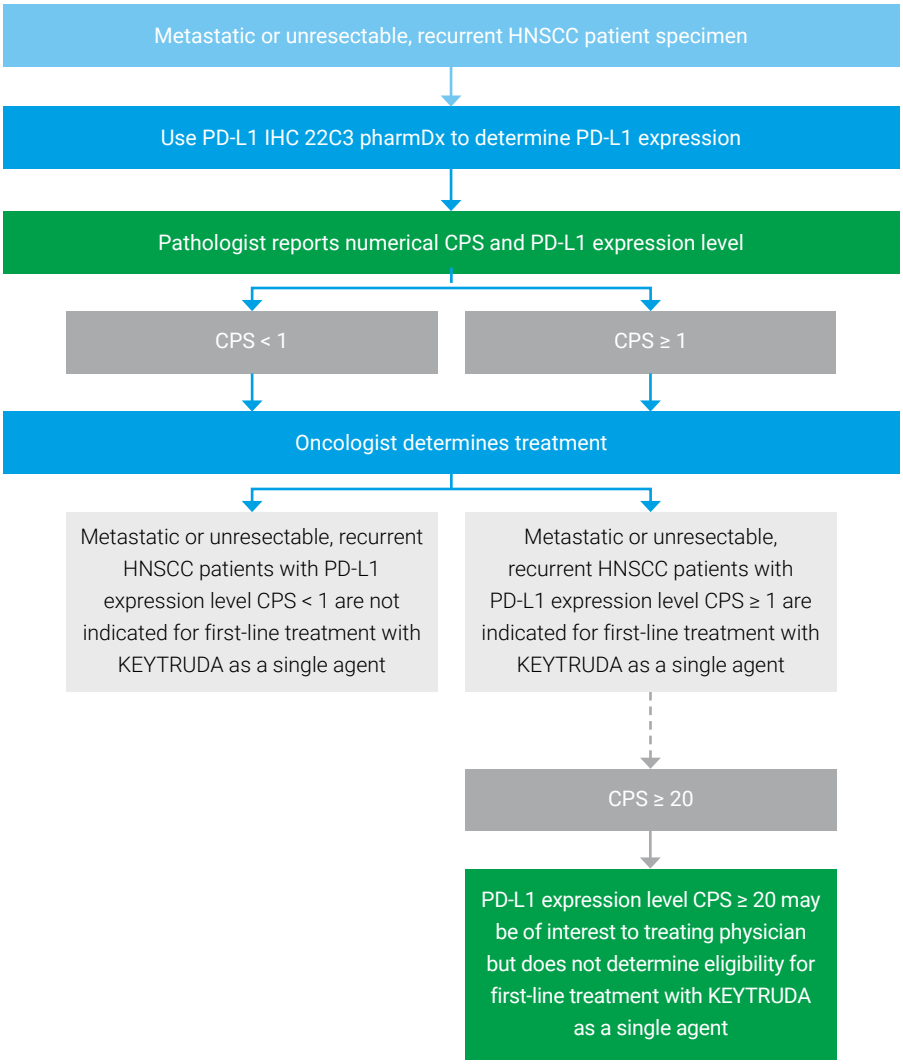


Figure 17: Testing scheme for PD-L1 IHC 22C3 pharmDx.

Reporting Results

Suggested information to include when reporting results with PD-L1 IHC 22C3 pharmDx.

PD-L1 IHC 22C3 pharmDx Summary of Sample Tested

Date of Run: _____

PD-L1 IHC 22C3 pharmDx Lot: _____

Staining Run Log ID: _____

Specimen ID: _____

Patient Identifiers: _____

Type of Service: IHC Stain with Manual Interpretation

Other: _____

PD-L1 Testing Results

Control Cell Line Slide Results: Pass: ☐ Fail: ☐

Adequate Tumor Cells Present (≥ 100 cells): Yes: ☐ No: ☐

PD-L1 IHC 22C3 pharmDx Result to Treating Physician

Combined Positive Score: _____

CPS < 1: ☐ CPS ≥ 1: ☐

CPS ≥ 20: ☐

Note: PD-L1 expression level CPS ≥ 20 may be of interest to treating physician but does not determine eligibility for first-line treatment with KEYTRUDA as a single agent.

Comments to Treating Physician:

– KEYTRUDA, as a single agent, is indicated for the first-line treatment of patients with metastatic or with unresectable, recurrent HNSCC whose tumors express PD-L1 (CPS ≥ 1) as determined by an FDA-approved test. See the KEYTRUDA prescribing information for details.

Combined Positive Score Summary and Examples

Key Considerations in Scoring PD-L1 IHC 22C3 pharmDx Stained Specimens

By definition, PD-L1 staining cells in HNSCC are:

- Viable tumor cells with perceptible and convincing partial or complete linear membrane staining (at any intensity) that is perceived distinct from cytoplasmic staining
- Lymphocytes and macrophages (mononuclear inflammatory cells, MICs) within the tumor nests and/or adjacent supporting stroma with membrane and/or cytoplasmic staining (at any intensity). MICs must be directly associated with the response against the tumor

PD-L1 expression status in HNSCC is determined by Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100.

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells (tumor cells, lymphocytes, macrophages)}}{\text{Total \# viable tumor cells}} \times 100$$

This section will define and illustrate scoring inclusions and exclusions for accurate determination of Combined Positive Score. All images are HNSCC, except for Figure 35, which is squamous cell carcinoma from the cervix.

Image Guide for Interpretation of PD-L1 IHC 22C3 pharmDx Staining in HNSCC

PD-L1 Staining Cells Included in the Combined Positive Score (CPS)

Tumor cells, lymphocytes, and macrophages exhibiting appropriate PD-L1 expression are defined as PD-L1 staining cells. All PD-L1 staining cells are included in the CPS numerator for determination of the Combined Positive Score (see Tables 1 and 2 on page 26 for additional CPS inclusion/exclusion criteria). All viable tumor cells should be included in the denominator. Below are common staining characteristics of PD-L1 staining cells that must be included in the CPS numerator. All images are HNSCC unless otherwise noted in the figure caption.

Tumor Cells

Linear Membrane Staining

Tumor cells exhibiting perceptible and convincing partial and/or complete smooth or granular linear membrane staining are considered PD-L1 staining cells. Linear membrane staining can be present at any intensity and must be perceptible and convincing at no higher than 20× magnification.

Perceptible and convincing staining of tumor cells (linear membrane staining) is often heterogeneous, with various staining intensities present.

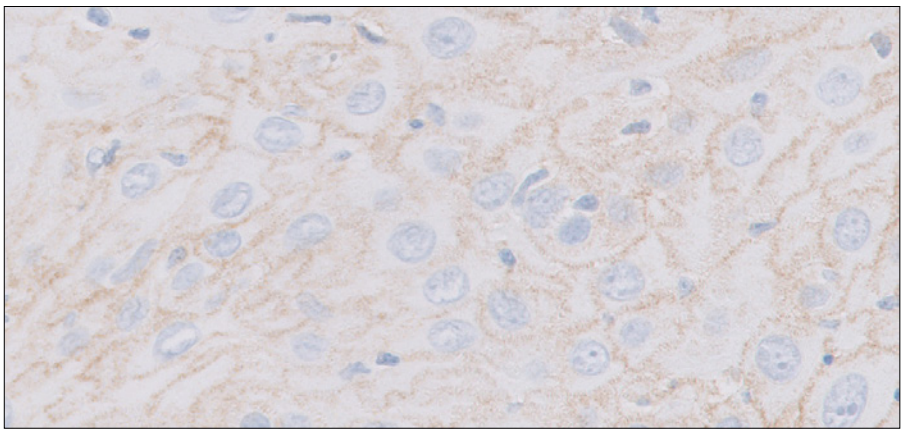


Figure 18a: HNSCC specimen stained with PD-L1 primary antibody exhibiting 1+ linear membrane staining of tumor cells (20× magnification).

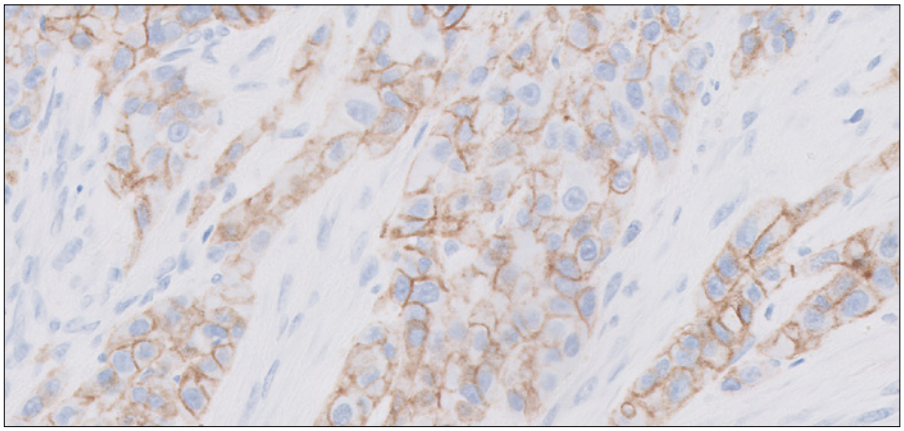


Figure 18b: HNSCC specimen stained with PD-L1 primary antibody exhibiting 2+ linear membrane staining of tumor cells (20× magnification).

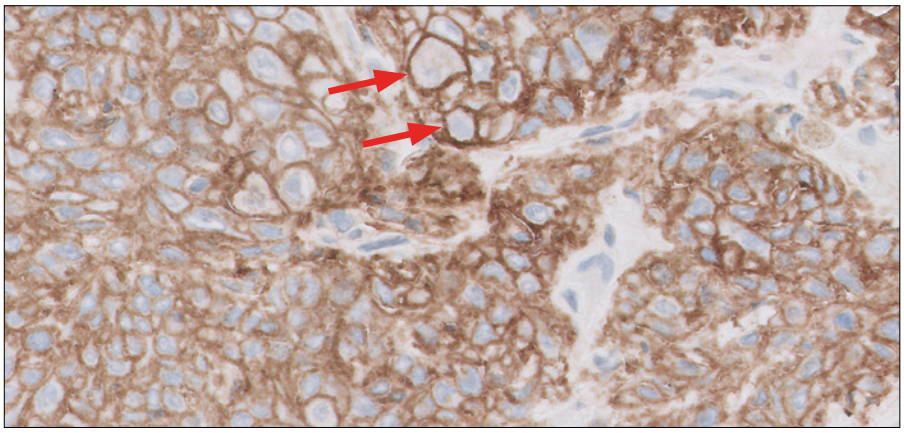


Figure 18c: HNSCC specimen stained with PD-L1 primary antibody exhibiting 3+ linear membrane staining of tumor cells (arrows) (20× magnification).

Key Point

Perceptible and convincing linear membrane staining of tumor cells at any intensity should be included in the CPS numerator

Partial Linear Membrane Staining

Tumor cells can exhibit partial linear membrane staining. Any partial linear membrane staining observed at any intensity at a 20× magnification must be included in the CPS numerator.

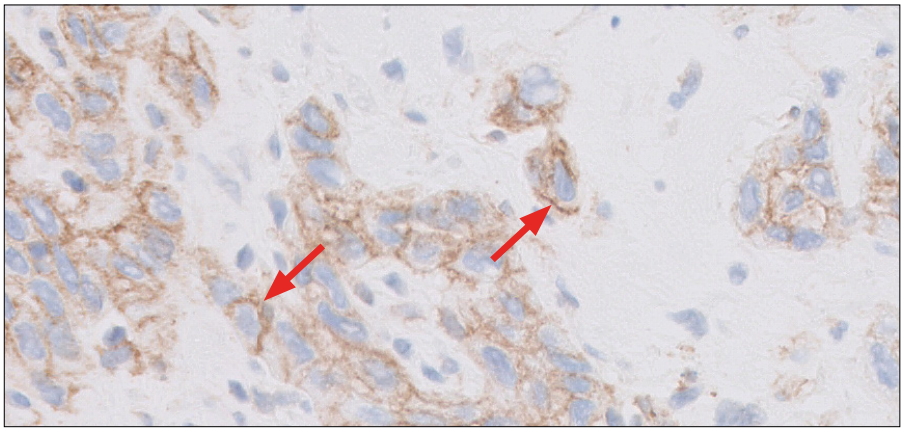


Figure 19: HNSCC specimen stained with PD-L1 primary antibody exhibiting partial linear membrane staining of tumor cells (arrows) (20× magnification).

Key Point

Perceptible and convincing partial linear membrane staining of tumor cells should be included in the CPS numerator

Linear Membrane and Cytoplasmic Staining

Tumor cells with both perceptible and convincing linear membrane staining (≥ 1+ intensity) and cytoplasmic staining at 20× magnification should be included in the CPS numerator. Tumor cells exhibiting only cytoplasmic staining are excluded from the CPS numerator, as this is considered non-specific staining. Additionally, linear PD-L1 staining of tumor cells can be smooth or granular. If partial or complete linear membrane staining is distinct from cytoplasmic staining, then the cell should be included in the CPS numerator.

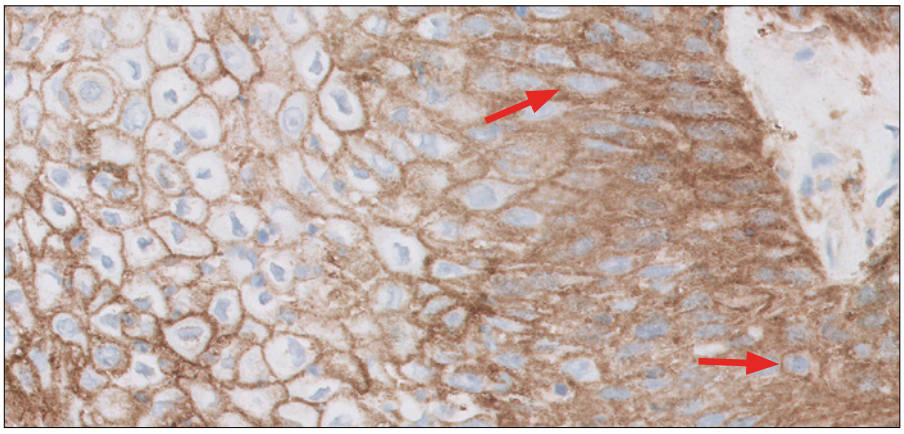


Figure 20: HNSCC specimen stained with PD-L1 primary antibody exhibiting linear membrane staining distinct from cytoplasmic staining (arrows) (20× magnification).

Key Point

Tumor cells exhibiting perceptible and convincing linear membrane staining that is distinct from cytoplasmic staining are included in the CPS numerator

Granular Staining

Tumor cells can exhibit a granular membrane staining pattern where membrane and cytoplasmic staining are indistinguishable. Only perceptible and convincing membrane staining of tumor cells (≥ 1+ intensity) observed at no higher than 20× magnification should be included in the CPS numerator.

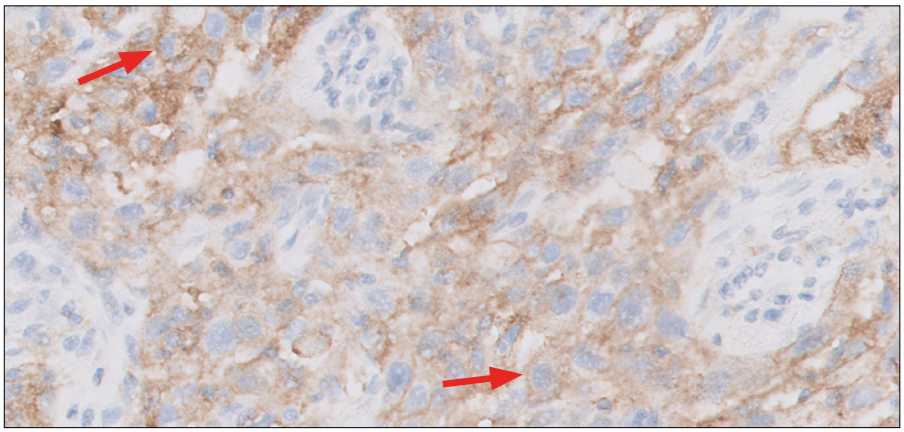


Figure 21: HNSCC specimen stained with PD-L1 primary antibody exhibiting granular linear membrane staining pattern (arrows) (20× magnification).

Key Point

Granular staining of tumor cells must exhibit a perceptible and convincing linear membrane pattern to be included in the CPS numerator

Multinucleate Tumor Cells

Some tumor cells in HNSCC may be multinucleate and each multinucleate tumor cell should be counted as one cell. The same rules should apply for inclusion in the numerator and denominator: all viable tumor cells should be included in the denominator and all tumor cells with partial or complete linear membrane staining should be included in the numerator. Additionally, multinucleate macrophages are commonly seen in HNSCC and, if PD-L1 staining is present on these cells, they should be counted as one cell and included in the numerator.

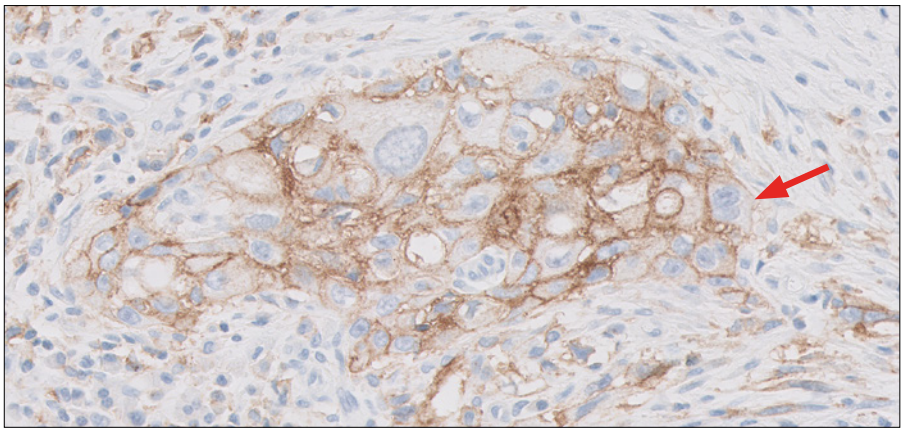


Figure 22: Multinucleate tumor cell (arrow) (20× magnification).

Key Point

Multinucleate tumor cells can be seen in HNSCC and follow the same criteria for inclusion/exclusion as mononucleate tumor cells

Immune Cells

Tumor-associated Mononuclear Inflammatory Cells (MICs)

Tumor-associated lymphocytes and macrophages (mononuclear inflammatory cells, MICs) exhibiting membrane and/or cytoplasmic staining at a 20× magnification (≥ 1+ intensity) are considered PD-L1 staining cells and should be included in the CPS numerator. Tumor-associated MICs are present within the tumor nests and/or adjacent supporting stroma and are directly associated with the response against the tumor.

Staining of tumor-associated lymphocytes and macrophages (membrane and/or cytoplasmic) is often heterogeneous, with various staining intensities present.

Note: PD-L1 staining lymphocytes often have indistinguishable membrane and cytoplasmic staining due to a high nuclear to cytoplasmic ratio; PD-L1 staining macrophages often have distinct membrane staining and low cytoplasmic staining. All PD-L1 staining tumor-associated MICs should be included in the CPS numerator.

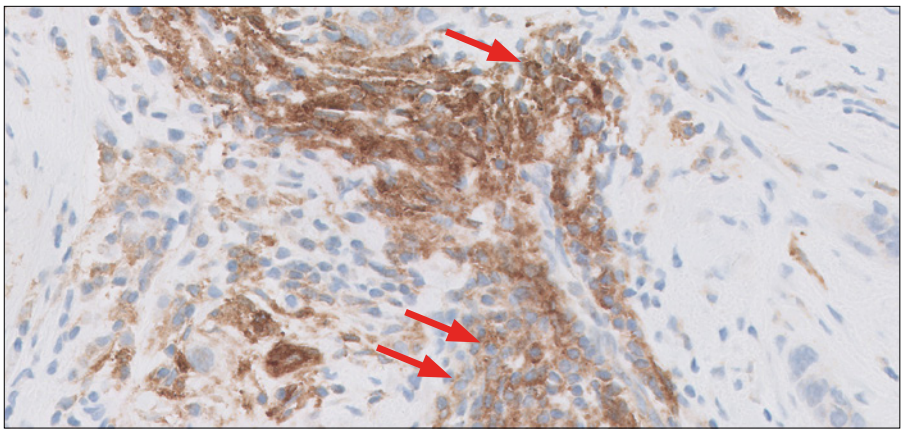


Figure 23a: HNSCC specimen stained with PD-L1 primary antibody exhibiting staining of tumor-associated lymphocytes (arrows) (20× magnification).

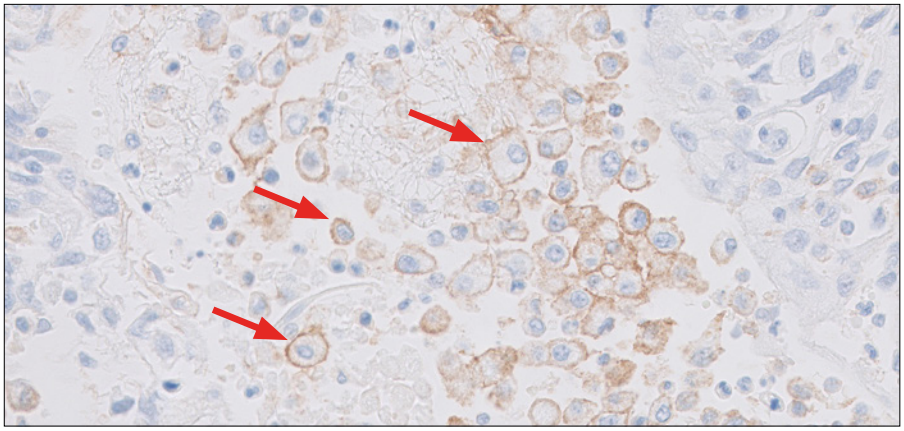


Figure 23b: HNSCC specimen stained with PD-L1 primary antibody exhibiting staining of tumor-associated macrophages (arrows) (20× magnification).

Key Point

Tumor-associated lymphocytes and macrophages with membrane and/or cytoplasmic staining should be included in the CPS numerator

Immune Cell Inclusion/Exclusion: 20× Rule

PD-L1 staining mononuclear inflammatory cells (MICs) must be directly associated with the response against the tumor to be included in the CPS numerator. MICs are considered tumor-associated if they are present within the tumor nests and/or adjacent supporting stroma within a 20× magnification field of view. In cases where it is difficult to tell if MICs are tumor-associated, the following is suggested as a guideline:

Move the slide so that the tumor is in the approximate center of a 20× field. Immune cells surrounding the tumor in this field should be included in scoring. Immune cells outside of this field should be excluded from scoring as long as they do not surround neighboring tumor cells. In general, include PD-L1 staining MICs that are within 0.5 mm of the tumor cells. This rule may be applied to tumors within lymph nodes that contain PD-L1 staining MICs. See Figures 24a–24c for an example of determining which MICs are included in the CPS numerator.

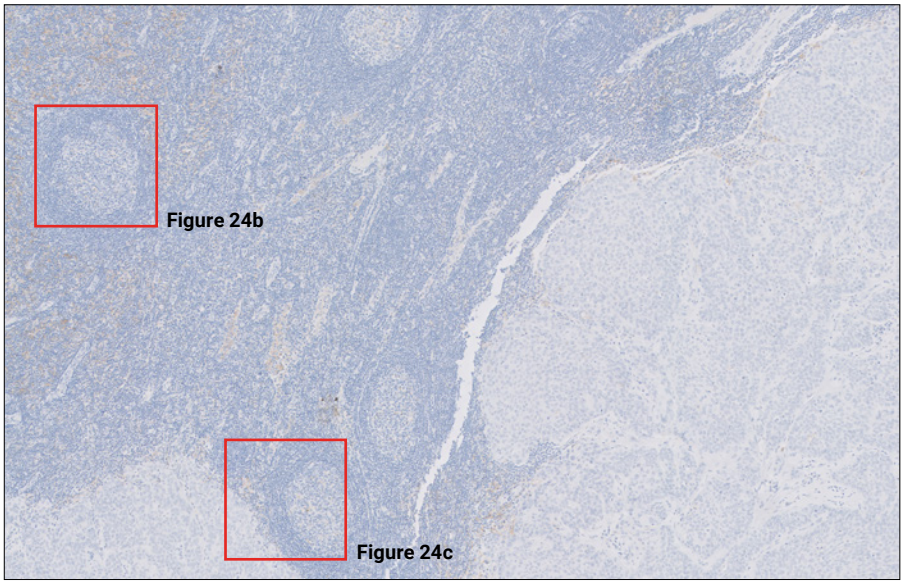


Figure 24a: At 5× magnification, several areas of PD-L1 staining mononuclear inflammatory cells are visible. To demonstrate which immune cells to include in the numerator, zoom in to 20× magnification on the boxed fields (5× magnification).

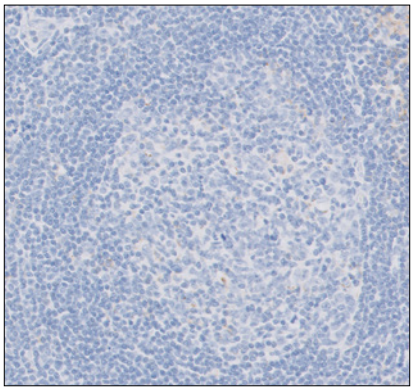


Figure 24b: Tumor cells are absent from this 20× field containing PD-L1 staining mononuclear inflammatory cells, thus none of these cells should be included in the numerator (20× magnification).

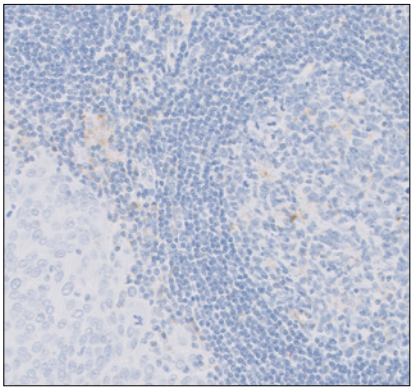


Figure 24c: When positioning the tumor cells in the approximate center of a 20× field, PD-L1 staining mononuclear inflammatory cells that are present within the same field should be included in the numerator (20× magnification).

Tumor Cell Density Patterns

HNSCC includes different morphologies that can impact the Combined Positive Score (CPS) by increasing or decreasing the total number of tumor cells that are included in the denominator. A squamous cell pattern with well-differentiated, cytoplasmic rich tumor cells will commonly have fewer cells per 20× field, whereas a poorly-differentiated, basaloid pattern will commonly have a higher number of tumor cells per 20× field. The more tumor cells included in the denominator, the greater the number of PD-L1 staining tumor cells, lymphocytes, and macrophages that are needed in the numerator to bring the overall score to CPS 1 or above. As a guideline, if tumor cells are 20 µm in diameter and fill a 20× field, there would be approximately 2500 tumor cells in that field.

Moderate Density Pattern

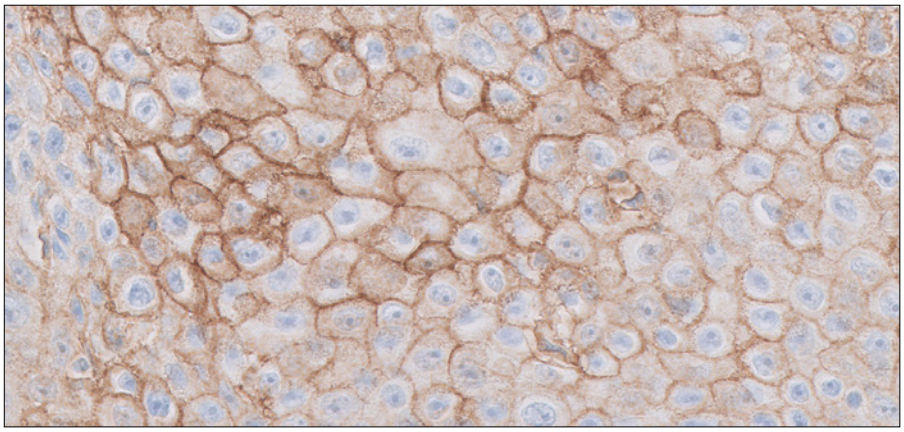


Figure 25: HNSCC specimen stained with PD-L1 primary antibody exhibiting moderately differentiated tumor cell pattern (20× magnification).

High Density Pattern

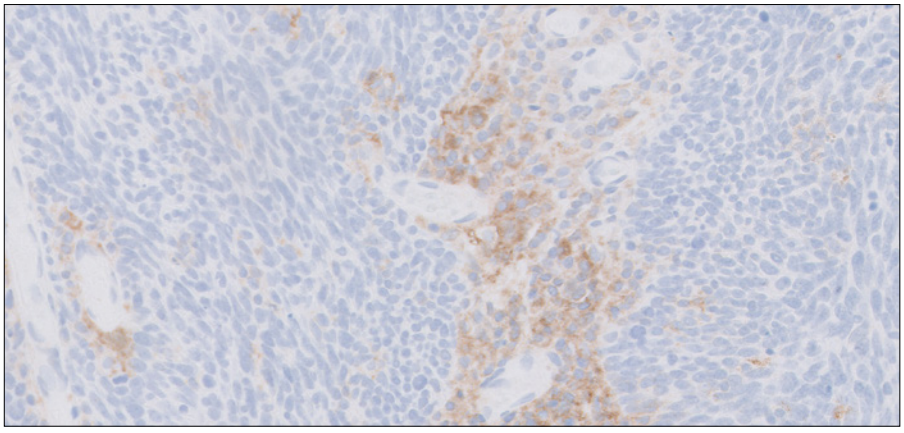


Figure 26: HNSCC specimen stained with PD-L1 primary antibody exhibiting poorly differentiated, basaloid tumor cell pattern (20× magnification).

Low Density Pattern

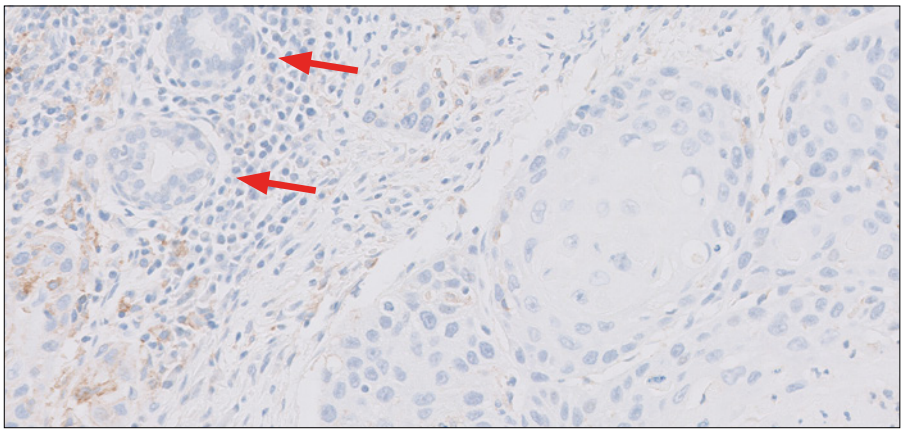


Figure 27: HNSCC specimen stained with PD-L1 primary antibody exhibiting well differentiated, highly cytoplasmic tumor cell pattern. Note the two salivary gland acini (arrows) that should be excluded from the score (20× magnification).

Key Point

The tumor cell density pattern can impact the CPS by increasing or decreasing the total number of tumor cells in the denominator

Cells Excluded from CPS

Only tumor cells exhibiting PD-L1 membrane staining and MICs exhibiting PD-L1 membrane and/or cytoplasmic staining should be included in the CPS numerator. Below are other cells that can exhibit PD-L1 expression but should be excluded from the CPS calculation (CPS numerator and/or denominator).

Note: Images that follow represent the most common exclusion elements, therefore not all exclusions are represented by images in this manual. Please refer to Tables 1 and 2 on page 26 to view all exclusion criteria.

Tumor Cells with Only Cytoplasmic Staining

Tumor cells exhibiting only cytoplasmic staining are excluded from the CPS numerator. They should, however, still be included in the CPS denominator.

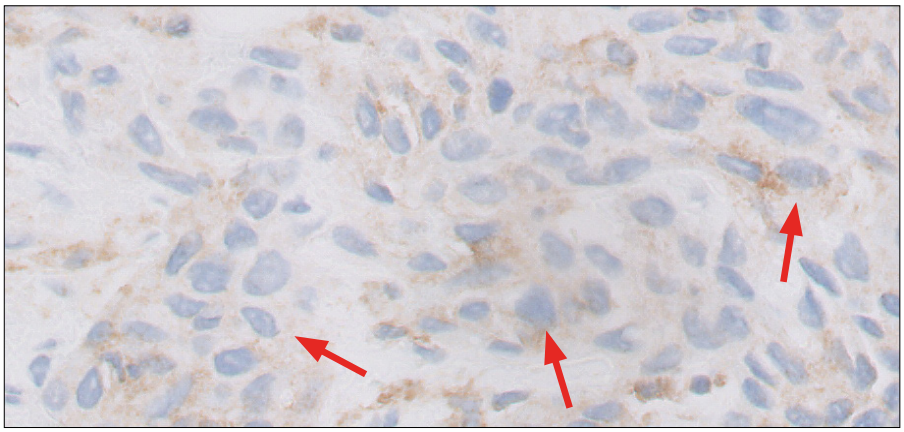


Figure 28: HNSCC specimen stained with PD-L1 primary antibody exhibiting only cytoplasmic staining (arrows) (20× magnification).

Key Point

Tumor cells exhibiting only cytoplasmic staining should not be included in the CPS numerator

Benign Glands

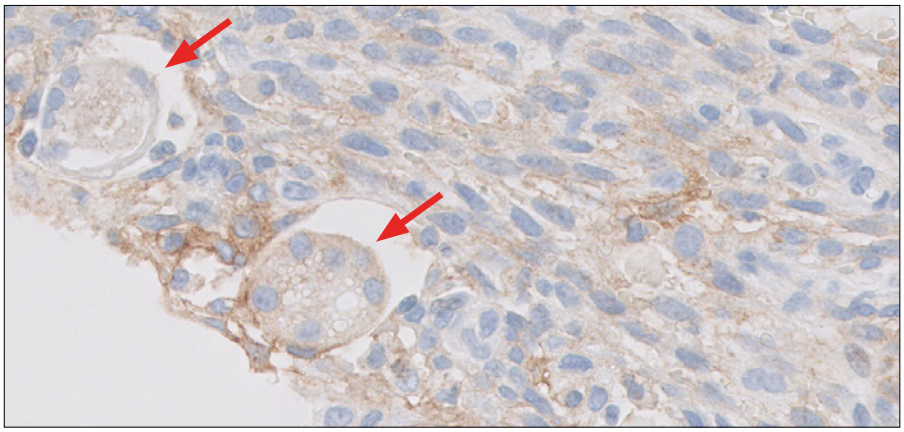


Figure 29: Entrapped salivary gland acini (arrows) (20× magnification).

Key Point

Entrapped salivary gland acini may exhibit PD-L1 staining and should be excluded from the score

Carcinoma In Situ (CIS)

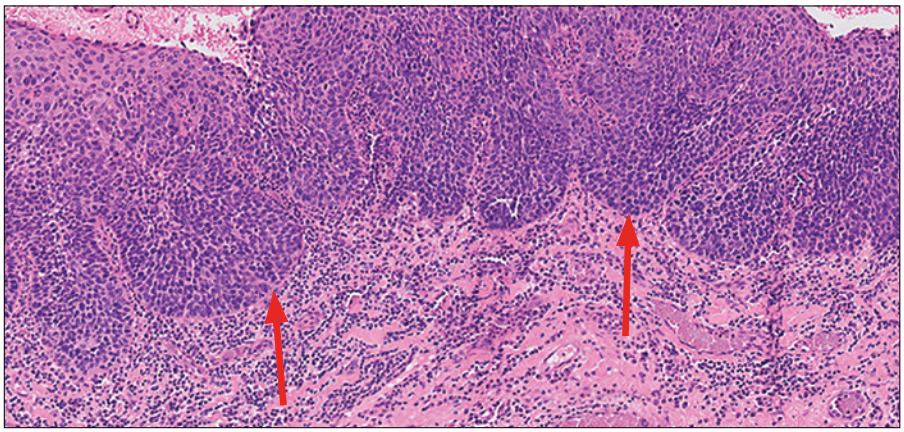


Figure 30a: Hematoxylin and eosin (H&E) section demonstrating HNSCC in situ (CIS) (arrows) (5× magnification).

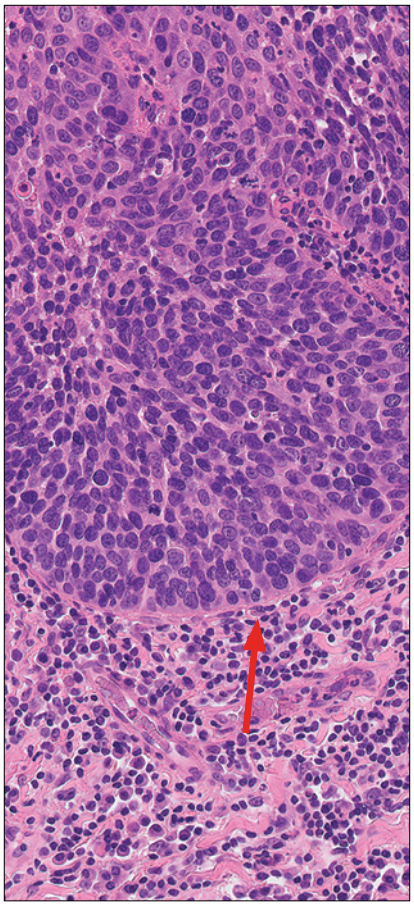


Figure 30b: Hematoxylin and eosin (H&E) section demonstrating HNSCC in situ (CIS) (arrow) (10× magnification).

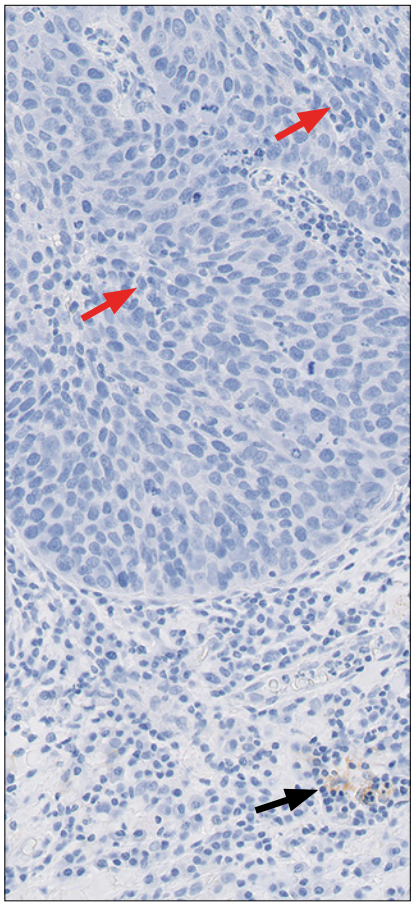


Figure 30c: Any tumor cells that are part of the CIS component should be excluded from the numerator and denominator (red arrows). Any mononuclear inflammatory cells (MICs) (black arrow) associated with the CIS component should be excluded from the numerator (10× magnification).

Key Point

Any tumor cells and MICs associated with the CIS component should be excluded from the score

Normal Tonsil

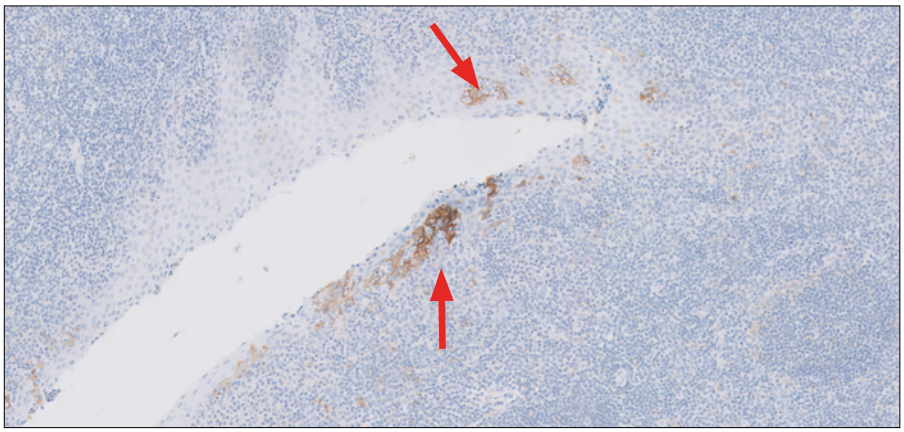


Figure 31: Normal tonsil tissue cells exhibiting PD-L1 staining (arrows) in the crypt epithelium (10× magnification).

Key Point

Normal tonsil can contain cells exhibiting PD-L1 staining in the crypt epithelium and may show considerable cytologic atypia, and should not be mistaken for cancer cells

Stromal Cells

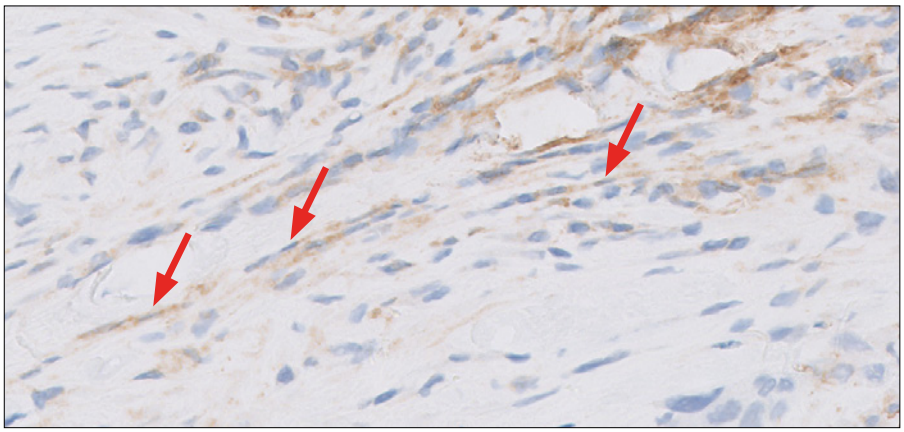


Figure 32: PD-L1 staining on stromal cells (arrows) (20× magnification).

Key Point

Stromal cells exhibiting PD-L1 staining should be excluded from the score

Other Immune Cells Excluded from CPS

Various types of immune cells can exhibit PD-L1 staining, but only tumor-associated lymphocytes and macrophages should be included in the CPS calculation. Refer to page 41 for the immune cell inclusion/exclusion 20× rule. PD-L1 staining neutrophils, eosinophils, and plasma cells should be excluded from the score.

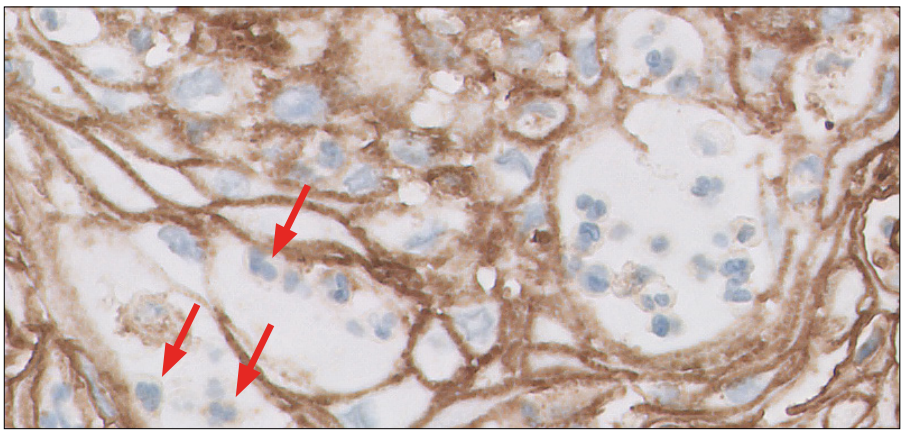


Figure 33a: PD-L1 staining on neutrophils (arrows) (20× magnification).

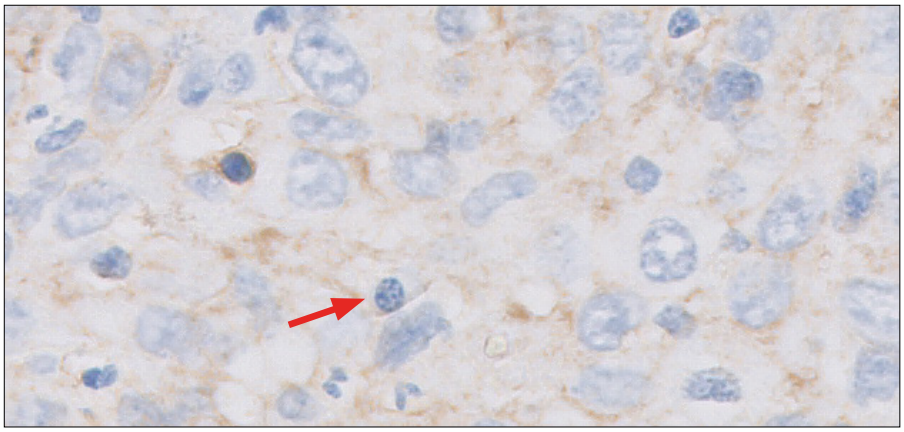


Figure 33b: PD-L1 staining on a plasma cell (arrow) (20× magnification).

Key Point

PD-L1 staining neutrophils, eosinophils, and plasma cells should be excluded from the score

Artifacts

The following pages provide examples of artifacts you may see when staining with PD-L1 IHC 22C3 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 the section
- Incomplete rinsing of slides during staining
- 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 NCR-stained test section is useful in determining the level of background staining in the PD-L1 stained test section. All specimens must have ≤ 1+ non-specific background staining.

The use of fixatives other than neutral buffered formalin may be a source of background staining and is not recommended. Background staining with PD-L1 IHC 22C3 pharmDx is rare.

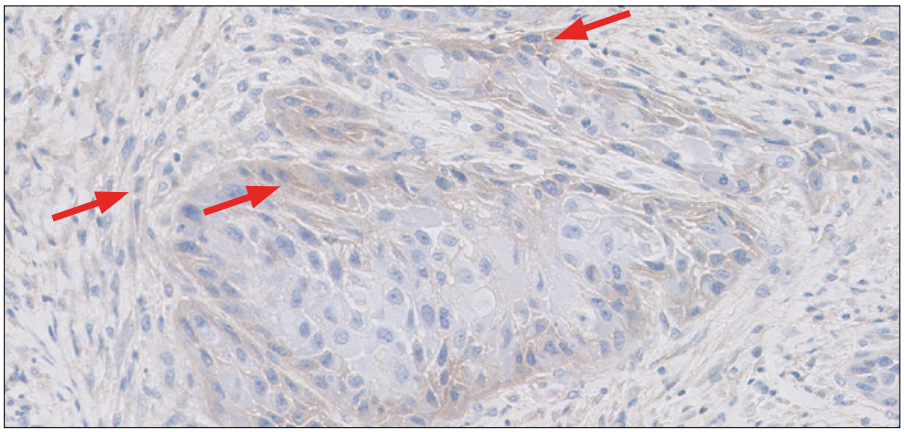


Figure 34a: HNSCC specimen stained with PD-L1 primary antibody exhibiting non-specific background staining; cells with non-specific background staining (arrows) should be excluded from the score (20× magnification).

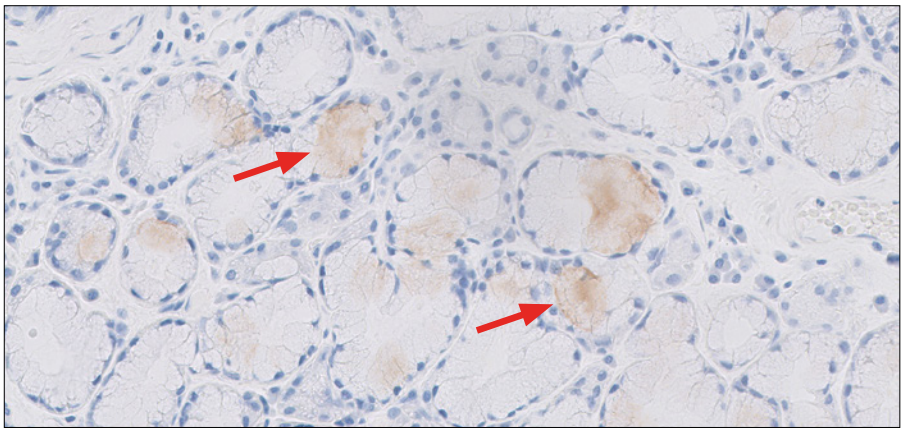


Figure 34b: HNSCC specimen stained with PD-L1 primary antibody exhibiting non-specific DAB staining; non-specific DAB staining (arrows) should be excluded from the score (20× magnification).

Key Point

All specimens must have ≤ 1+ non-specific background staining

Edge Artifact

Commonly, edge artifact is linked to the following pre-analytic factors:

- Thick tissue sections
- Drying of tissue prior to fixation or during staining procedure

Both factors can lead to accentuation of staining at the periphery of the section, and minimal staining or non-staining in the central portion. In this case, only PD-L1 staining at the edge of the tissue section is excluded from scoring.

Note: Although edge artifact can be present, it is not as commonly seen as in other IHC stains.

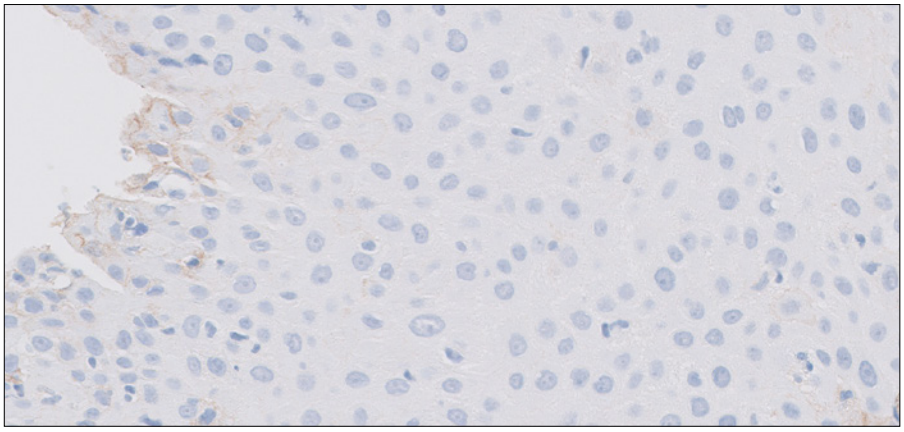


Figure 35: Edge staining should be excluded from the score (20× magnification). **Note:** Squamous cell carcinoma from the cervix is depicted.

Key Point

Scoring of the edge of a specimen should be avoided if staining is inconsistent with the rest of the specimen

Crush Artifact

Areas of the examined section exhibiting cytologically and morphologically distorted secondary crush artifact may show exaggerated staining and should be excluded from the score.

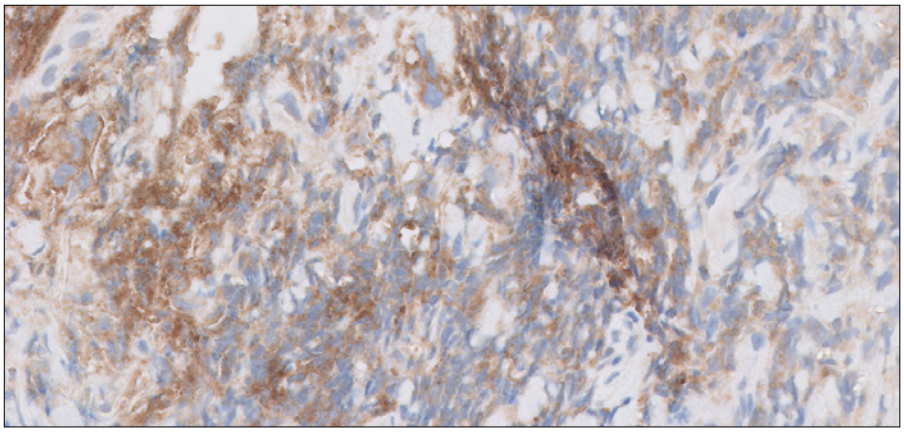


Figure 36: HNSCC specimen stained with PD-L1 primary antibody exhibiting crush artifact; crush artifact should be excluded from the score (20× magnification).

Key Point

Scoring of crush artifact should be avoided

Necrosis

Necrosis can be described as morphological changes indicative of cell death with undefined cellular detail. PD-L1 staining necrosis is often present in HNSCC specimens and should be excluded from the score.

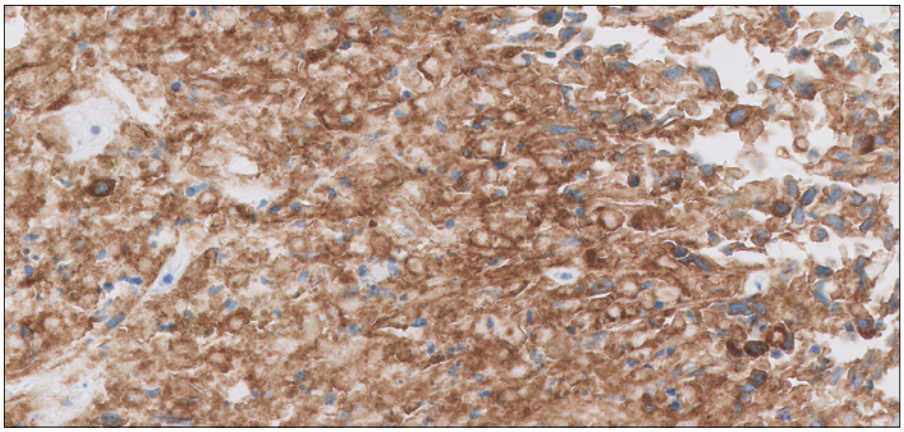


Figure 37: HNSCC specimen stained with PD-L1 primary antibody exhibiting staining of necrosis; necrosis staining should be excluded from the score (20× magnification).

Key Point

Scoring of necrotic areas should be excluded from the CPS calculation

PD-L1 IHC 22C3 pharmDx CPS Case Examples

CPS < 1 Case Examples

Case 1: CPS 0

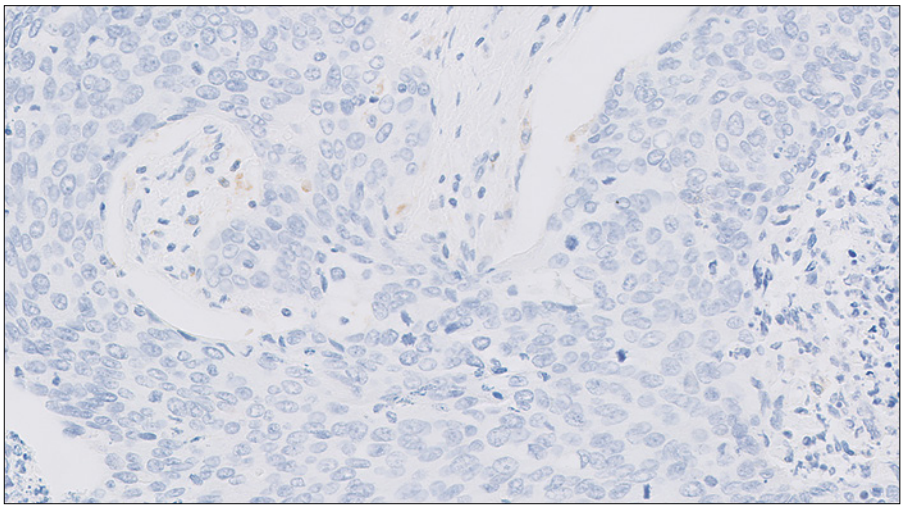


Figure 38: HNSCC specimen stained with PD-L1 antibody exhibiting CPS 0 (20× magnification).

Case 2: CPS 0

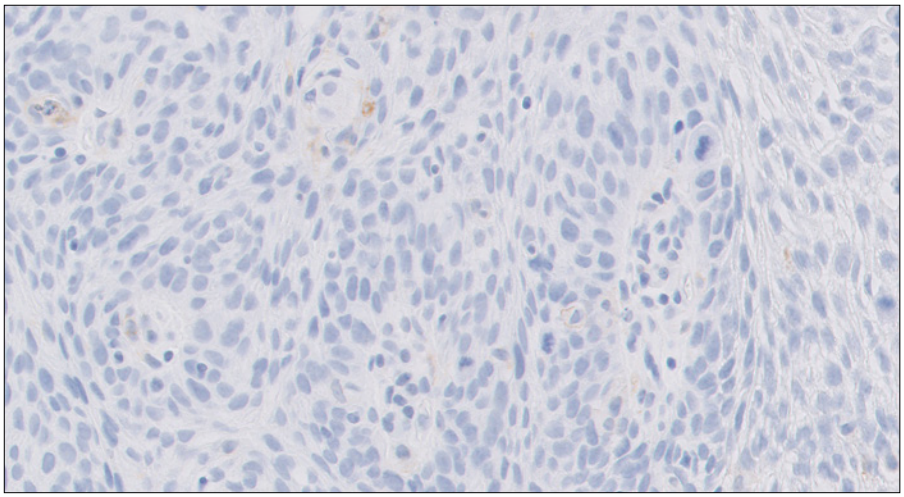


Figure 39: HNSCC specimen stained with PD-L1 antibody exhibiting CPS 0 (20× magnification).

Near Cut-off Case Examples (CPS Range of Greater Than 0 but Less Than or Equal to 10)

Challenging Case 1: Near Cut-off (CPS Range of Greater Than 0 but Less Than or Equal to 10)

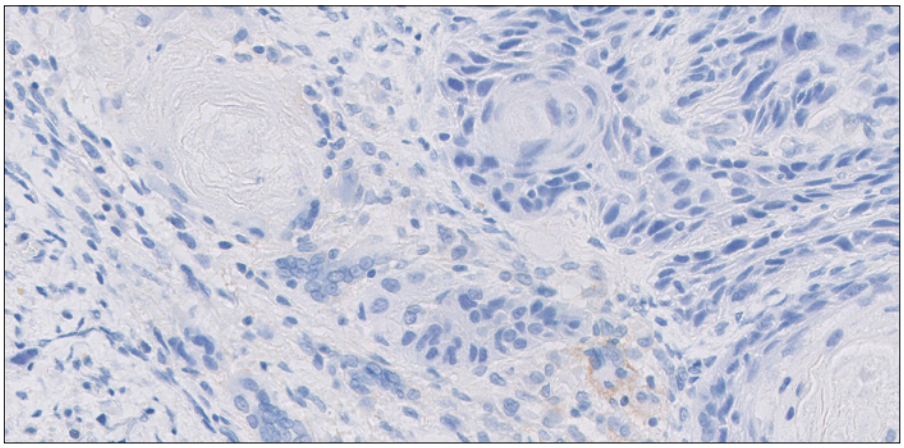


Figure 40: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 1, however any numerical CPS between 1–2 could be assigned to this image (20× magnification).

Challenging Case 2: Near Cut-off (CPS Range of Greater Than 0 but Less Than or Equal to 10)

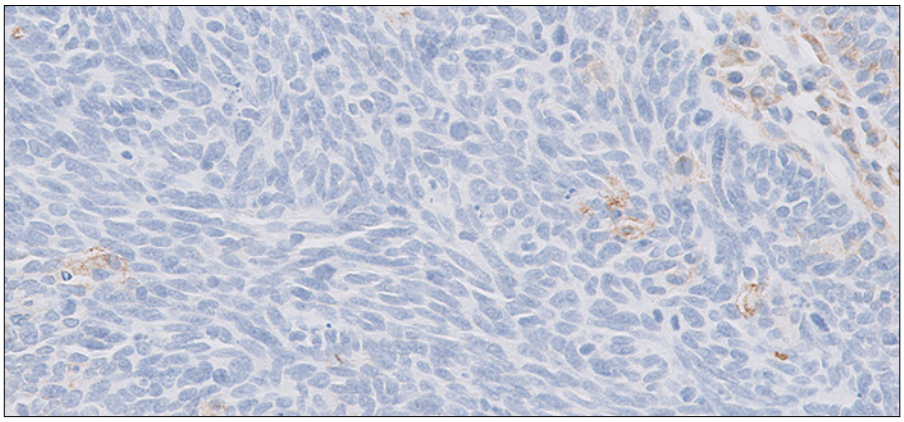


Figure 41: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 2, however any numerical CPS between 1–3 could be assigned to this image (20× magnification).

Challenging Case 3: Near Cut-off (CPS Range of Greater Than 0 but Less Than or Equal to 10)

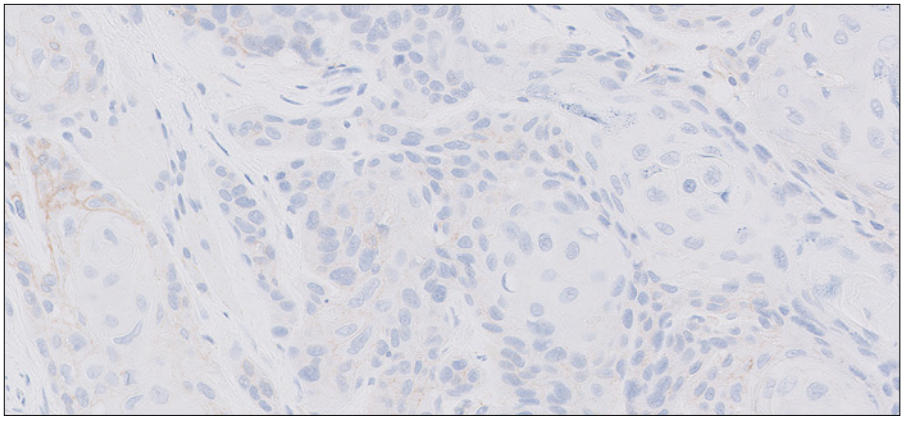


Figure 42: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).

Challenging Case 4: Near Cut-off (CPS Range of Greater Than 0 but Less Than or Equal to 10)

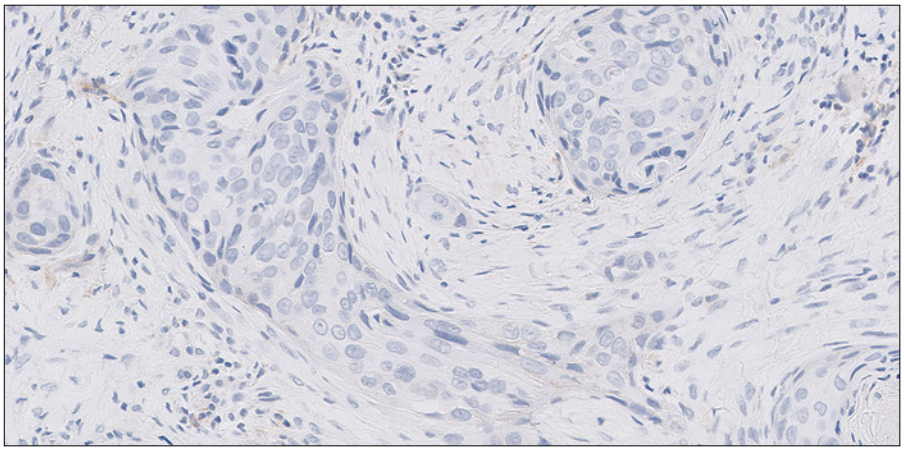


Figure 43: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 5, however any numerical CPS between 3–7 could be assigned to this image (20× magnification).

Challenging Case 5: Near Cut-off (CPS Range of Greater Than 0 but Less Than or Equal to 10)

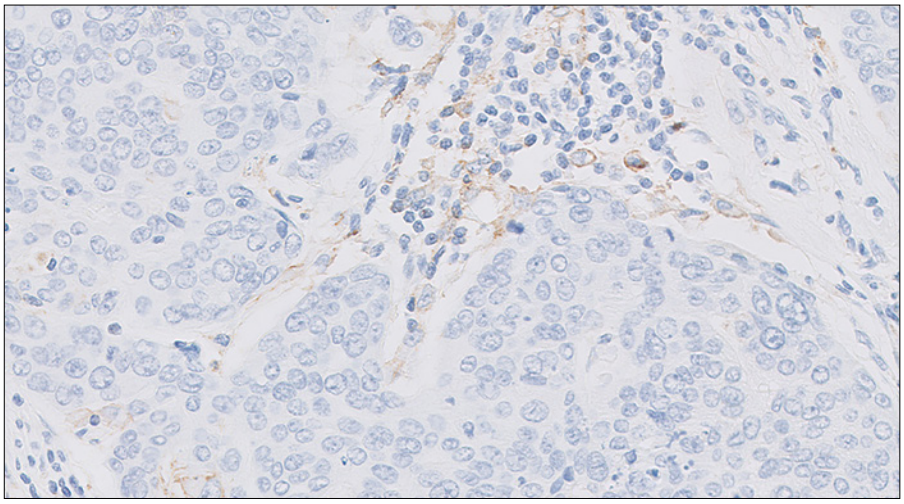


Figure 44: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 8, however any numerical CPS between 6–10 could be assigned to this image (20× magnification).

Challenging Case 6: Near Cut-off (CPS Range of Greater Than 0 but Less Than or Equal to 10)

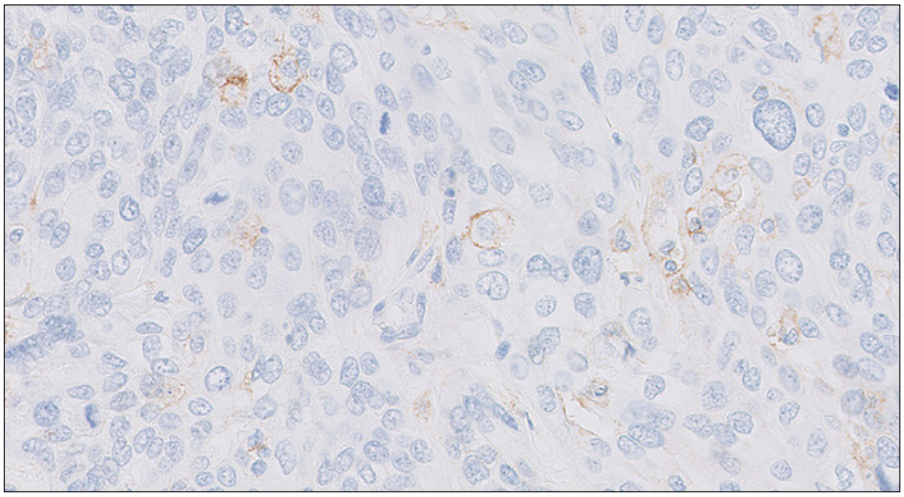


Figure 45: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 6, however any numerical CPS between 4–8 could be assigned to this image (20× magnification).

Challenging Case 7: Near Cut-off (CPS Range of Greater Than 0 but Less Than or Equal to 10)

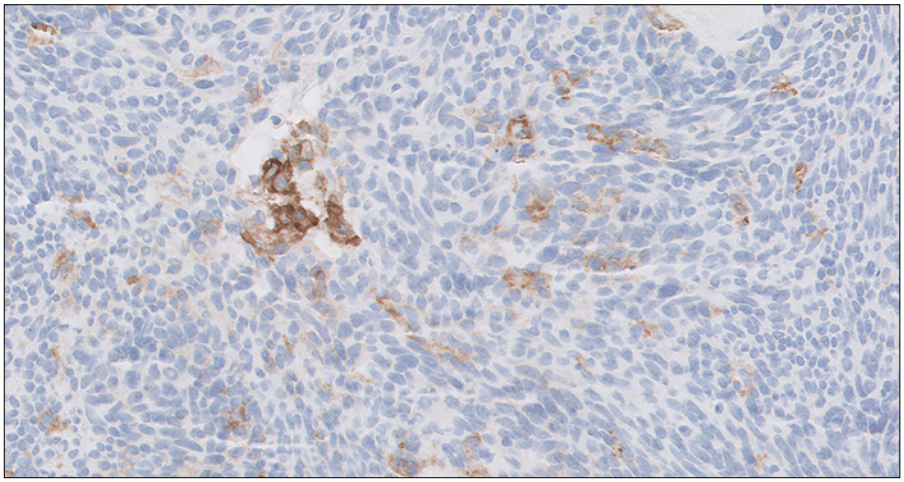


Figure 46: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 4, however any numerical CPS between 2–6 could be assigned to this image (20× magnification).

Challenging Case 8: Near Cut-off (CPS Range of Greater Than 0 but Less Than or Equal to 10)

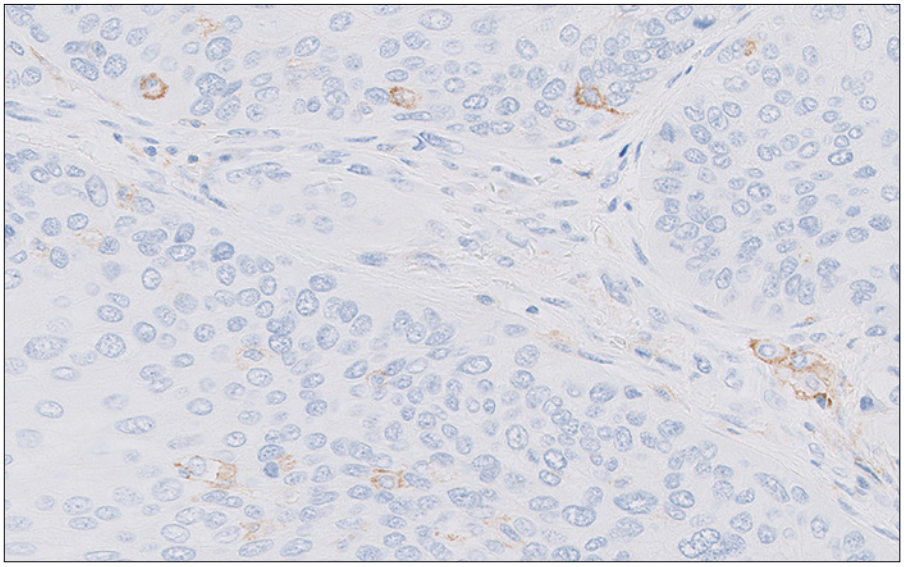


Figure 47: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 4, however any numerical CPS between 2–6 could be assigned to this image (20× magnification).

Challenging Case 9: Near Cut-off (CPS Range of Greater Than 0 but Less Than or Equal to 10)

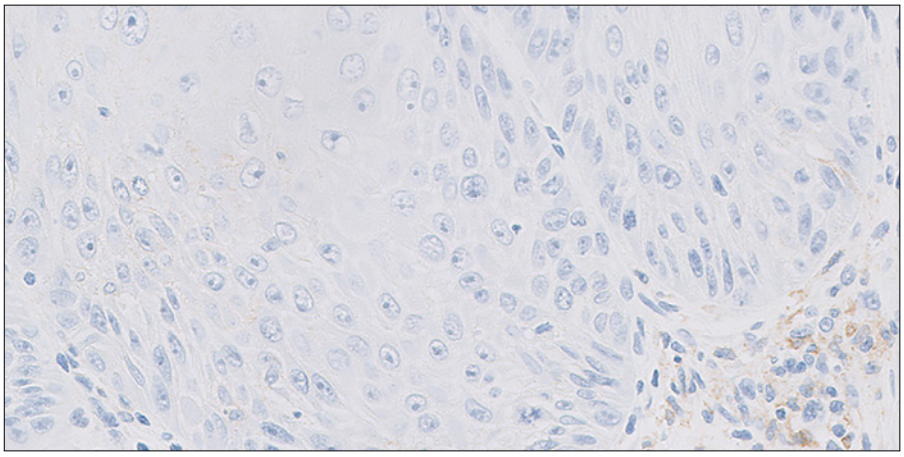


Figure 48: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 6, however any numerical CPS between 4–8 could be assigned to this image (20× magnification).

CPS Range of Greater Than 10 but Less Than or Equal to 30

Challenging Case 10: CPS Range Greater Than 10 but Less Than or Equal to 30

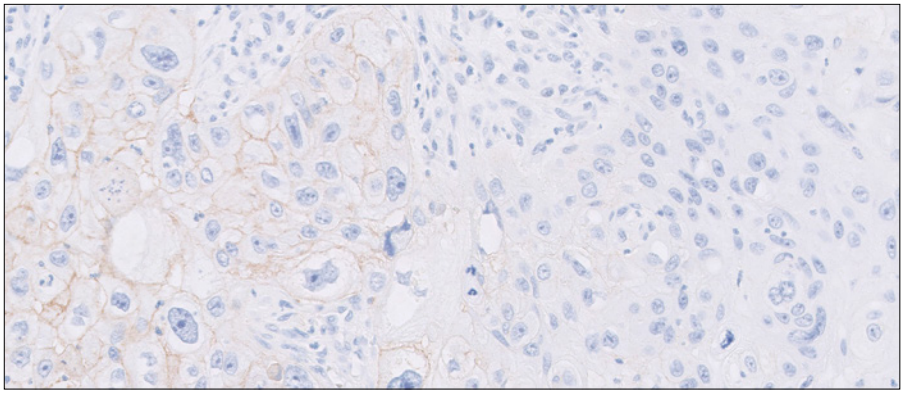


Figure 49: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 22, however any numerical CPS between 20–25 could be assigned to this image (20× magnification).

Challenging Case 11: CPS Range Greater Than 10 but Less Than or Equal to 30

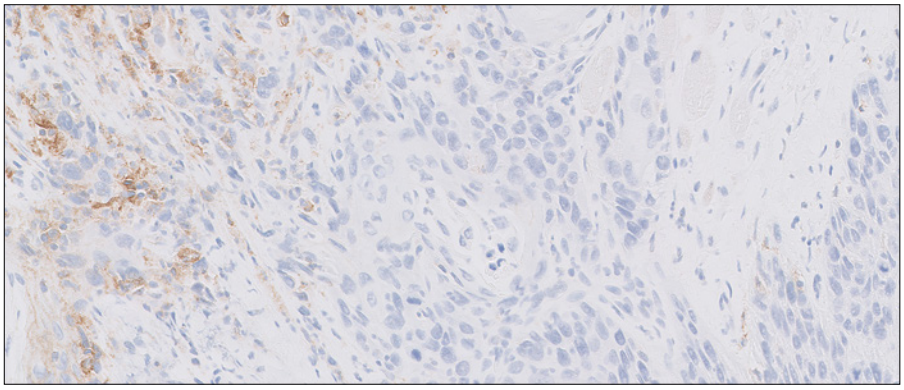


Figure 50: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 25, however any numerical CPS between 20–30 could be assigned to this image (20× magnification).

Challenging Case 12: CPS Range Greater Than 10 but Less Than or Equal to 30

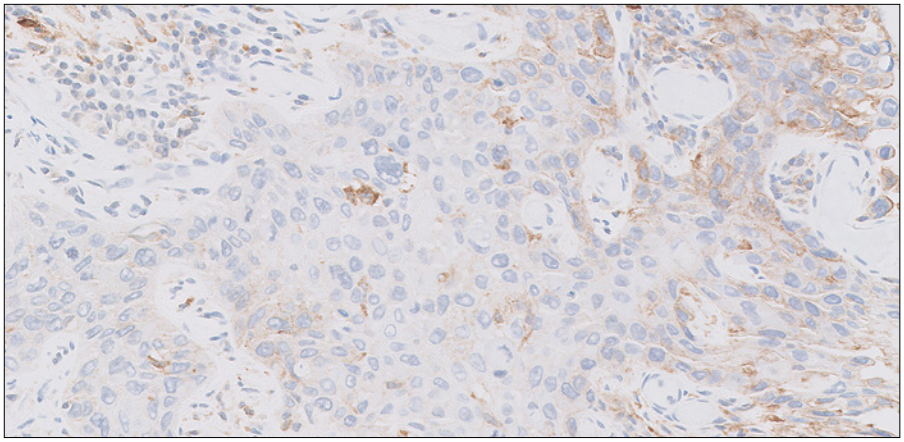


Figure 51: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 25, however any numerical CPS between 20–30 could be assigned to this image. PD-L1 staining plasma cells in the upper left corner of the field should be excluded from scoring (20× magnification).

Challenging Case 13: CPS Range Greater Than 10 but Less Than or Equal to 30

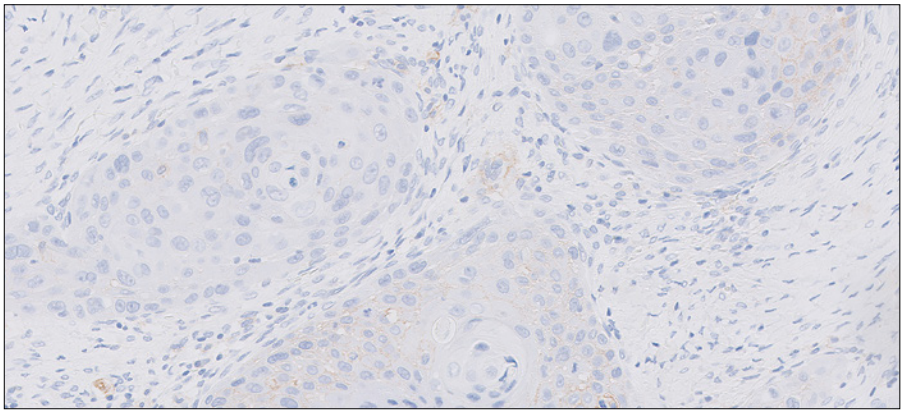


Figure 52: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 22, however any numerical CPS between 20–25 could be assigned to this image (20× magnification).

CPS ≥ 20 Case Examples

Case 3: CPS ≥ 20

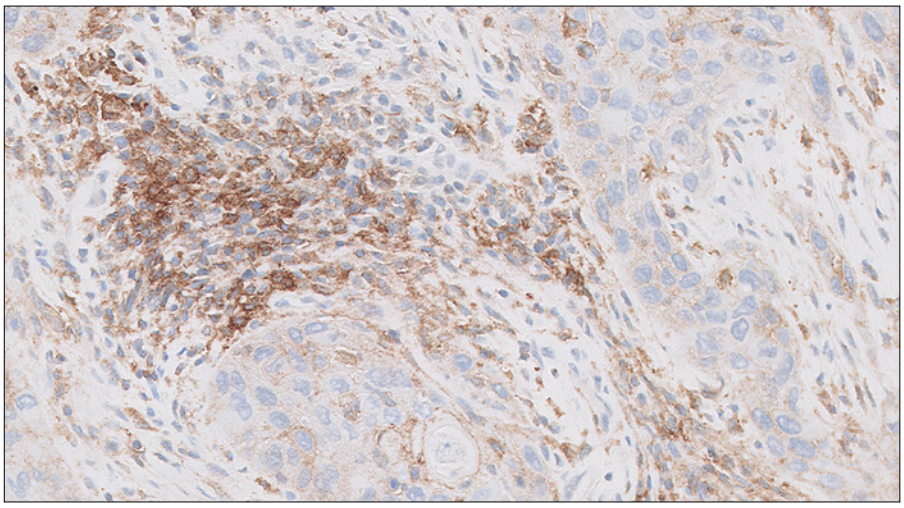


Figure 53: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 100 (20× magnification).

Case 4: CPS ≥ 20

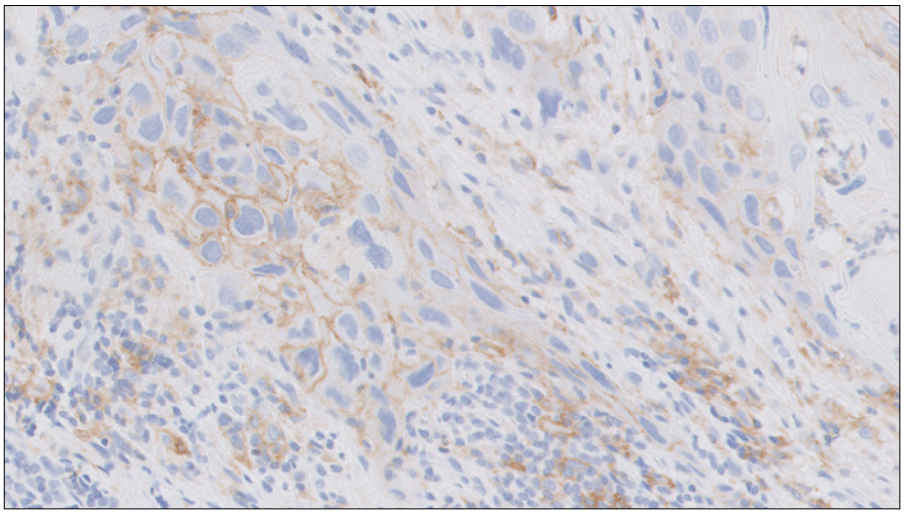


Figure 54: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 100 (20× magnification).

Case 5: CPS ≥ 20

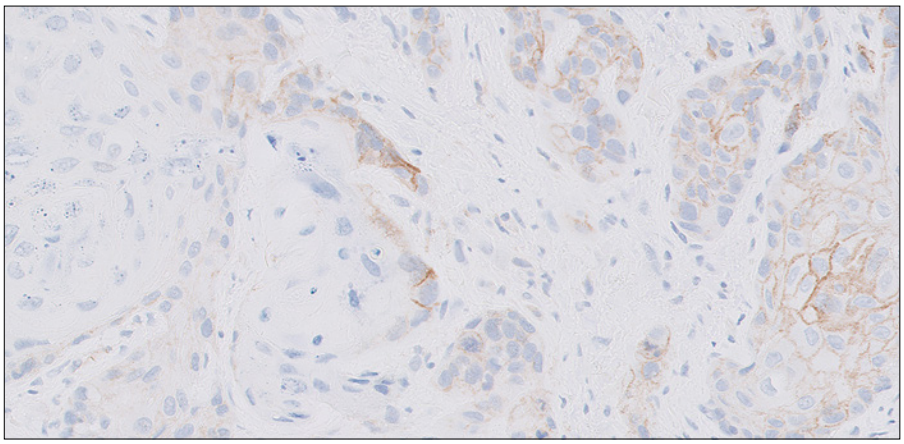


Figure 55: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 50, however any numerical CPS between 45–55 could be assigned to this image (20× magnification).

Case 6: CPS ≥ 20

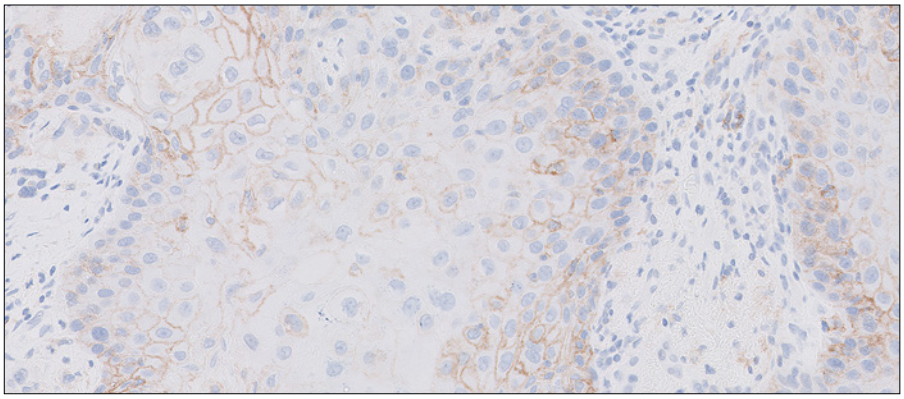


Figure 56: HNSCC specimen stained with PD-L1 antibody exhibiting a CPS of 55, however any numerical CPS between 50–60 could be assigned to this image (20× magnification).

Troubleshooting Guide

Troubleshooting Guidelines for PD-L1 IHC 22C3 pharmDx

For further troubleshooting help, contact your local Agilent representative.

Problem	Probable Cause	Suggested Action
No staining of slides	Programming error	Verify that the PD-L1 IHC 22C3 pharmDx program was selected for programming of slides
	Lack of reaction with DAB+ Substrate-Chromogen Solution (DAB)	Verify that DAB+ Substrate-Chromogen Solution was prepared properly
	Sodium azide in wash buffer	Use only Dako Wash Buffer (Code K8007)
	Degradation of Control Slide	Check kit expiration date and kit storage conditions on outside of package
Weak staining of specimen slides	Inappropriate fixation method used	Ensure that only neutral buffered formalin fixative and approved fixation methods are used
	Insufficient reagent volume applied	Check size of tissue section and reagent volume applied
	Inappropriate wash buffer used	Use only Dako Wash Buffer, Code K8007
Weak staining of specimen slides or of the positive cell line pellet on the Control Cell Line Slide provided with the kit	Inadequate target retrieval	Verify that the 3-in-1 pre-treatment procedure was correctly performed
	Inappropriate wash buffer used	Use only Dako Wash Buffer, Code K8007
Excessive background staining of slides	Paraffin incompletely removed	Verify that the 3-in-1 pre-treatment procedure was correctly performed
	Slides dried while loading onto Autostainer Link 48	Ensure slides remain wet with buffer while loading and prior to initiating run
	Nonspecific binding of reagents to tissue section	Check for proper fixation of the specimen and/or the presence of necrosis
	Inappropriate fixation method used	Ensure that only neutral buffered formalin fixative and recommended fixation methods are used
Tissue detached from slides	Use of incorrect microscope slides	Use Dako FLEX IHC Microscope Slides, (Code K8020), or Superfrost Plus slides
	Inadequate preparation of specimens	Cut sections should be placed in a 58 ± 2 °C oven for 1 hour prior to staining
Excessively strong specific staining	Inappropriate fixation method used	Ensure that only approved fixatives and fixation methods are used
	Inappropriate wash buffer used	Only use Dako Wash Buffer, Code K8007
Target Retrieval Solution is cloudy in appearance when heated	When heated, the Target Retrieval Solution turns cloudy in appearance	This is normal and does not influence staining

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 call Agilent Technical Support for further assistance. Additional information on staining techniques and specimen preparation can be found in Dako Education Guide: Immunohistochemical Staining Methods (available from Agilent).

Clinical Performance Evaluation

The efficacy of KEYTRUDA was investigated in KEYNOTE-048 (NCT02358031), a randomized, multicenter, open label, active controlled trial conducted in 882 patients with metastatic or recurrent HNSCC who had not previously received systemic therapy for metastatic disease or with recurrent disease who were considered incurable by local therapies. Patients with active autoimmune disease that required systemic therapy within two years of treatment or a medical condition that required immunosuppression were ineligible. Randomization was stratified by tumor PD-L1 expression (TPS ≥ 50% or < 50%) according to the PD-L1 IHC 22C3 pharmDx Kit, HPV status according to p16 IHC (positive or negative), and ECOG PS (0 vs. 1). Patients were randomized 1:1:1 to one of the following treatment arms:

- KEYTRUDA 200 mg intravenously every 3 weeks
- KEYTRUDA 200 mg intravenously every 3 weeks, carboplatin AUC 5 mg/mL/min intravenously every 3 weeks or cisplatin 100 mg/m^2 intravenously every 3 weeks, and FU 1000 mg/m^2/day as a continuous intravenous infusion over 96 hours every 3 weeks (maximum of 6 cycles of platinum and FU)
- Cetuximab 400 mg/m^2 intravenously as the initial dose then 250 mg/m^2 intravenously once weekly, carboplatin AUC 5 mg/mL/min intravenously every 3 weeks or cisplatin 100 mg/m^2 intravenously every 3 weeks, and FU 1000 mg/m^2/day as a continuous intravenous infusion over 96 hours every 3 weeks (maximum of 6 cycles of platinum and FU)

Treatment with KEYTRUDA continued until RECIST v1.1-defined progression of disease as determined by the investigator, unacceptable toxicity, or a maximum of 24 months. Administration of KEYTRUDA was permitted beyond RECIST-defined disease progression if the patient was clinically stable and considered to be deriving clinical benefit by the investigator. Assessment of tumor status was performed at Week 9 and then every 6 weeks for the first year, followed by every 9 weeks through 24 months. A retrospective re-classification of patients' tumor PD-L1 status according to CPS according to the PD-L1 IHC 22C3 pharmDx Kit was conducted using the tumor specimens used for randomization.

The main efficacy outcome measures were OS and PFS as assessed by BICR according to RECIST v1.1 (modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ) sequentially tested in the subgroup of patients with CPS ≥ 20, the subgroup of patients with CPS ≥ 1, and the overall population.

A total of 601 patients were randomized to the KEYTRUDA as a single agent and cetuximab in combination with chemotherapy arms; 301 patients to the KEYTRUDA as a single agent arm and 300 patients to the cetuximab in combination with chemotherapy arm. The study population characteristics were: median age of 61 years (range: 22 to 94); 36% age 65 or older; 85% male; 74% White and 19% Asian, and 1.7% Black; 61% ECOG PS of 1; and 79% were former/current smokers. Twenty-two percent of patients' tumors were HPV-positive, and 96% had Stage IV disease (Stage IVA 20%, Stage IVB 6%, and Stage IVC 70%).

For the subgroup of patients randomized to KEYTRUDA as a single agent or to cetuximab in combination with chemotherapy, PD-L1 expression level for 601 patient tumor biopsy or resection tissue (159 archival and 442 newly obtained; refer to definition in Table 5) was determined using PD-L1 IHC 22C3 pharmDx. Overall, 85% (512/601) of the patients had tumors that expressed PD-L1 with CPS ≥ 1. Eighty-six percent (380/442) of patients whose tumors were newly obtained for PD-L1 testing and 83% (132/159) of patients whose archival tumors were tested expressed PD-L1 at CPS ≥ 1. Forty-three percent (255/597) of the patients had tumors that expressed PD-L1 with CPS ≥ 20; four patients had unknown PD-L1 expression status (one specimen was archival tissue and three specimens were newly obtained tissue). Forty-two percent (186/439) of patients whose tumors were newly obtained for PD-L1 testing and 44% (69/158) of patients whose archival tumors were tested expressed PD-L1 at CPS ≥ 20 (Table 5).

Table 5: Tumor PD-L1 Expression by Specimen Type

Tumor Tissue	Number (%) with CPS < 1	Number (%) with CPS ≥ 1	Number (%) with CPS ≥ 20
Overall Study n=601	89 (15)	512 (85)	255 (43)**
Archival Tissue* n=159	27 (17)	132 (83)	69 (44)**
Newly Obtained Tissue* n= 442	62 (14)	380 (86)	186 (42)**

** In the context of clinical trial KEYNOTE-048, newly obtained tissue biopsy was defined as the biopsy collected within 90 days of initiation of treatment with pembrolizumab. Specimens that were > 90 days were classified as archival.*

*** Based on patients with known PD-L1 expression; 4 patients had unknown PD-L1 expression status (one specimen was archival tissue and three specimens were newly obtained tissue).*

The trial demonstrated a statistically significant improvement in OS for the subgroup of patients with PD-L1 CPS ≥ 1 randomized to KEYTRUDA as a single agent compared to those randomized to cetuximab in combination with chemotherapy. At the time of the interim analysis, there was no significant difference in OS between the KEYTRUDA single agent arm and the control arm for the overall population.

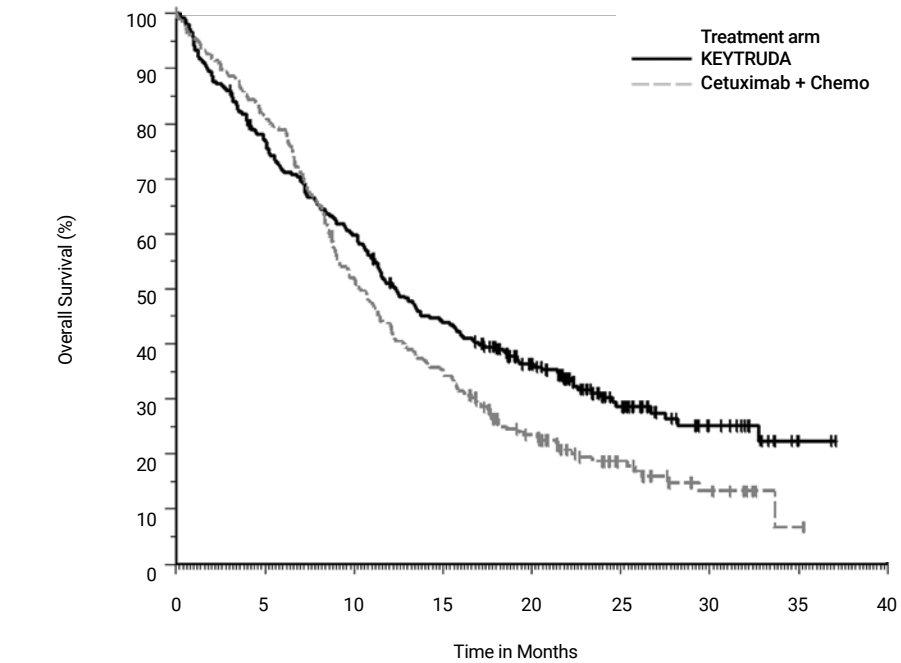
Table 6 summarizes efficacy results for KEYTRUDA as a single agent in the subgroup of patients with CPS ≥ 1 HNSCC and CPS ≥ 20 HNSCC. Figure 57 summarizes the OS results in the subgroup of patients with CPS ≥ 1 HNSCC.

Table 6: Efficacy Results for KEYTRUDA as a Single Agent in KEYNOTE-048 (CPS ≥ 1 and CPS ≥ 20)

	CPS ≥ 1		**CPS ≥ 20**	
Endpoint	**KEYTRUDA 200 mg every 3 weeks n=257**	**Cetuximab Platinum FU n=255**	**KEYTRUDA 200 mg every 3 weeks n=133**	**Cetuximab Platinum FU n=122**
OS				
Number of events (%)	177 (69%)	206 (81%)	82 (62%)	95 (78%)
Median in months (95% CI)	12.3 (10.8, 14.9)	10.3 (9.0,11.5)	14.9 (11.6, 21.5)	10.7 (8.8, 12.8)
Hazard ratio* (95% CI)	0.78 (0.64, 0.96)		0.61 (0.45, 0.83)	
p-Value†	0.0171		0.0015	
PFS				
Number of events (%)	225 (88%)	231 (91%)	113 (85%)	111 (91%)
Median in months (95% CI)	3.2 (2.2, 3.4)	5.0 (4.8, 5.8)	3.4 (3.2, 3.8)	5.0 (4.8, 6.2)
Hazard ratio‡ (95% CI)	1.15 (0.95, 1.38)		0.99 (0.75, 1.29)	
Objective Response Rate				
ORR‡ (95% CI)	19% (14.5, 24.4)	35% (29.1, 41.1)	23% (16.4, 31.4)	36% (27.6, 45.3)
Complete response rate	5%	3%	8%	3%
Partial response rate	14%	32%	16%	33%
Duration of Response				
Median in months (range)	20.9 (1.5+, 34.8+)	4.5 (1.2+, 28.6+)	20.9 (2.7, 34.8+)	4.2 (1.2+, 22.3+)

** Based on the stratified Cox proportional hazard model*
† Based on stratified log-rank test
‡ Response: Best objective response as confirmed complete response or partial response

In an exploratory subgroup analysis for patients with CPS 1–19 HNSCC, the median OS was 10.8 months (95% CI: 9.0, 12.6) for KEYTRUDA as a single agent and 10.1 months (95% CI: 8.7, 12.1) for cetuximab in combination with chemotherapy, with an HR of 0.90 (95% CI: 0.68, 1.18).



Number at Risk									
KEYTRUDA:	257	196	152	110	74	34	17	2	0
Cetuximab + Chemo:	255	207	131	89	47	21	9	1	0

Figure 57: Kaplan-Meier Curve for Overall Survival for KEYTRUDA as a Single Agent in KEYNOTE-048 (CPS ≥ 1)

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1. PD-L1 IHC 22C3 pharmDx [package insert]. Carpinteria, CA: Dako, Agilent Pathology Solutions; 2019. **2.** Keytruda [package insert]. Kenilworth, NJ: Merck & Co., Inc.; 2019. **3.** Data on file. Agilent Technologies, Inc.

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