Agilent
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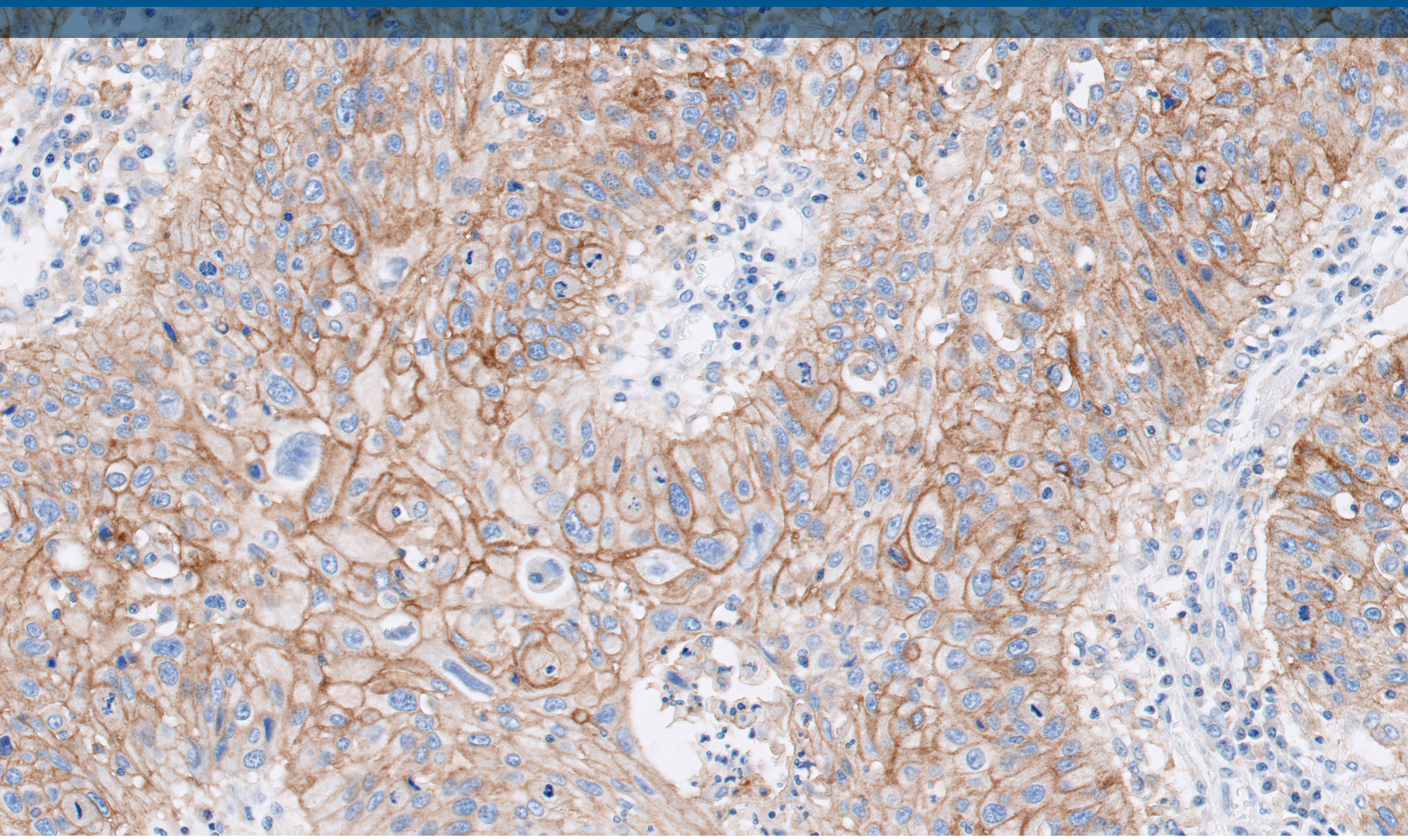
PD-L1 IHC 28-8 pharmDx Interpretation Manual - Esophageal Squamous Cell Carcinoma

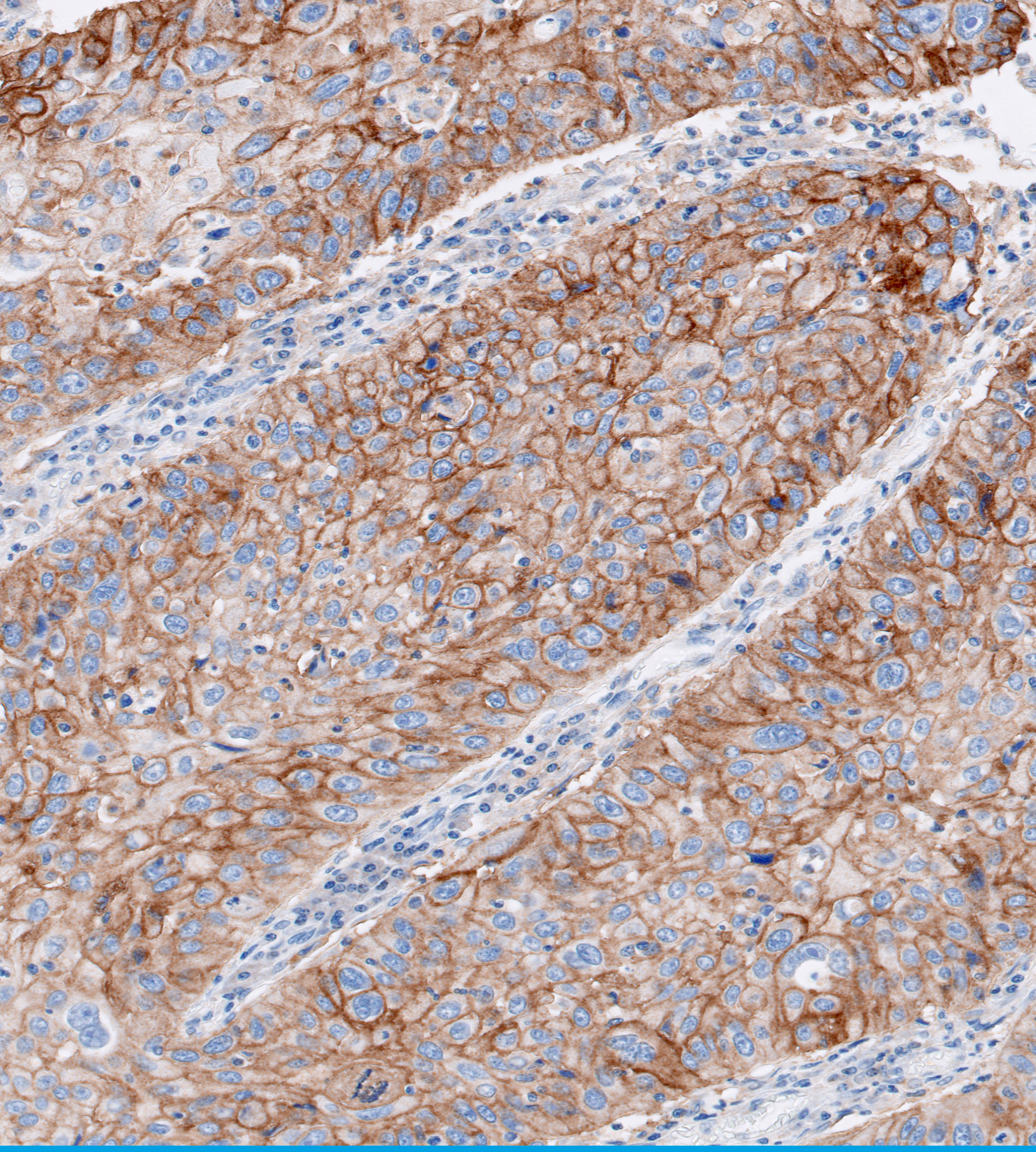
CE-IVD marked for in vitro diagnostic use



Agilent
Trusted Answers

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

Table of Contents



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

Introduction

Intended Use

For In Vitro Diagnostic Use

PD-L1 IHC 28-8 pharmDx is a qualitative immunohistochemical assay using Monoclonal Rabbit Anti-PD-L1, Clone 28-8 intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) esophageal squamous cell carcinoma (ESCC) tissues using EnVision FLEX visualization system on Autostainer Link 48.

PD-L1 protein expression in esophageal squamous cell carcinoma (ESCC) is determined by using % tumor cell expression, which is the percentage of evaluable tumor cells exhibiting partial or complete membrane staining at any intensity.

Companion Diagnostic Indication

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

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

PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in ESCC demonstrates clinically meaningful improvements in overall survival (OS) and progression-free survival (PFS) with OPDIVO® (nivolumab) in combination with chemotherapy and a clinically meaningful improvement in OS with OPDIVO® (nivolumab) in combination with YERVOY® (ipilimumab).

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

This ESCC 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 ESCC specimens stained with PD-L1 IHC 28-8 pharmDx. The PD-L1 IHC 28-8 pharmDx package insert contains guidelines and technical tips for ensuring high-quality staining in your laboratory.

A review of this ESCC PD-L1 IHC 28-8 pharmDx Interpretation Manual will provide a solid foundation for evaluating ESCC slides stained with PD-L1 IHC 28-8 pharmDx. For more details, please refer to the current version of the package insert provided with PD-L1 IHC 28-8 pharmDx or visit www.agilent.com.

The included photomicrographs are ESCC unless otherwise noted.

Acknowledgment

Photomicrographs

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

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

- Tissue samples supplied by BioIVT (Hicksville, NY, USA).
- The data and ESCC specimens used in this project were provided by National BioService LLC (Saint Petersburg, Russia) with appropriate ethics approval and through Trans-Hit Biomarkers Inc.
- The data and ESCC specimens used in this project were provided by Nottingham University Hospitals NHS Trust (Nottingham, UK) with appropriate ethics approval and through Trans-Hit Biomarkers Inc.

Assay Interpretation

The clinical interpretation of any staining, or the absence of staining, must be complemented by the evaluation of proper controls. An evaluation must be made by a qualified pathologist within the context of the patient's clinical history and other diagnostic tests. This product is intended for in vitro diagnostic (IVD) use.

Reporting Results

To help understand what information should be reported to the treating physician, please refer to the Reporting Results section of this manual on page 27.

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

Limiting damage to healthy tissue

Inactivation of T-cells limits damage to healthy tissue.

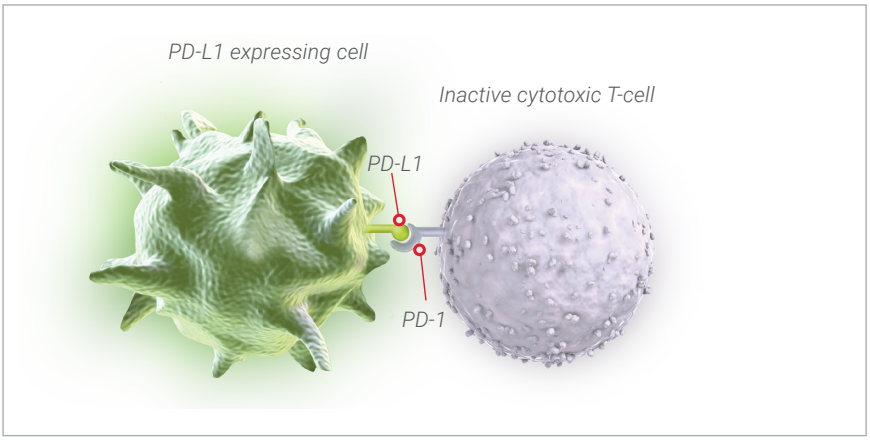


Figure a.

The tumor escapes detection

Inactivation of T-cells reduces tumor cell killing.

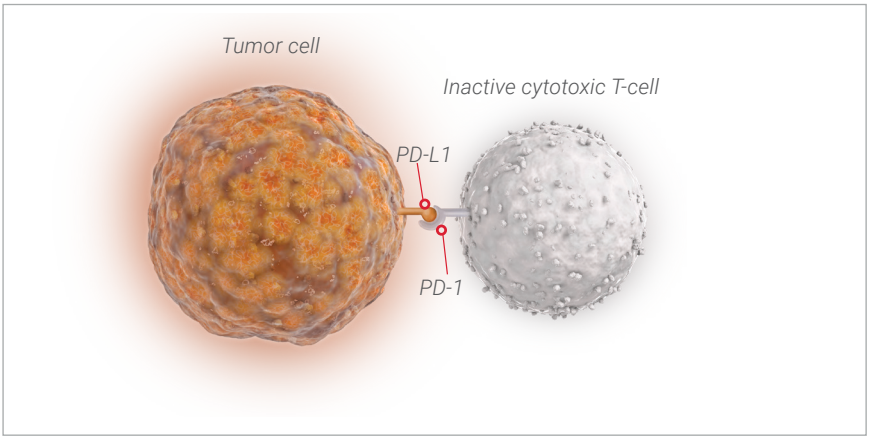


Figure b.

Immuno-oncology therapies harness the immune response to fight tumors

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

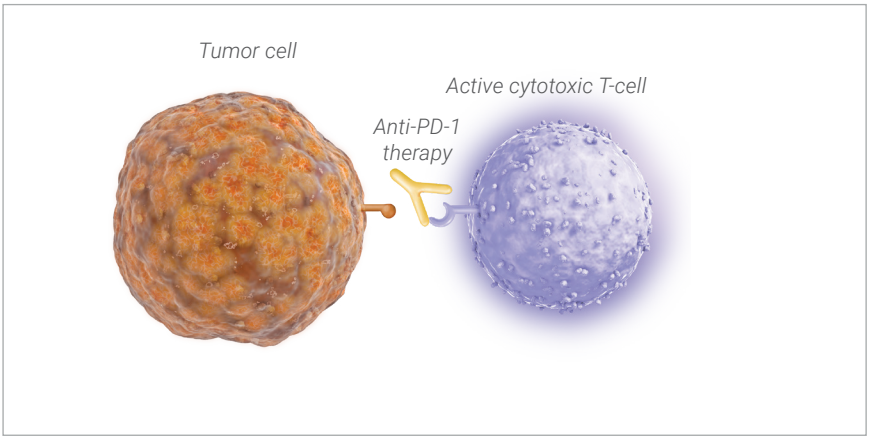


Figure c.

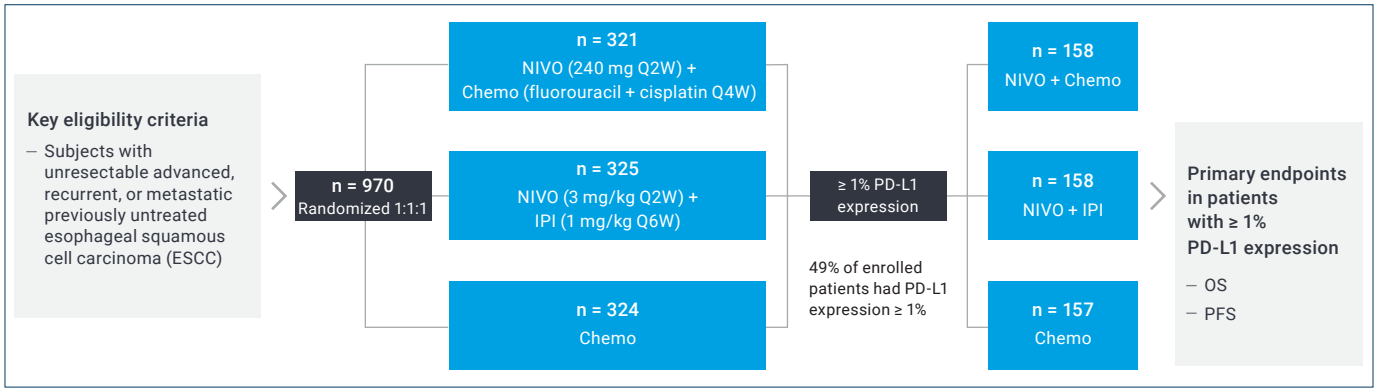
Study Data for PD-L1 IHC 28-8 pharmDx in ESCC

In CheckMate-648, among all randomized subjects with ≥ 1% PD-L1 expression, OPDIVO (nivolumab) in combination with chemotherapy demonstrated clinically meaningful improvements in OS and PFS, and also demonstrated a clinically meaningful improvement in OS with OPDIVO (nivolumab) + YERVOY (ipilimumab), compared with chemotherapy alone.

- CheckMate-648 investigated the validity of PD-L1 IHC 28-8 pharmDx for the assessment of PD-L1 status in ESCC patients treated with OPDIVO (nivolumab) in combination with fluorouracil plus cisplatin chemotherapy or with OPDIVO (nivolumab) + YERVOY (ipilimumab) versus chemotherapy alone.
- This was a randomized phase 3 study in patients with unresectable advanced, recurrent, or metastatic previously untreated ESCC.

In the landmark phase 3 study, OPDIVO (nivolumab) in combination with fluorouracil plus cisplatin chemotherapy or OPDIVO (nivolumab) + YERVOY (ipilimumab) versus chemotherapy alone was assessed in patients with ESCC.

CheckMate-648* Study Design



OS = overall survival; PFS = progression-free survival

*Doki, Y.; Ajani, J.A.; Kato, K.; et al. Nivolumab Combination Therapy in Advanced Esophageal Squamous-Cell Carcinoma. *N. Engl. J. Med.* **2022**, *386(5)*, 449–462.

The Clinical Value of PD-L1 IHC 28-8 pharmDx Expression in ESCC

As per CheckMate-648 study results, in patients with a PD-L1 expression ≥ 1%,

- the Median Overall Survival (OS) is 15.44 months with OPDIVO (nivolumab) + chemotherapy, 13.70 months with OPDIVO (nivolumab) + YERVOY (ipilimumab), and 9.07 months with chemotherapy alone.
- the Median Progression-free Survival (PFS) is 6.93 months with OPDIVO (nivolumab) + chemotherapy, and 4.44 months with chemotherapy alone.

Table 1. Efficacy of Nivolumab + Chemotherapy vs Chemotherapy by Baseline Tumor Cell PD-L1 Levels - in All Randomized Subjects with ≥ 1% PD-L1 Expression

	PD-L1 ≥ 1%	
	Nivo + Chemo N = 158	Chemo N = 157
Overall Survival		
Events, n (%)	98 (62.0)	121 (77.1)
HR (99.5% CI)[a]	0.54 (0.37, 0.80)	
p-value[b]	< 0.0001	
Median OS, (months)[c] (95% CI)	15.44 (11.93, 19.52)	9.07 (7.69, 9.95)
Progression-free Survival per BICR		
Events, n (%)	117 (74.1)	100 (63.7)
HR (98.5% CI)[a]	0.65 (0.46, 0.92)	
p-value[b]	0.0023	
Median PFS, (months)[c] (95% CI)	6.93 (5.68, 8.34)	4.44 (2.89, 5.82)

[a] Stratified Cox proportional hazards model. HR is Nivo + Chemo over Chemo.
[b] Log-rank test stratified by ECOG PS (0 vs 1) and number of organs with metastases (≤ 1 vs ≥ 2) for All Randomized Subjects with ≥ 1% PD-L1 Expression.
[c] Based on Kaplan-Meier estimates.

Table 2. Efficacy of Nivolumab + Ipilimumab vs Chemotherapy by Baseline Tumor Cell PD-L1 Levels - in All Randomized Subjects with ≥ 1% PD-L1 Expression

	PD-L1 ≥ 1%	
	Nivo + Ipi N = 158	Chemo N = 157
Overall Survival		
Events, n (%)	106 (67.1)	121 (77.1)
HR (98.6% CI)[a]	0.64 (0.46, 0.90)	
p-value[b]	0.0010	
Median OS, (months)[c] (95% CI)	13.70 (11.24, 17.02)	9.07 (7.69, 9.95)

[a] Stratified Cox proportional hazards model. HR is Nivo + Ipi over Chemo.
[b] Log-rank test stratified by ECOG PS (0 vs 1) and number of organs with metastases (≤ 1 vs ≥ 2) for All Randomized Subjects with ≥ 1% PD-L1 Expression.
[c] Based on Kaplan-Meier estimates.

PD-L1 IHC 28-8 pharmDx Overview

Code SK005

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

Following incubation with the primary monoclonal antibody to PD-L1 or the Negative Control Reagent (NCR), specimens are incubated with a 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 may then be counterstained and coverslipped. Results are interpreted using a light microscope. Control Slides containing two FFPE human cell lines are provided to validate staining runs.

PD-L1 IHC 28-8 pharmDx staining procedure

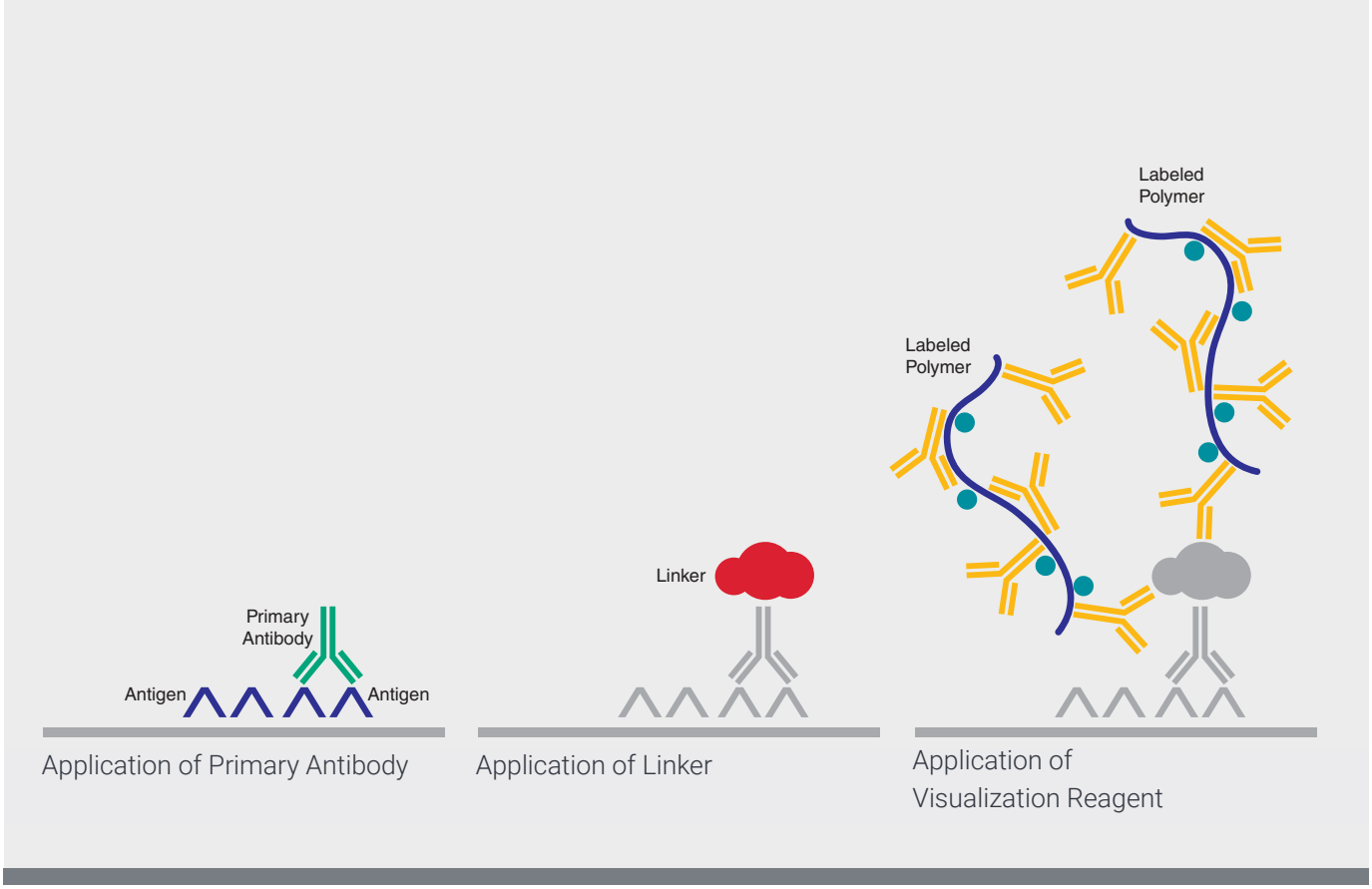


Figure 1a. PD-L1 IHC 28-8 pharmDx staining procedure.

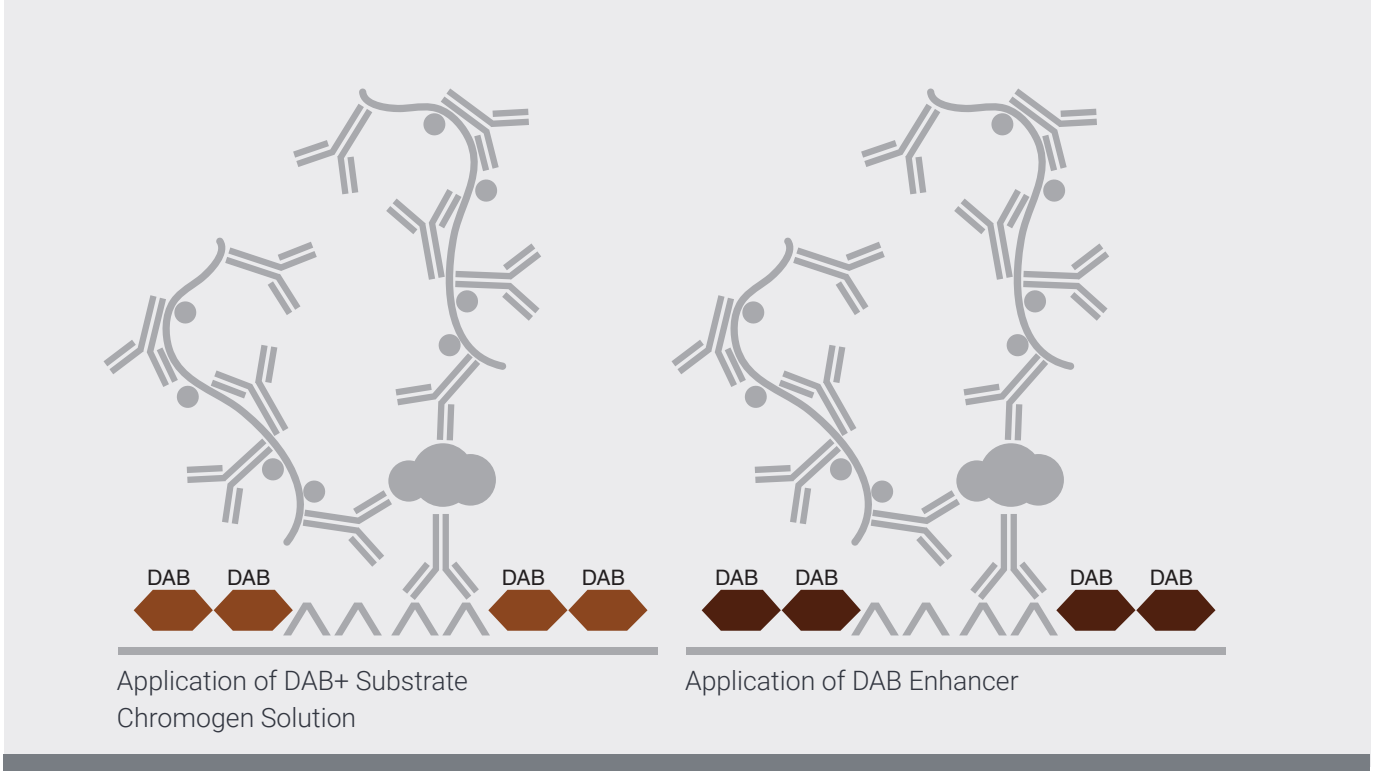


Figure 1b. PD-L1 IHC 28-8 pharmDx staining procedure.

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

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

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

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

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

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

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

Specimen Collection and Preparation

Specimens must be handled in a way that preserves the tissue for immunohistochemical staining. Tissue should be stained and interpreted as close to the time of biopsy as possible. Use the recommended methods of tissue processing for all specimens.

Tissue Processing

FFPE tissues are suitable for use. Recommended handling and processing conditions are: < 30 minutes ischemia time prior to immersion in fixative, and 24–48 hours fixation time in 10% neutral buffered formalin. Alternative fixatives have not been validated and may give erroneous results. Specimens should be blocked into a thickness of 3 or 4 mm, fixed in 10% neutral buffered formalin, and dehydrated and cleared in a series of alcohols and xylene, followed by infiltration with melted paraffin. The paraffin temperature should not exceed 60 °C. The use of PD-L1 IHC 28-8 pharmDx on decalcified tissues has not been validated and is not recommended.

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

Positive and Negative Control Tissues (Lab-Supplied)

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

Control tissue must represent one of the approved tumor indications for PD-L1 IHC 28-8 pharmDx as listed in the Intended Use of the IFU. Fix, process, and embed the control tissue in the same manner as patient specimens. Control tissue processed differently from the patient specimen validates reagent performance only and does not verify tissue preparation. The ideal positive control tissue gives weak to moderate positive staining. The variety of different cell types present in most tissue sections offers internal negative control sites; this should be verified by the user.

PD-L1 IHC 28-8 pharmDx Staining Procedure

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

Reagent Storage

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

Reagent Preparation

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

EnVision FLEX Target Retrieval Solution, Low pH

Prepare a sufficient quantity of 1x EnVision FLEX Target Retrieval Solution, Low pH by diluting EnVision FLEX Target Retrieval Solution, Low pH (50x) 1:50 using distilled or deionized water; the pH of 1x EnVision FLEX Target Retrieval Solution must be 6.1 ± 0.2. One 30 mL bottle of EnVision FLEX Target Retrieval Solution, Low pH (50x), diluted 1:50 will provide 1.5 L of 1x reagent, sufficient to fill one PT Link tank, which will treat up to 24 slides per use. Discard 1x EnVision FLEX Target Retrieval Solution after three uses and do not use after 5 days following dilution. Note, the EnVision FLEX Target Retrieval Solution, Low pH (50x) is a red-colored solution.

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

EnVision FLEX Wash Buffer (20x)

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

DAB+ Substrate-Chromogen Solution

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

- If using an entire bottle of DAB+ Substrate Buffer, add 9 drops of DAB+ Chromogen. Although the DAB+ Substrate Buffer label states 7.2 mL, this is the usable volume and does not account for the "dead volume" of DAB+ Substrate Buffer in the bottle.
- The color of the DAB+ Chromogen may vary from clear to lavender-brown. This will not affect the performance of the product. Dilute as per the guidelines in this section. Adding excess DAB+ Chromogen to the DAB+ Substrate Buffer results in deterioration of the positive signal.

Control Slides

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

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

Staining Protocol

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

Deparaffinization, Rehydration, and Target Retrieval

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

- Set Pre-heat and Cool to 65 °C, and set Heat to 97 °C for 20 minutes.
- Fill PT Link tanks with 1.5 L per tank of EnVision FLEX Target Retrieval Solution, Low pH, 1x working solution to cover the tissue sections.
- Pre-heat the EnVision FLEX Target Retrieval Solution, Low pH to 65 °C.
- Immerse Autostainer racks containing mounted, FFPE tissue sections into the pre-heated EnVision FLEX Target Retrieval Solution, Low pH (1x working solution) in PT Link tank. Incubate for 20 minutes at 97 °C.
- As soon as Target Retrieval incubation has been completed and the temperature has cooled to 65 °C, remove each Autostainer slide rack with slides from the PT Link tank and immediately place the slides into a tank (e.g., PT Link Rinse Station, Code PT109) containing room temperature EnVision FLEX Wash Buffer working solution.
- Immerse Autostainer rack with slides in room temperature EnVision FLEX Wash Buffer for 5 minutes.

Staining and Counterstaining

- Place the Autostainer rack with slides on the Autostainer Link 48. Ensure slides remain wet with buffer while loading and prior to initiating the run. Dried tissue sections may display increased non-specific staining.
- The instrument performs the staining and counterstaining procedures by applying the appropriate reagent, monitoring the incubation time, and rinsing slides between reagents. Counterstaining using EnVision FLEX Hematoxylin (Code K8008), for 7 minutes, is included in the staining protocol. Do not allow slides to dry prior to mounting.

Mounting

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

PD-L1 IHC 28-8 pharmDx Technical Checklist

Customer Name / Institution: _____

Name and Title: _____

Autostainer Link 48 Serial Number: _____ Software Version: _____

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

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

Additional Observations or Comments:

_____

_____

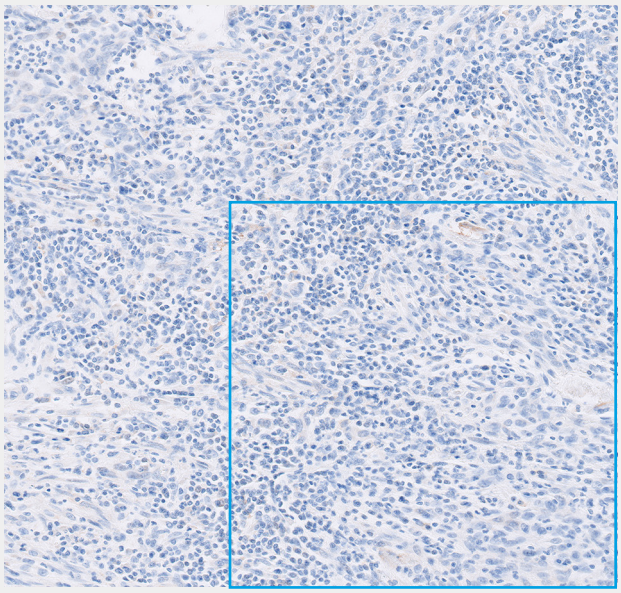
_____

Guidelines for Scoring PD-L1 IHC 28-8 pharmDx

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.

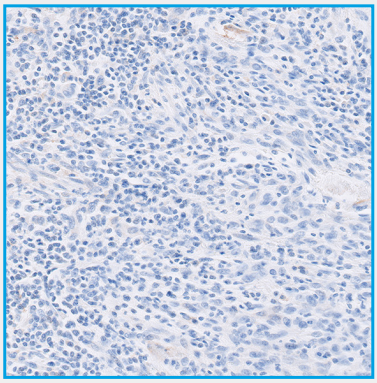
The percentage of viable tumor cells exhibiting circumferential or partial linear plasma membrane PD-L1 staining at any intensity determines PD-L1 IHC 28-8 pharmDx result. Scoring guidelines and reporting recommendations are presented in Figure 3. See page 27 for an example of a reporting results form for PD-L1 IHC 28-8 pharmDx.

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

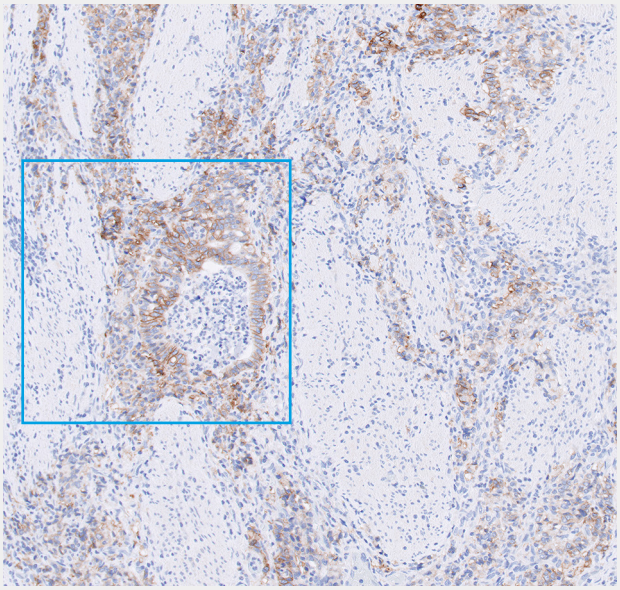


PD-L1 expression

< 1%



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



PD-L1 expression

≥ 1%

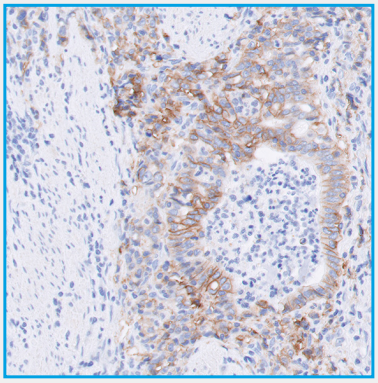


Figure 3. Guidelines for scoring and reporting PD-L1 IHC pharmDx results.

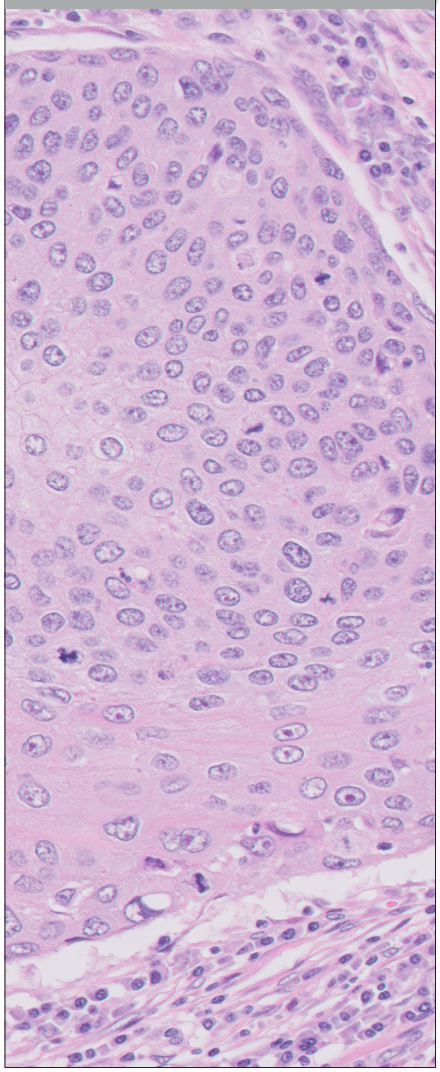
Recommended Slide Order for Interpretation of PD-L1 IHC 28-8 pharmDx

The following flow of slide review is recommended when conducting interpretation of PD-L1 IHC 28-8 pharmDx.
Refer to detailed description on pages 20–23.

1

Patient Specimen stained with H&E
Histology and Preservation Quality

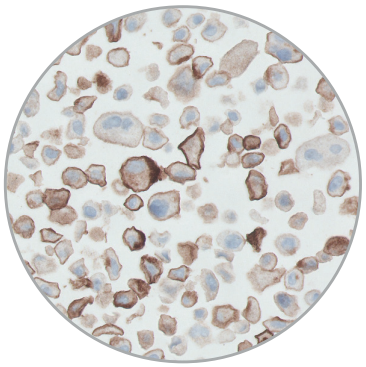
Acceptable



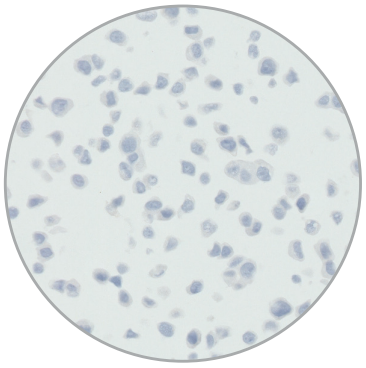
2

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

Acceptable



PD-L1 Positive Control

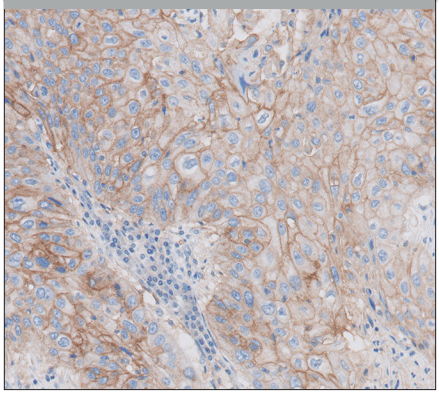


PD-L1 Negative Control

3A

Positive Control Tissue
Stained with PD-L1 Primary Antibody

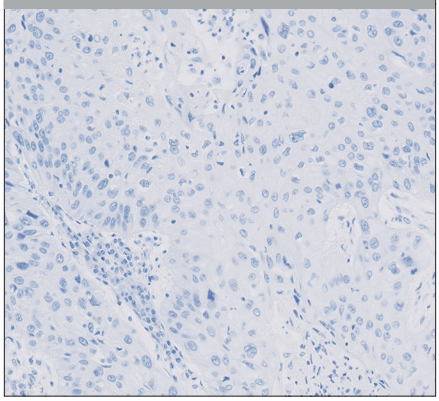
Acceptable



3B

Positive Control Tissue
Stained with Negative Control Reagent

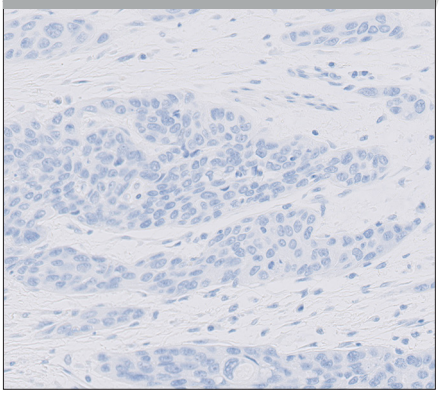
Acceptable



4A

Negative Control Tissue
Stained with PD-L1 Primary Antibody

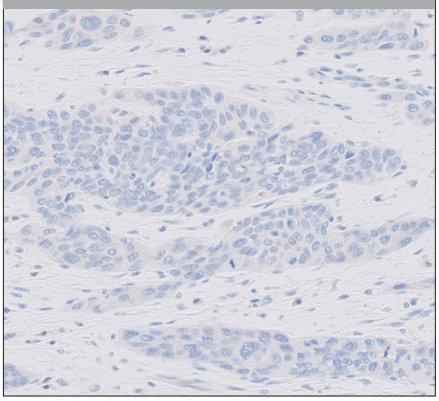
Acceptable



4B

Negative Control Tissue
Stained with Negative Control Reagent

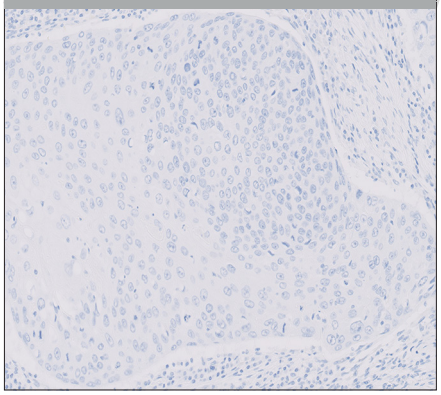
Acceptable



5

Patient Specimen
Stained with Negative Control Reagent

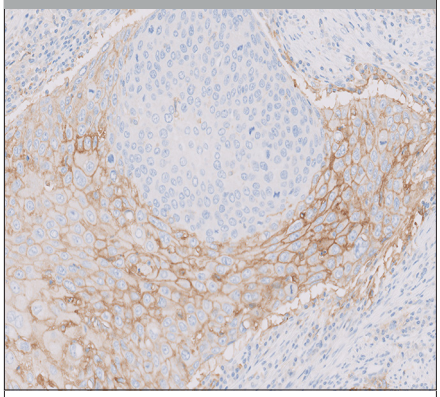
Acceptable



6

Patient Specimen
Stained with PD-L1 Primary Antibody

Acceptable



≥ 100 viable tumor cells should be present for scoring.

Include when scoring:

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

Exclude from scoring:

- Cytoplasmic staining
- Immune cells
- Normal cells
- Necrotic cells
- Cellular debris
- Foci of dysplasia
- Carcinoma *in situ*

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

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

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

Positive control tissue slides and negative control tissue slides should be supplied by the laboratory. Only the Control Slide is provided in the PD-L1 IHC 28-8 pharmDx.

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.

PD-L1 IHC 28-8 pharmDx Control Slide

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

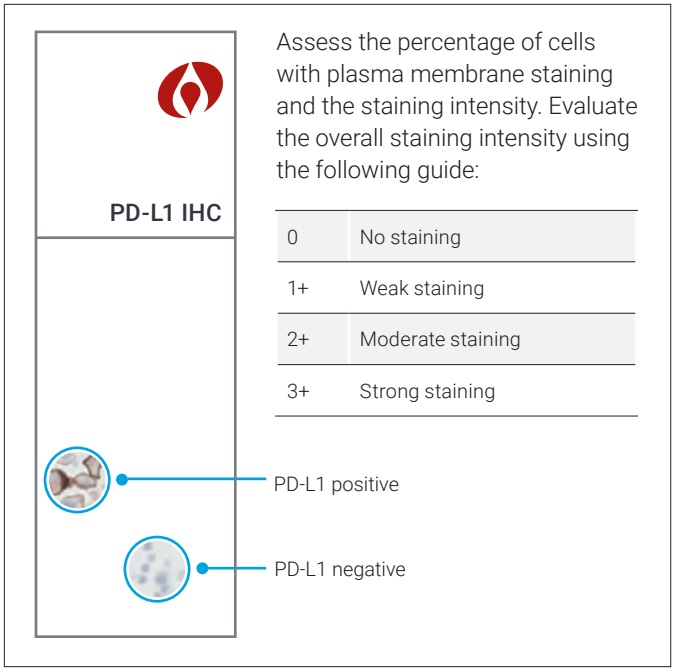


Figure 4. Each Control Slide contains sections of cell pellets with positive and negative PD-L1 expression.

For the PD-L1 positive cell pellet, the following staining is acceptable, see Figure 5.

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

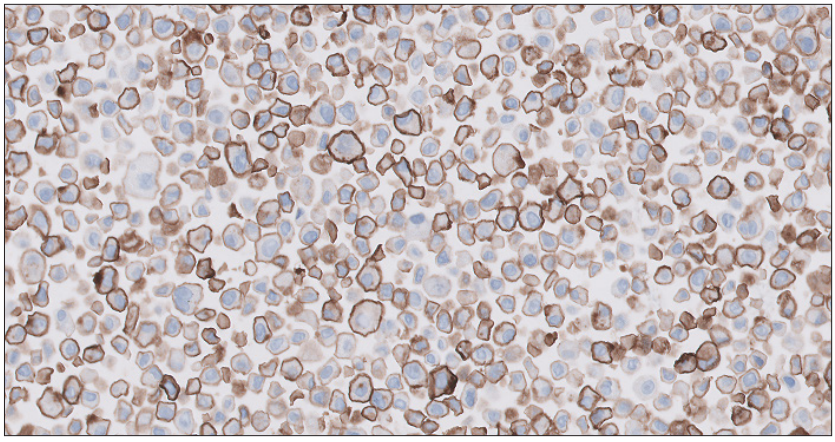


Figure 5. Acceptable Positive PD-L1 Control.

For the PD-L1 negative cell pellet, the following staining is acceptable, see Figure 6.

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

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

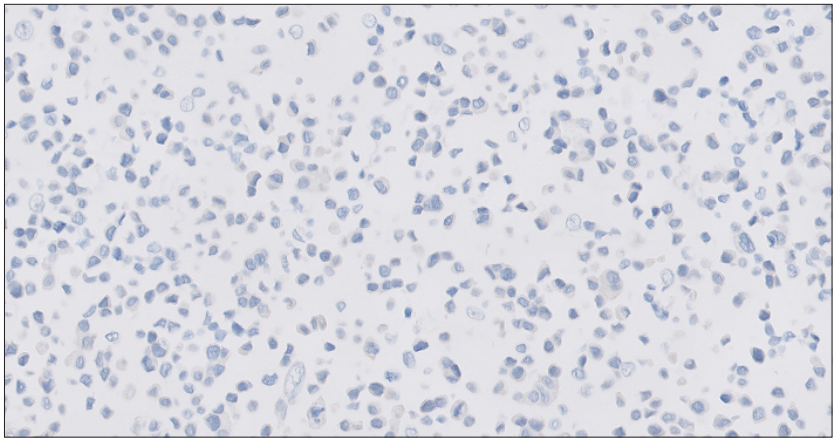


Figure 6. Acceptable Negative PD-L1 Control.

Positive Control Tissue Slides

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

Negative Control Tissue Slides

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

Patient Specimen Stained with Negative Control Reagent

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

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

Patient Specimen Stained with Primary Antibody

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

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

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

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
- Disregard non-specific cytoplasmic staining
- Necrotic tissue may stain but should be excluded
- Exclude any non-malignant cells and immune cells
- Granular staining must demonstrate a perceptible and convincing membrane pattern

Indeterminate Specimen

The recognition of tumor cell membrane staining 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.

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

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

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

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

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

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

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

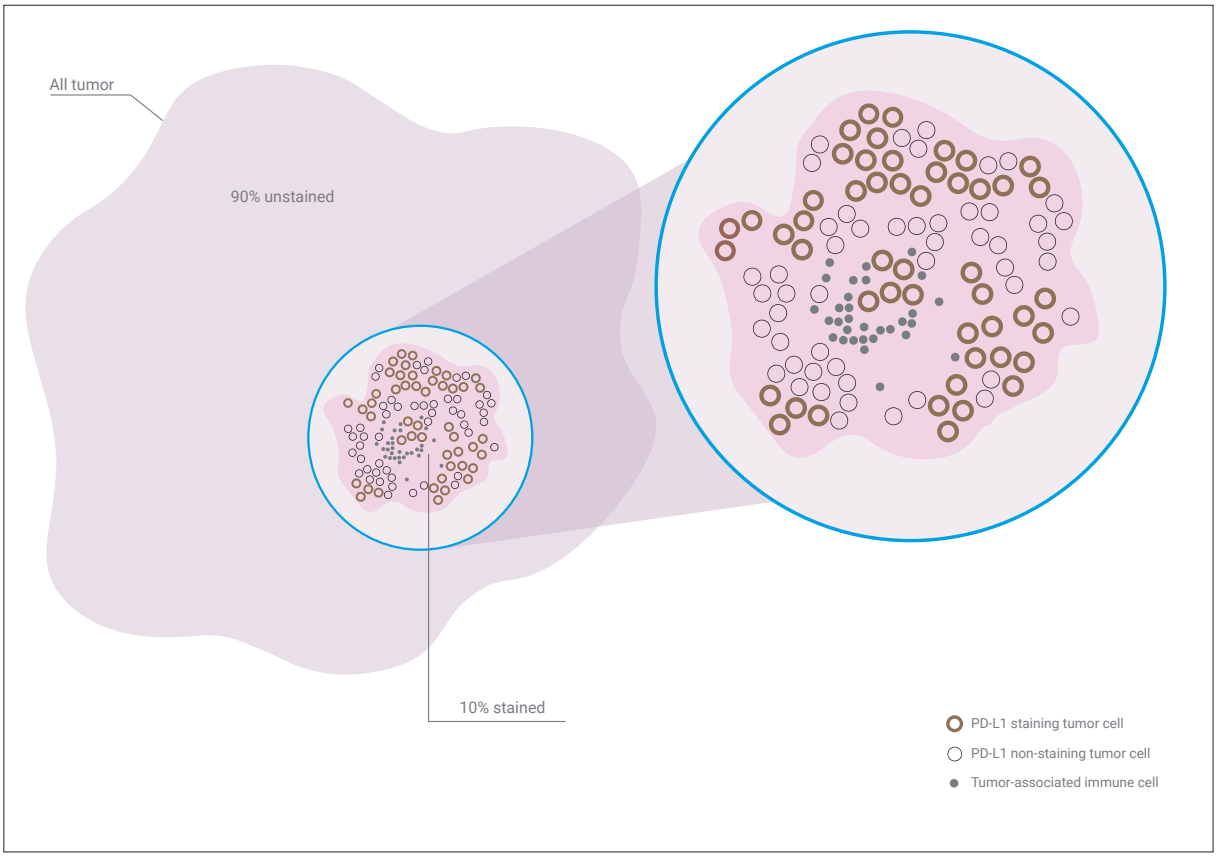


Figure 7. Example of tumor with small PD-L1 staining area.

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

Method 1

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

Method 2

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

Example 2: Calculating % PD-L1 expression in a specimen with heterogeneous staining

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

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

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

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

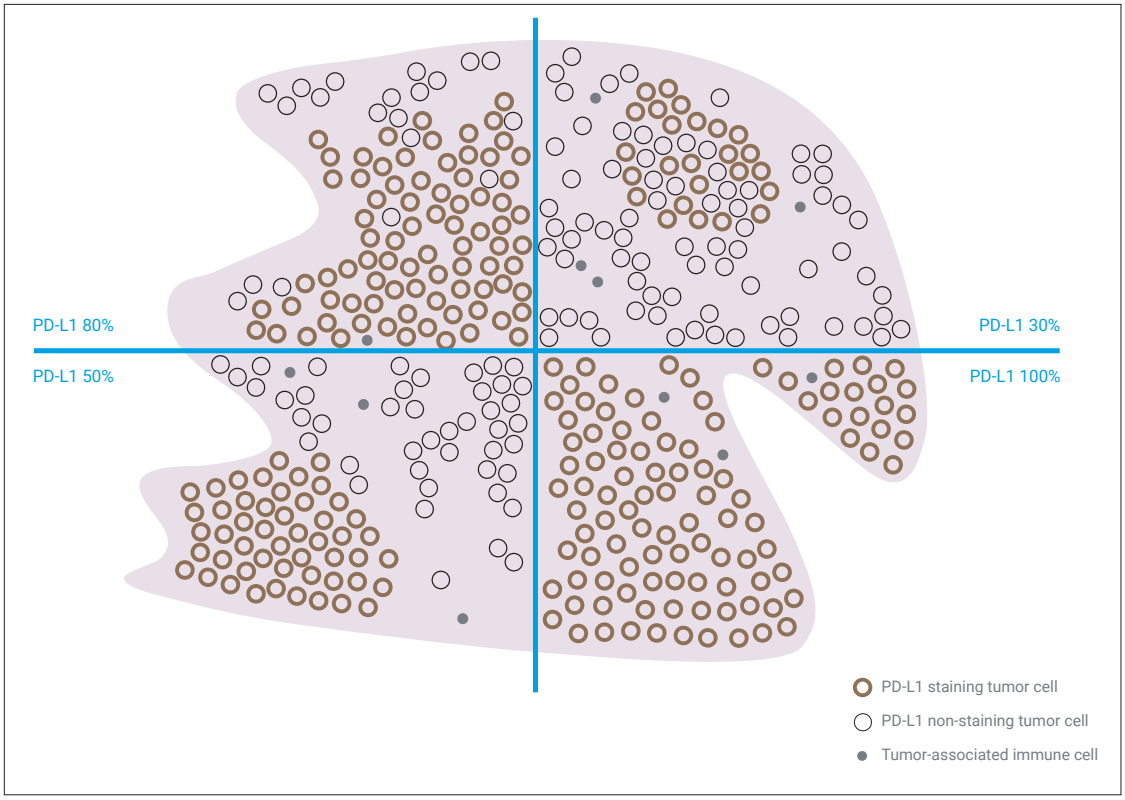


Figure 8. Example with heterogenous PD-L1 staining area.

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

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

PD-L1 IHC 28-8 pharmDX Reporting Results: ESCC

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

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 Cell Slide:	Pass ☐	Fail ☐
Positive Control Tissue Slides:	Pass ☐	Fail ☐
Negative Control Tissue Slides:	Pass ☐	Fail ☐
Patient Specimen, Negative Control Reagent:	Pass ☐	Fail ☐

PD-L1 Results: PD-L1 IHC 28-8 pharmDx is indicated as an aid in identifying ESCC patients for treatment with OPDIVO (nivolumab) in combination with fluoropyrimidine and platinum-based chemotherapy or OPDIVO (nivolumab) in combination with YERVOY (ipilimumab).

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

☐ PD-L1 expression is ≥ 1% ☐ PD-L1 expression is < 1%

Pathologist's comments: _____

OPDIVO and YERVOY are registered trademarks of Bristol Myers Squibb Company.

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

Positive Control Specimen

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

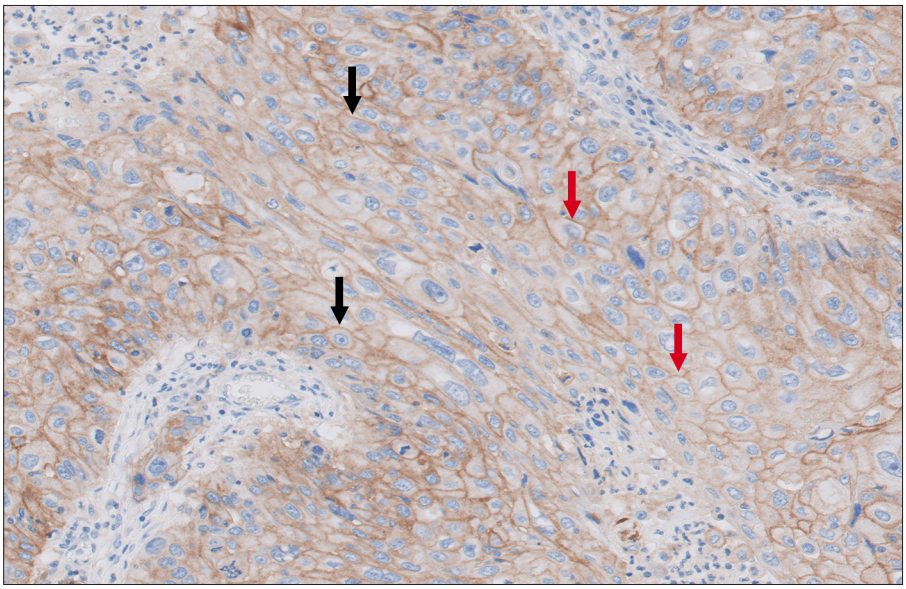


Figure 9. 20x magnification.

Distinguishing Tumor Cells from Immune Cells

ESCC specimen showing strong staining of tumor associated lymphocytes (**red arrows**), while the tumor cells are negative (**black arrows**) for PD-L1 positivity. Note the staining of immune cells are not included in determining the percent PD-L1 expression.

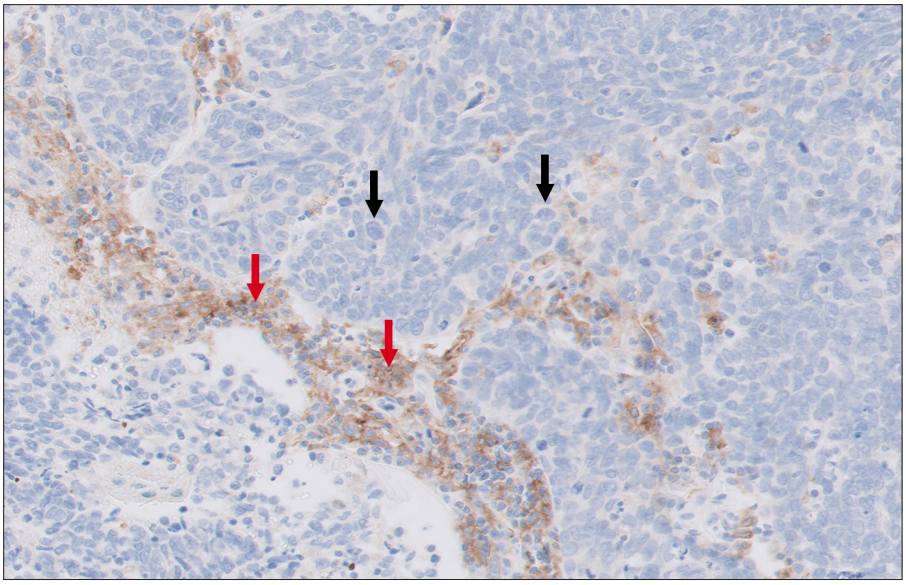


Figure 10. 20x magnification.

Distinguishing Tumor Cells from Immune Cells

Specimen showing strong staining of tumor associated macrophages (**red arrows**), while the tumor cells are negative (**black arrows**) for PD-L1 positivity. Note the staining of immune cells are not included in determining the percent PD-L1 expression.

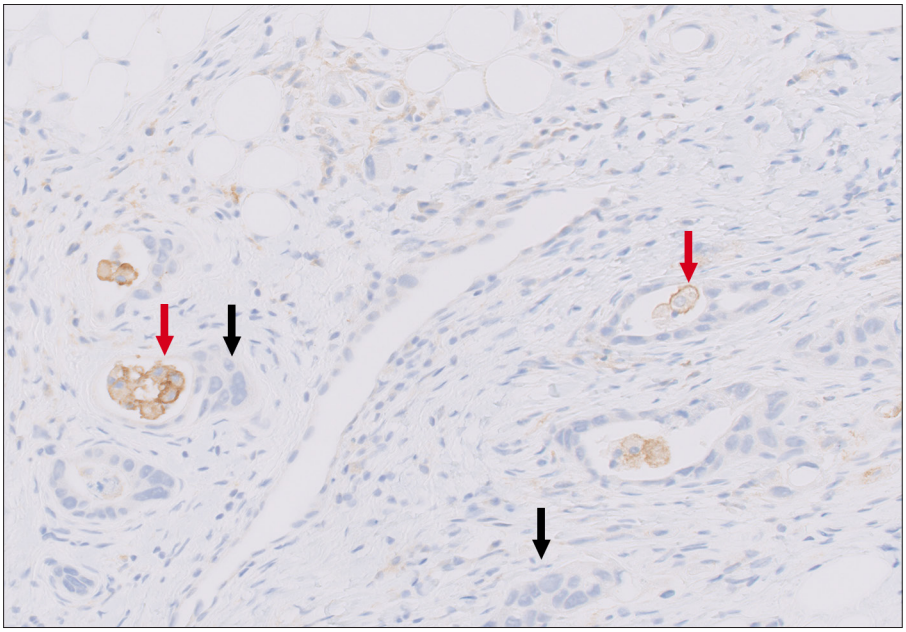


Figure 11. 20x magnification; esophageal adenocarcinoma specimen pictured.

Distinguishing Tumor Cells from Immune Cells

ESCC showing PD-L1 positive staining of macrophage (**red arrows**) and lymphocyte (**blue arrow**) immune cells and tumor cells (**black arrows**). Note the staining of immune cells are not included in determining the percent PD-L1 expression.

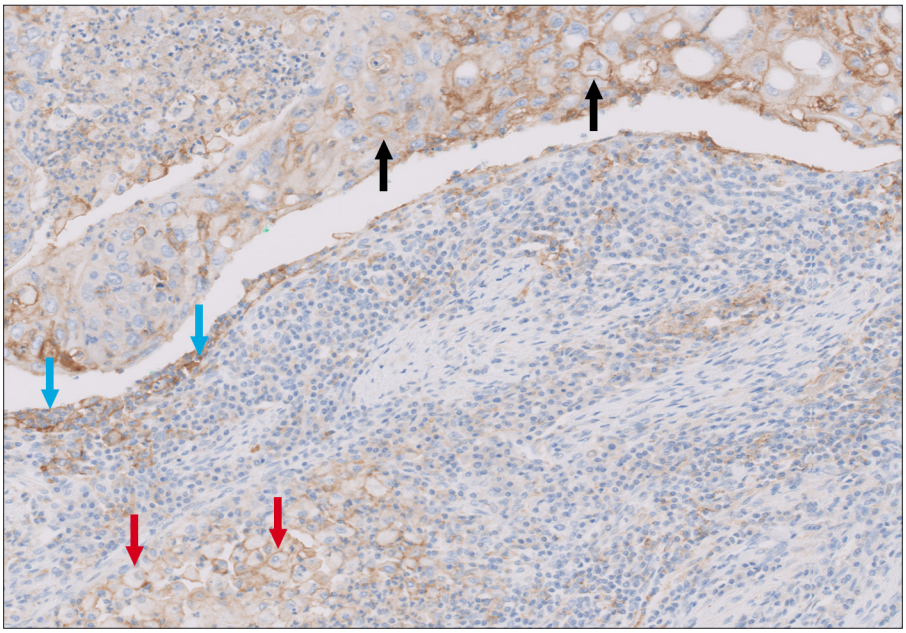


Figure 12. 20x magnification.

Cytoplasmic Staining

Positive linear membrane staining of tumor cells (**black arrows**) is observed and is distinguishable from the cytoplasmic staining (**red arrows**).

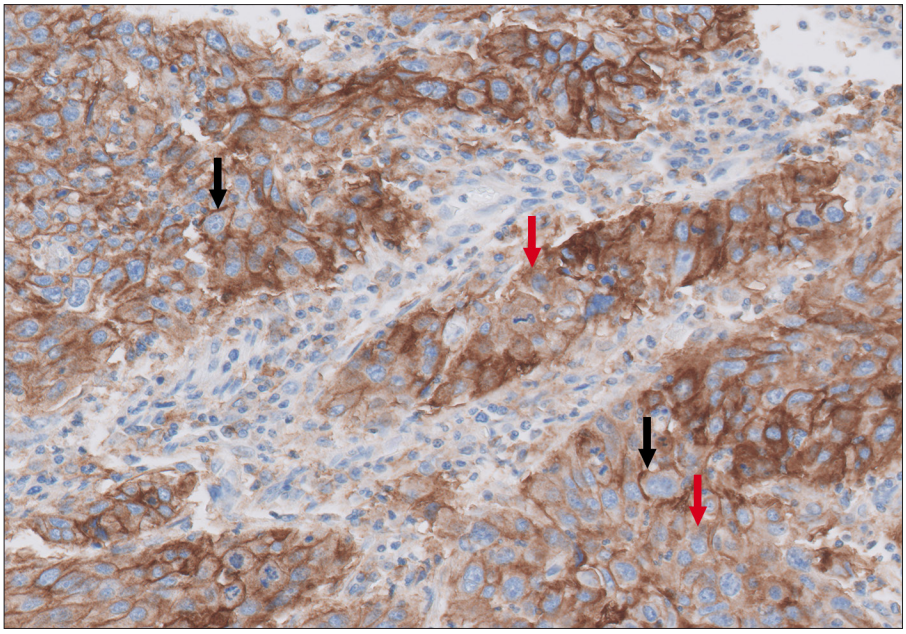


Figure 13. 20x magnification.

Granular Staining

Granular staining (**red arrows**) is present in the cytoplasm of tumor cells. Positive linear membrane staining of the tumor cells is observed (**black arrows**).

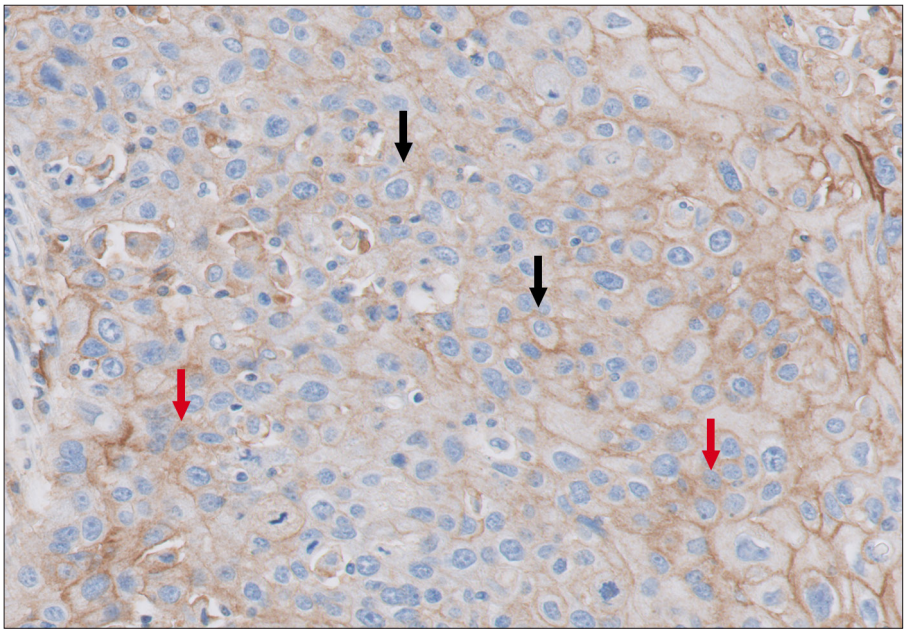


Figure 14. 20x magnification.

PD-L1 IHC 28-8 pharmDx ESCC Case Examples

Case 1: PD-L1 expression < 1%

No tumor cells are exhibiting PD-L1 staining in this case example. The PD-L1 expression is 0%.

Figure 15a. 10x magnification.

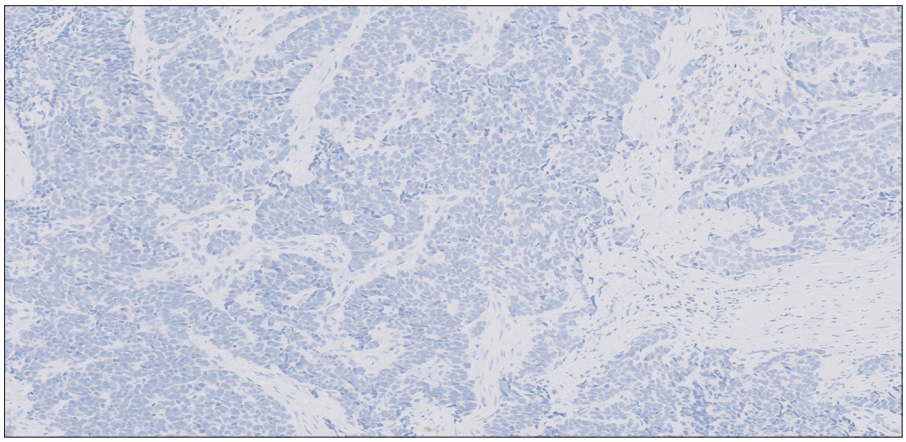


Figure 15b. 20x magnification.

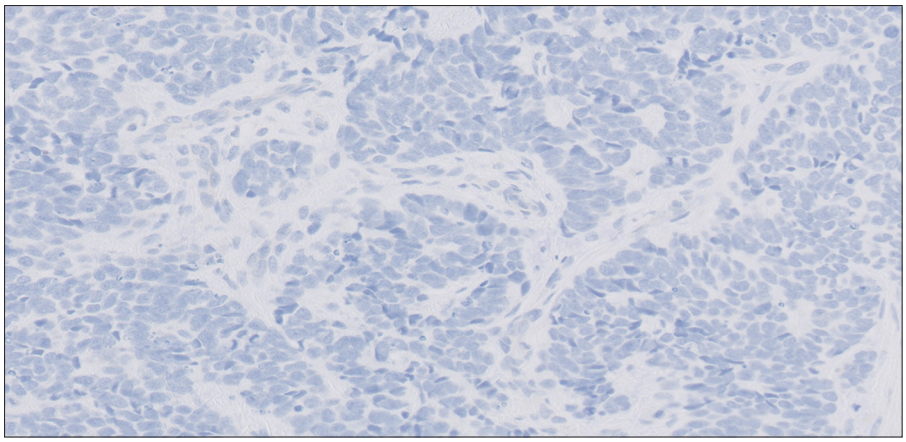
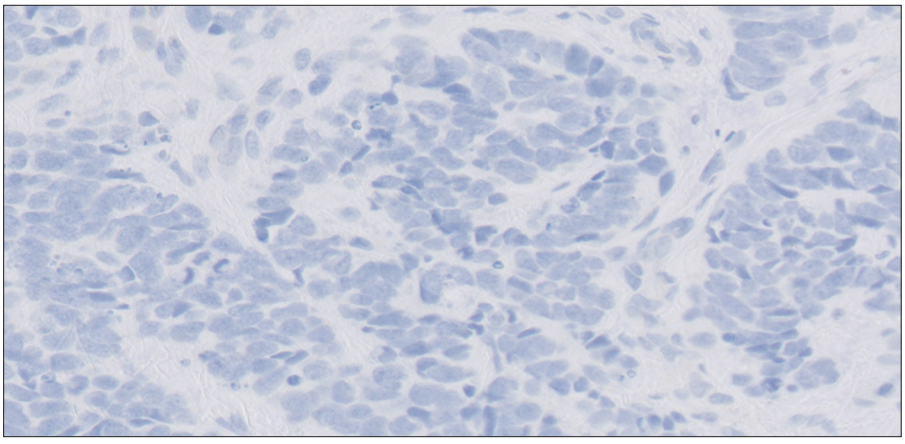


Figure 15c. 40x magnification.



Case 2: PD-L1 expression < 1%

There is presence of staining in this case example. Most of the staining appears to be non-specific, with the exception of a few tumor cells exhibiting partial linear membrane staining. The PD-L1 expression is < 1%.

Figure 16a. 10x magnification.

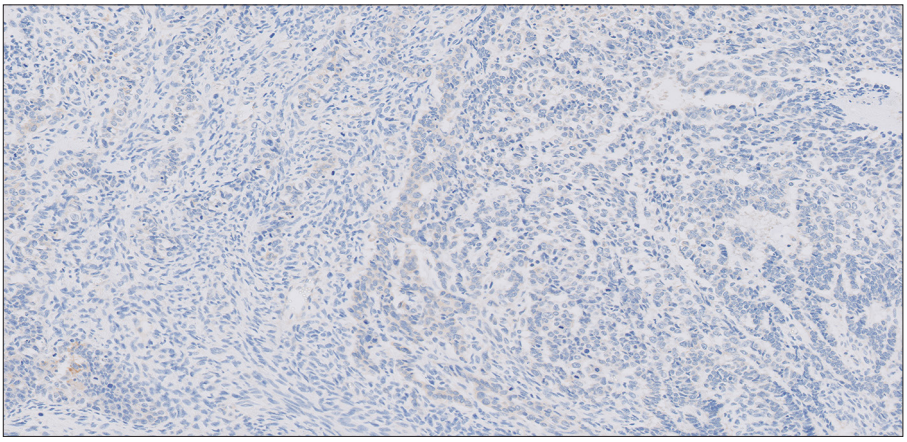


Figure 16b. 20x magnification.

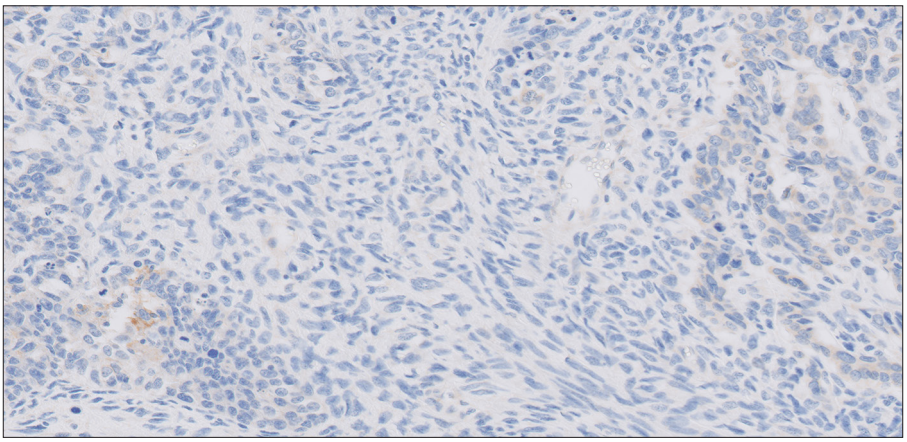
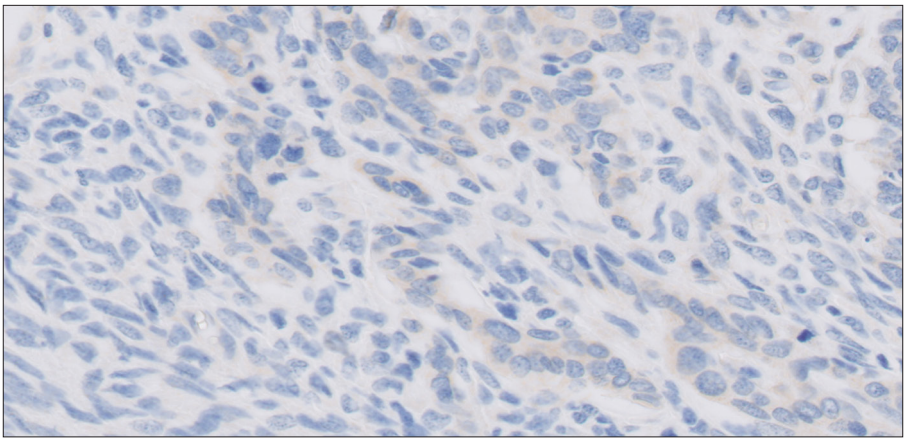


Figure 16c. 40x magnification.



Case 3: PD-L1 expression < 1%

There is presence of staining in this case example. Most of the PD-L1 staining is exhibited in the immune cells. A few tumors cells exhibit PD-L1 expression. The PD-L1 expression is < 1%.

Figure 17a. 10x magnification.

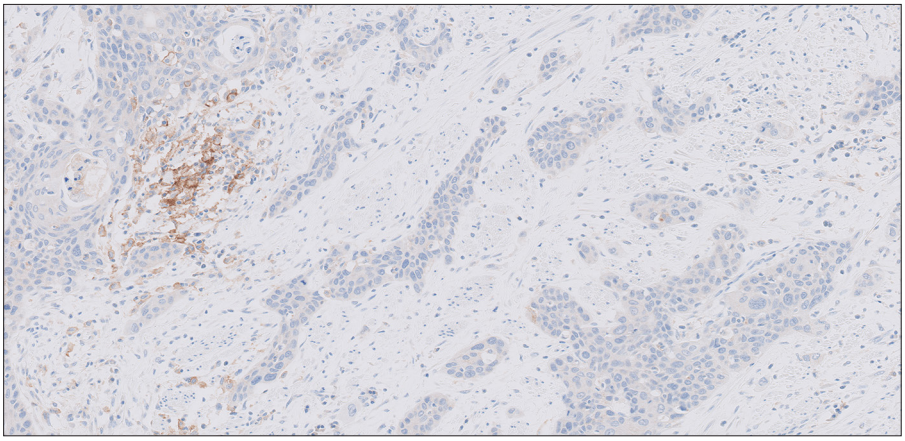


Figure 17b. 20x magnification.

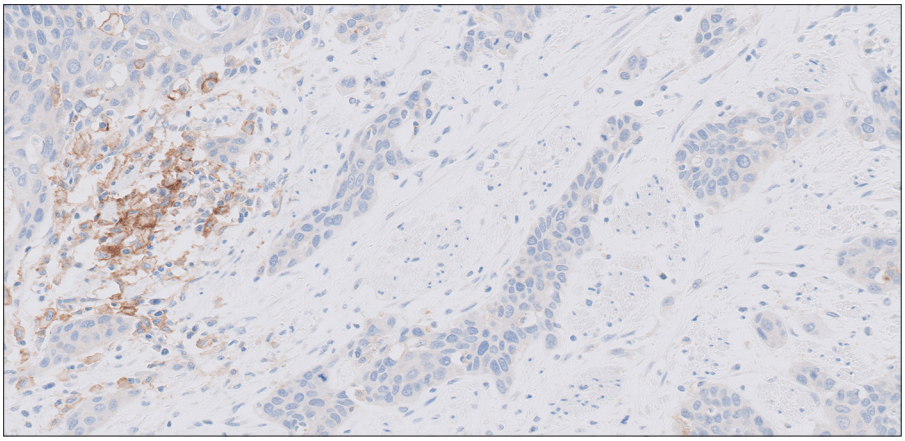
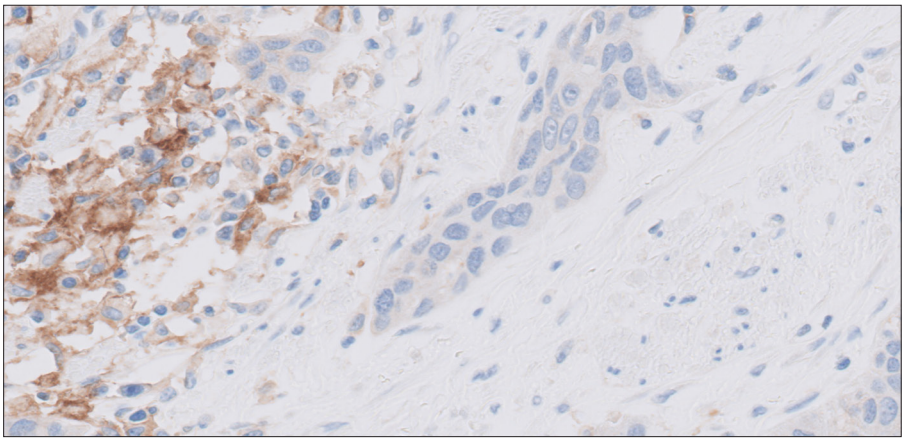


Figure 17c. 40x magnification.



Case 4: PD-L1 expression ≥ 1%

This case example demonstrates partial and complete membrane staining in the tumor cells. The PD-L1 expression is near and above the 1% clinical cutoff (PD-L1 expression 1–3%).

Figure 18a. 10x magnification.

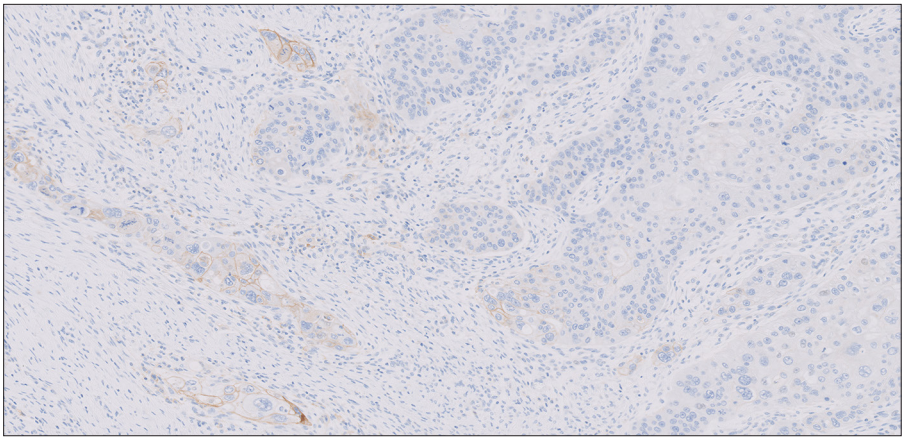


Figure 18b. 20x magnification.

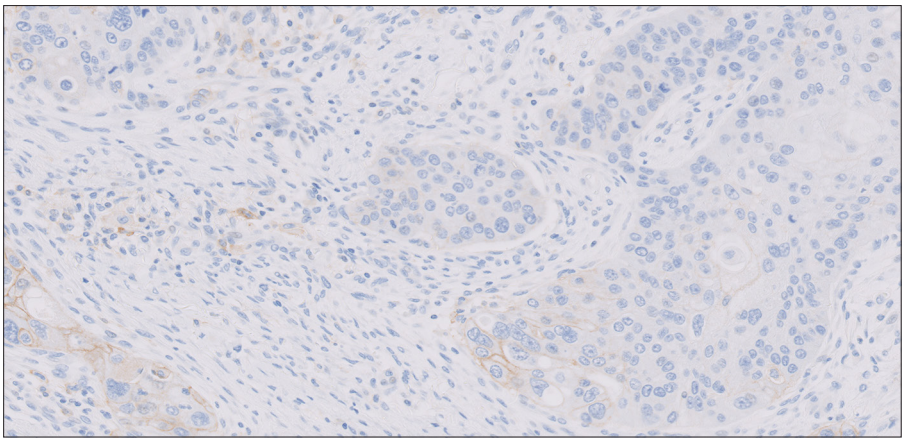
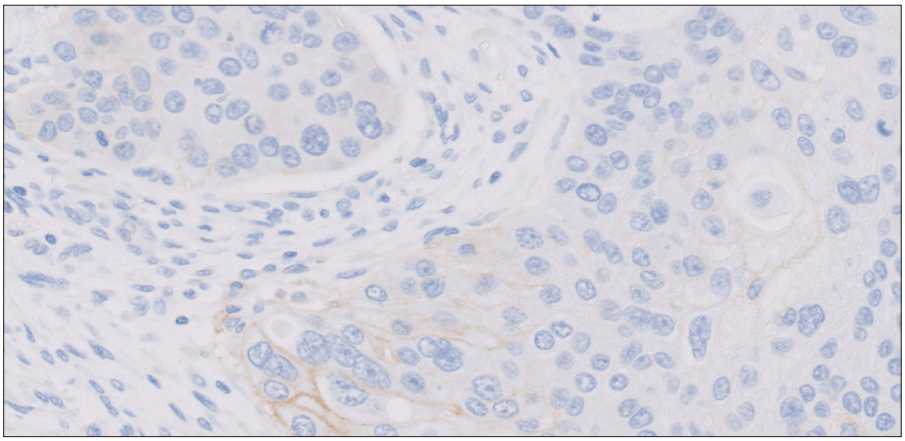


Figure 18c. 40x magnification.



Case 5: PD-L1 expression ≥ 1%

This case example demonstrates partial and complete membrane staining in the tumor cells. The PD-L1 expression is near and above the 1% clinical cutoff (PD-L1 expression 5–10%).

Figure 19a. 10x magnification.

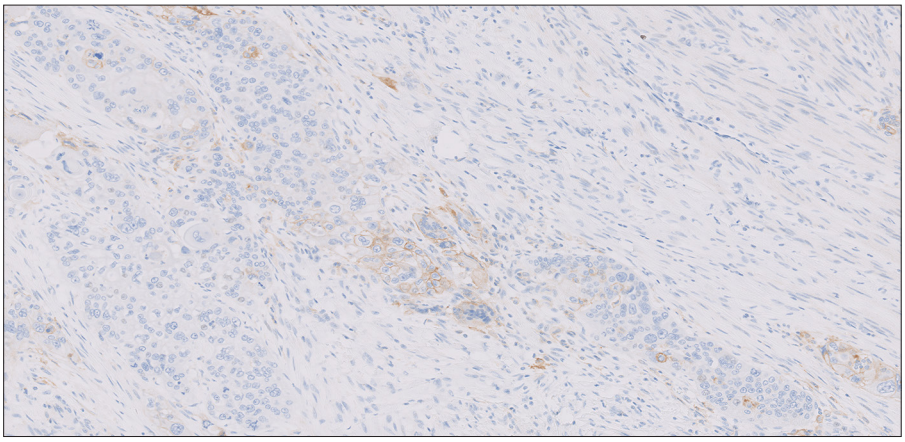


Figure 19b. 20x magnification.

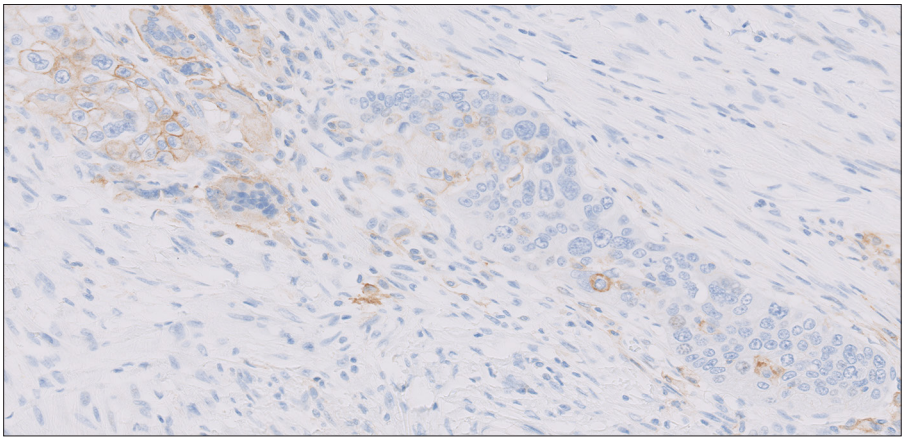
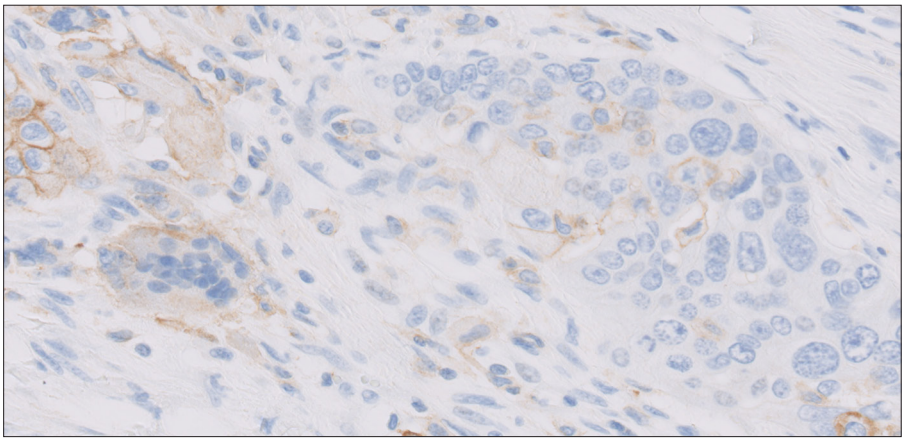


Figure 19c. 40x magnification.



Case 6: PD-L1 expression ≥ 1%

This case example demonstrates partial and complete membrane staining in the tumor cells. This case represents moderate PD-L1 expression of 20–30%.

Figure 20a. 10x magnification.

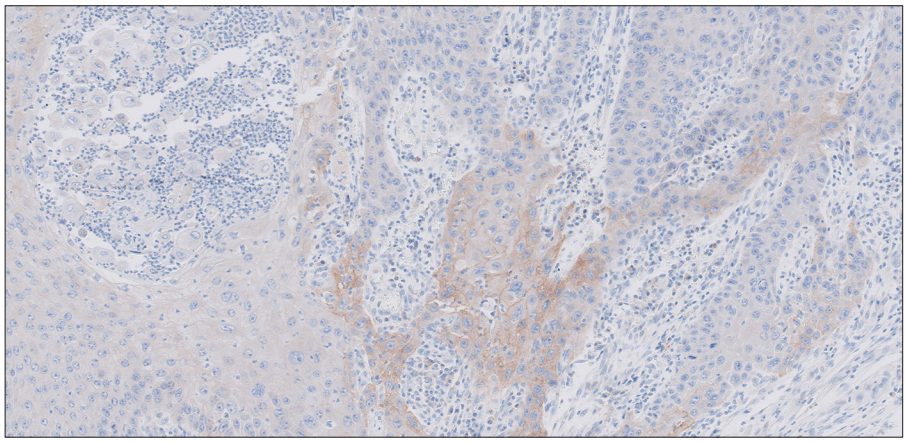


Figure 20b. 20x magnification.

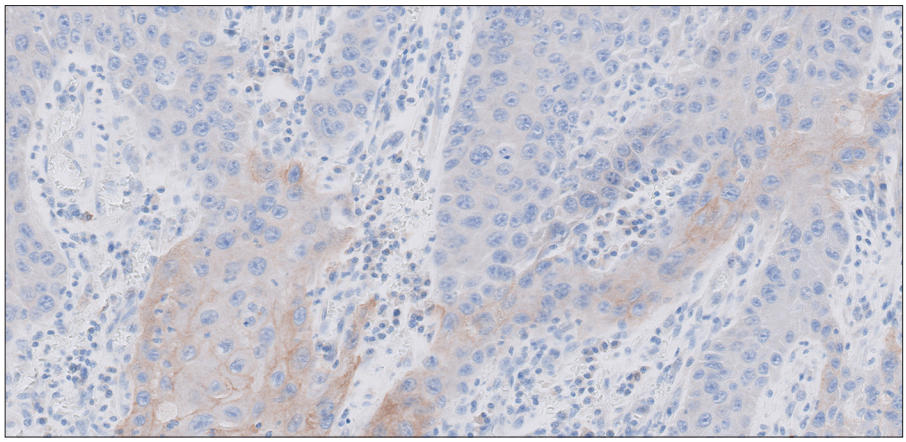
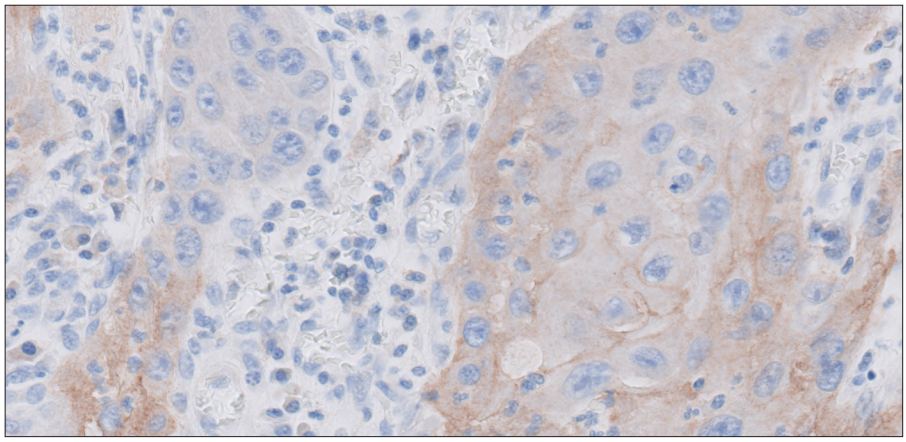


Figure 20c. 40x magnification.



Case 7: PD-L1 expression ≥ 1%

This case example demonstrates partial and complete membrane staining in the tumor cells. This case represents moderate to high PD-L1 expression of 50–60%.

Figure 21a. 10x magnification.

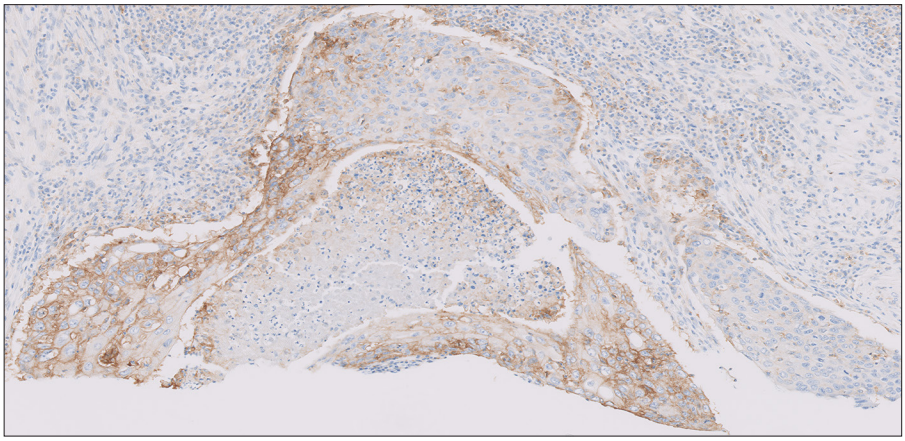


Figure 21b. 20x magnification.

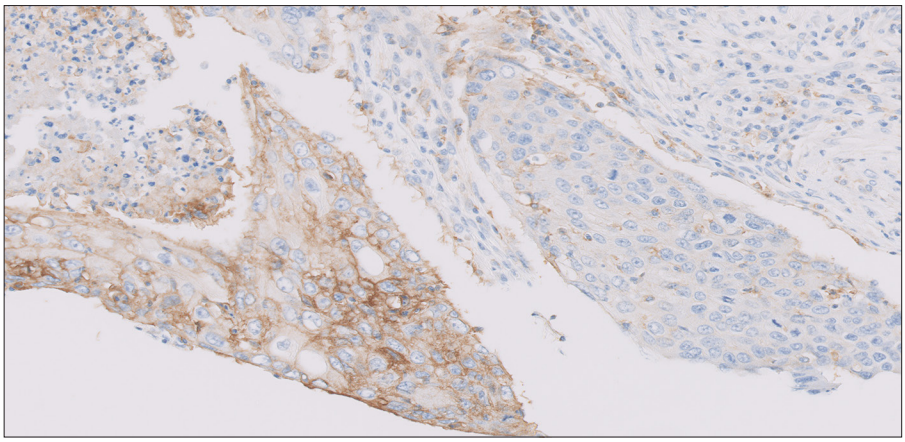
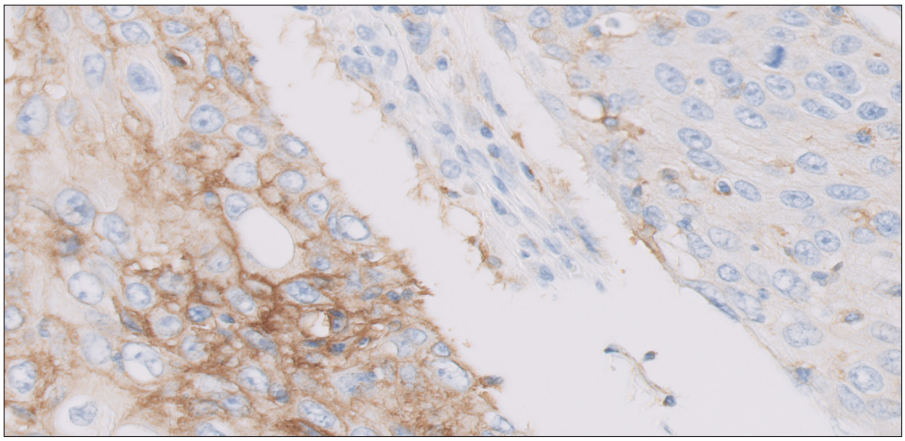


Figure 21c. 40x magnification.



Case 8: PD-L1 expression ≥ 1%

This case example demonstrates partial and complete membrane staining in the tumor cells. This case represents high PD-L1 expression of 95–100%.

Figure 22a. 10x magnification.

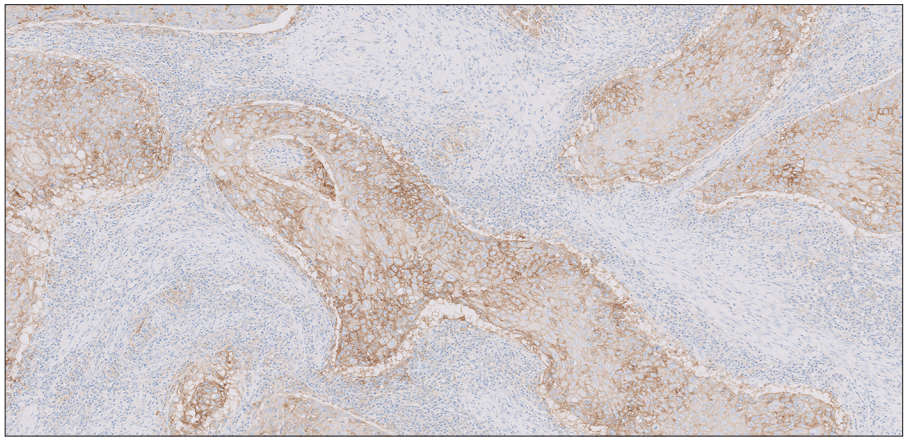


Figure 22b. 20x magnification.

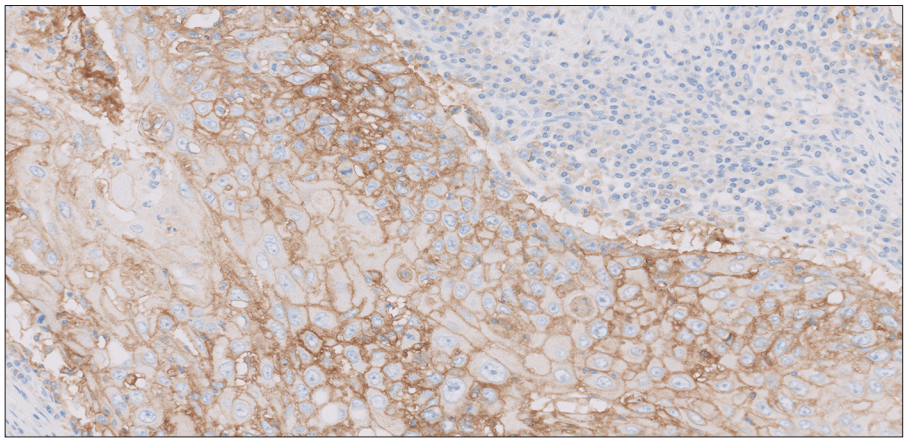
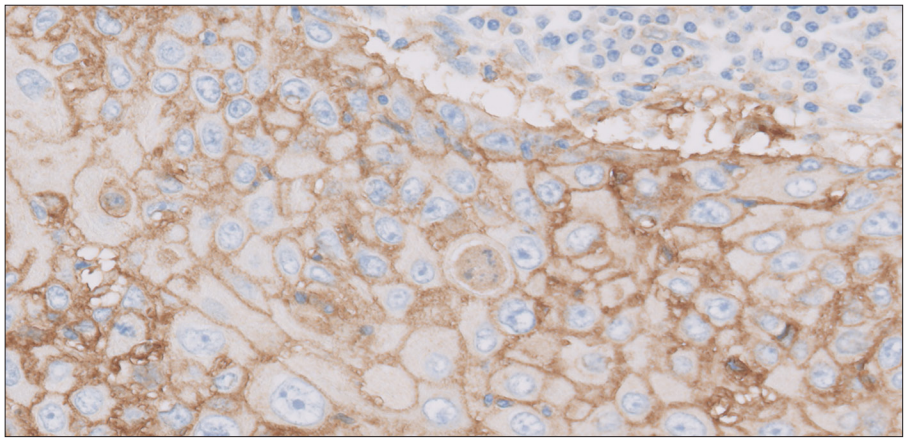


Figure 22c. 40x magnification.



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

Case 1: PD-L1 expression < 1%

This example shows a high number of PD-L1 positive immune cells staining which is not included in determining the % PD-L1 expression.

Figure 23a. 10x magnification.

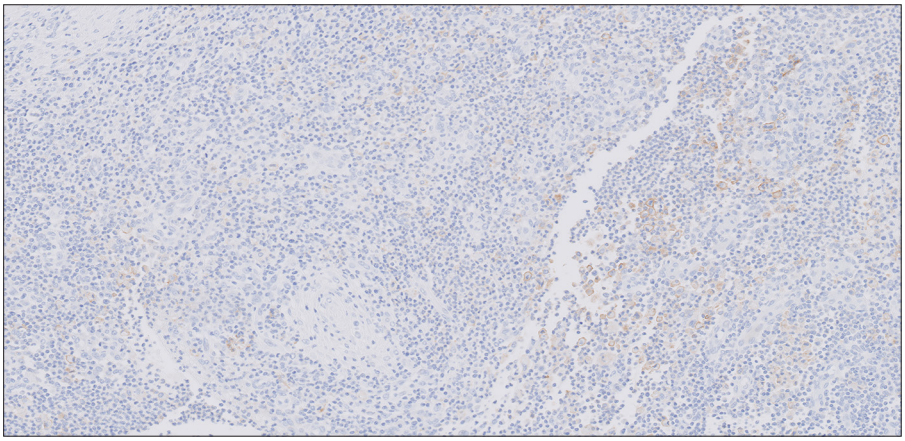


Figure 23b. 20x magnification.

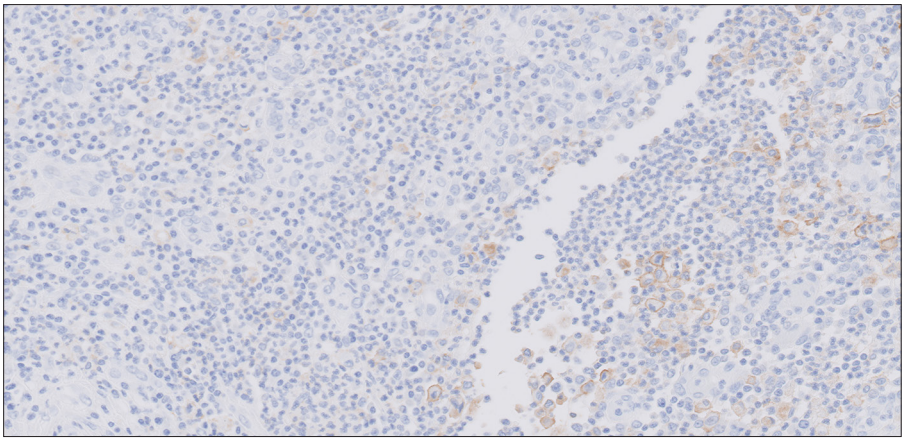
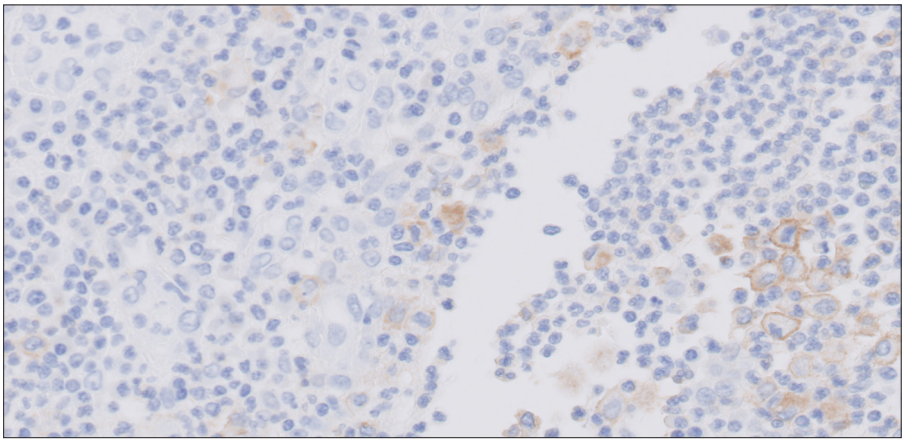


Figure 23c. 40x magnification.



Case 2: PD-L1 expression < 1%

This example shows a few PD-L1 positive tumor cells, which are confirmed at a higher objective (20x, 40x). However, the number of tumor cells staining PD-L1 positive are < 1% when divided by the total number of viable tumor cells in the entire specimen.

Figure 24a. 10x magnification.

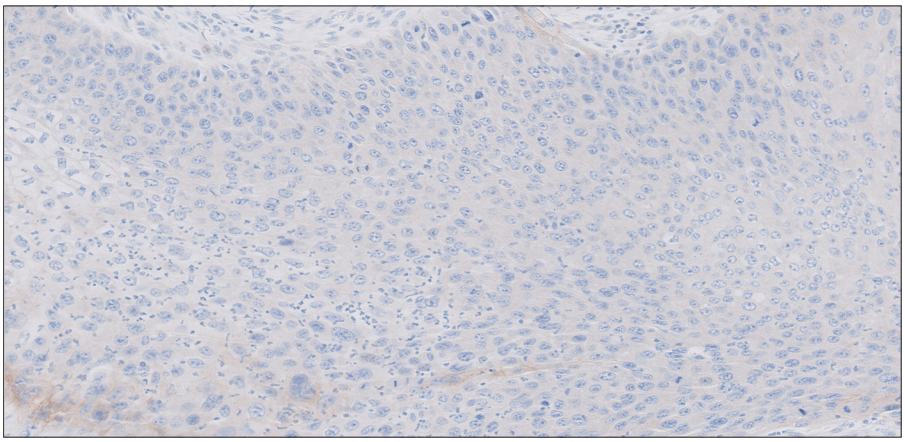


Figure 24b. 20x magnification.
.

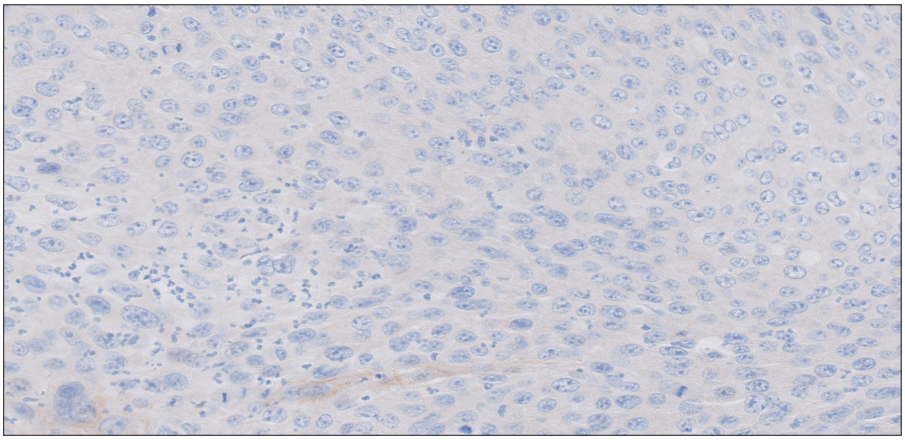
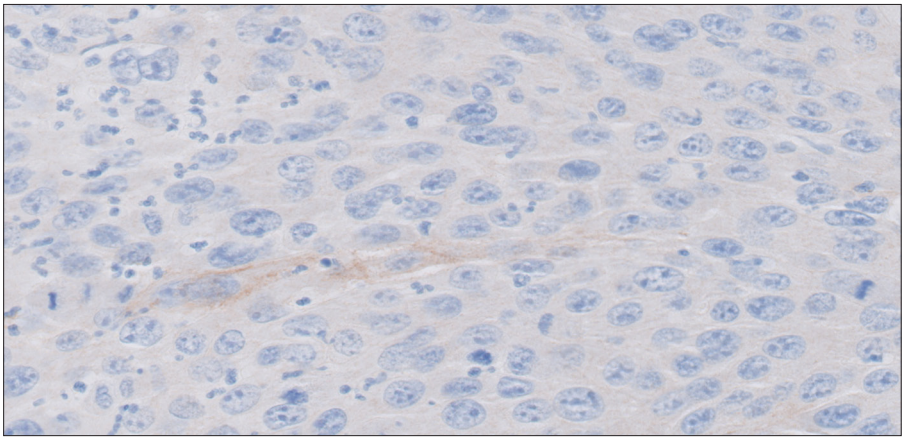


Figure 24c. 40x magnification.



Case 3: PD-L1 expression ≥ 1%

This example shows weak PD-L1 positive tumor cells, which may be missed at a lower objective (4x, 10x) but is confirmed at a higher objective (20x, 40x).

Figure 25a. 10x magnification.

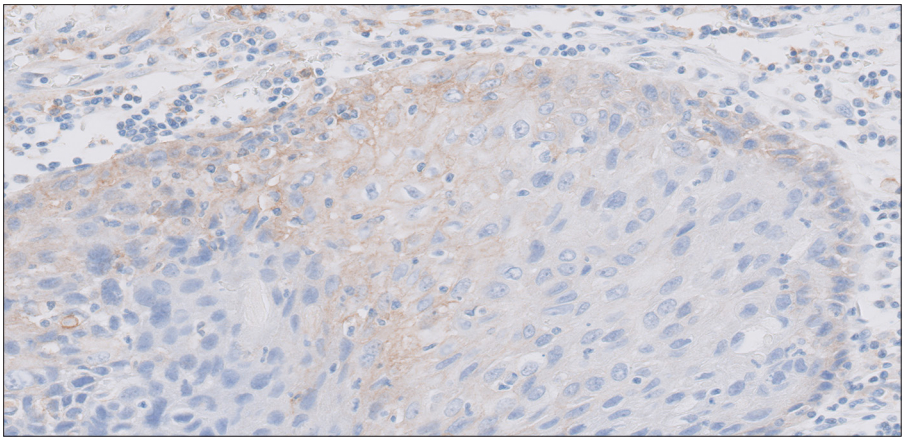


Figure 25b. 20x magnification.

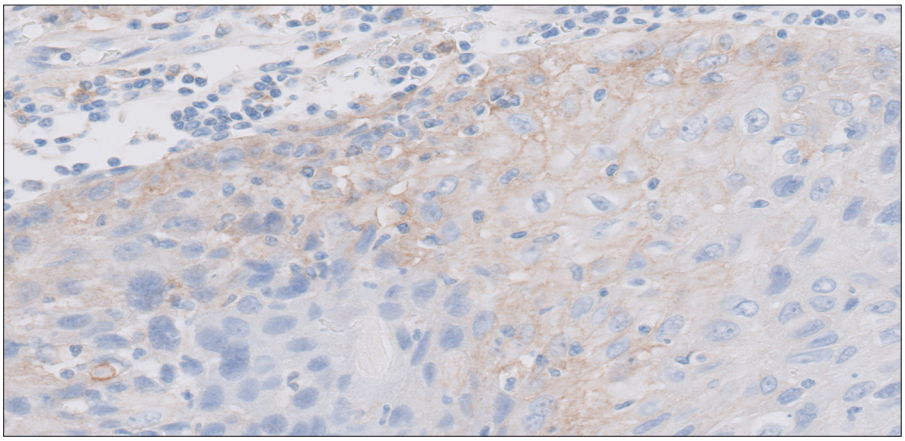
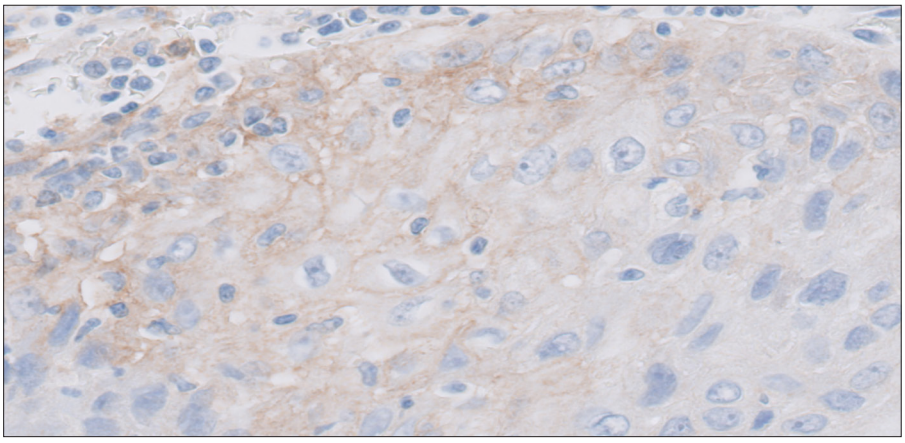


Figure 25c. 40x magnification.



Case 4: PD-L1 expression ≥ 1%

This example shows positive PD-L1 staining in the tumor cells in the presence of non-malignant cells. Reactive fibroblasts must not be mistaken as tumor cells or included in the denominator when determining the % PD-L1 expression. Reference to the H&E may provide assistance when differentiating between reactive fibroblasts and tumor cells. Note that cytoplasmic staining is also present.

Figure 26a. 10x magnification.

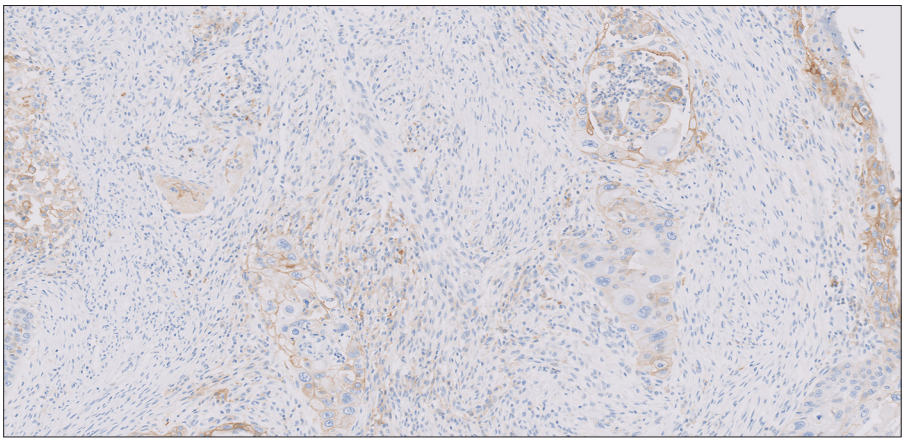


Figure 26b. 20x magnification.

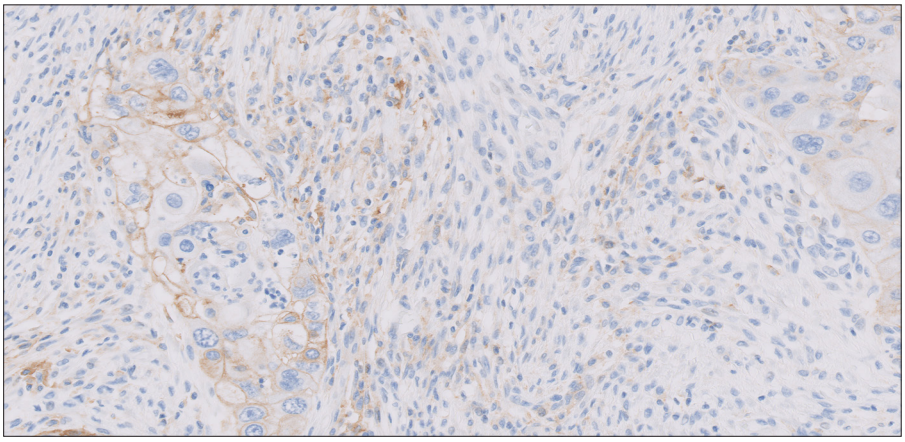
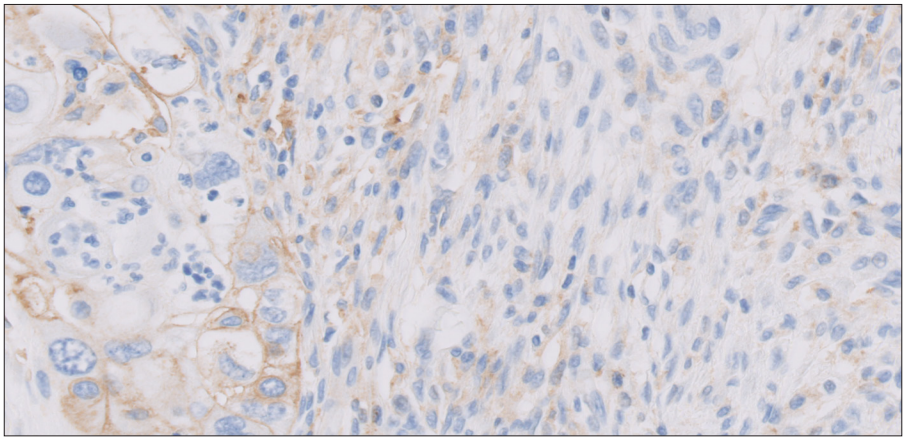


Figure 26c. 40x magnification.



Artifacts

Non-specific Staining

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

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

Possible Cause of Non-specific Staining

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

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

This ESCC example may be considered an indeterminate case if the excess cytoplasmic staining hampers scoring. Positive linear membrane staining of the tumor is observed (**black arrow**), however cytoplasmic staining is excessive in some of the specimen (**red arrows**).

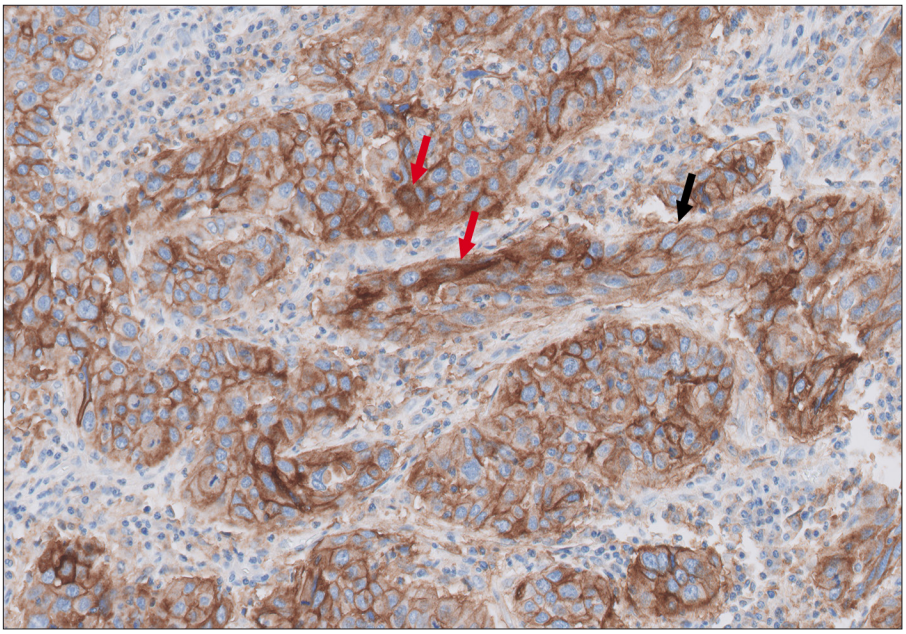


Figure 27. 20x magnification.

Necrosis

Necrotic tissue may show non-specific staining and should not be included in the scoring.
Note: If the specimen is excessively necrotic and contains < 100 viable tumor cells, the specimen is considered not evaluable.

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.

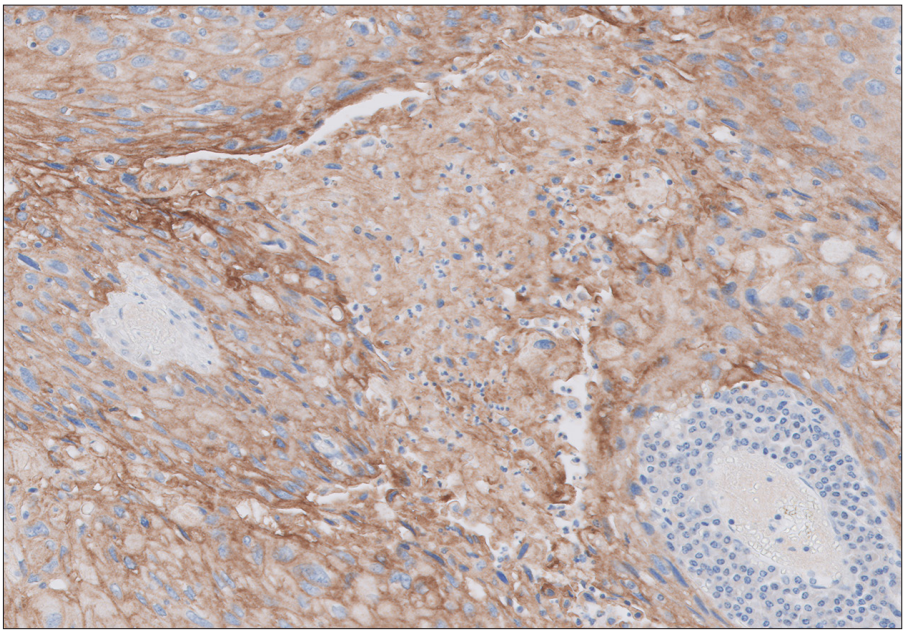


Figure 28. 20x magnification.

Hemosiderin 20X magnification

Specimen stained with PD-L1 antibody exhibiting negative tumor cells and hemosiderin artifact (**black arrows**). Hemosiderin should not be included in the scoring of PD-L1 expression. Note the staining of immune cells are not included in determining the percent PD-L1 expression.

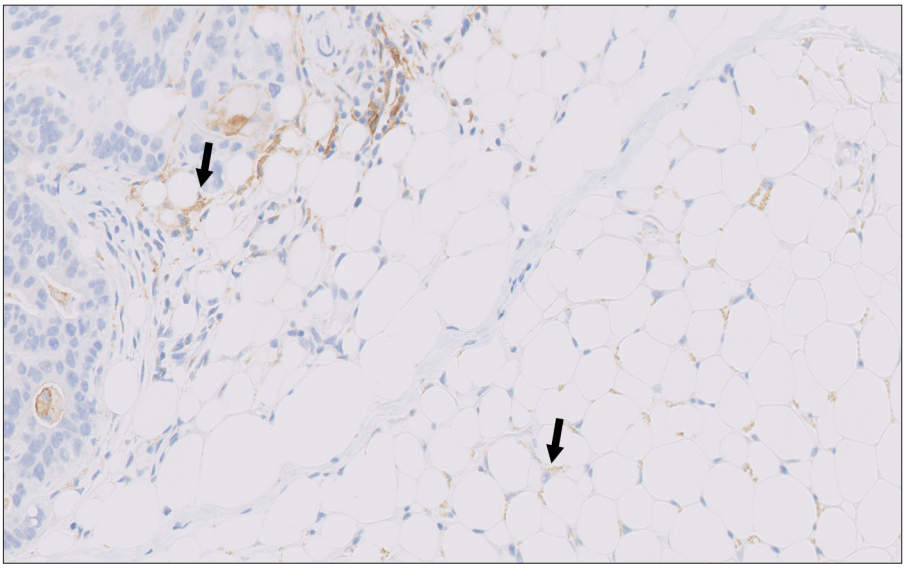


Figure 29. 20x magnification; esophageal adenocarcinoma specimen pictured.

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 on Autostainer Link 48	1a. Verify that the SK005 PD-L1 IHC 28-8 pharmDx program was selected for programming of slides
	1b. Lack of reaction with DAB+ Substrate - Chromogen Solution (DAB)	1b. Verify that DAB+ Substrate-Chromogen Solution was prepared properly
	1c. Sodium azide in wash buffer	1c. Use only EnVision FLEX Wash Buffer, Code K8007
	1d. Degradation of Control Slide	1d. Check kit expiration date and kit storage conditions on outside of package
2. Weak staining of specimen slides	2a. Inappropriate fixation method used	2a. Ensure that only neutral buffered formalin fixative and approved fixation methods are used
	2b. Insufficient reagent volume applied	2b. Check size of tissue section and reagent volume applied
	2c. Inappropriate wash buffer used	2c. Use only EnVision FLEX Wash Buffer, Code K8007
3. Weak staining of specimen slides or the positive cell line on the Agilent-supplied Control Slide	3a. Inadequate Target Retrieval	3a. Verify that the 3-in-1 pre-treatment procedure was correctly performed
	3b. Inappropriate wash buffer used	3b. Use only EnVision FLEX Wash Buffer, Code K8007
4. Excessive non-specific staining of slides	4a. Paraffin incompletely removed	4a. Verify that the 3-in-1 pre-treatment procedure was correctly performed
	4b. Slides dried while loading onto the Autostainer Link 48	4b. Ensure slides remain wet with buffer while loading and prior to initiating run
	4c. Non-specific binding of reagents to tissue section	4c. Check for proper fixation of the specimen and/or the presence of necrosis
	4d. Inappropriate fixation method used	4d. Ensure that only neutral buffered formalin fixative and approved fixation methods are used
	4e. Inadequate mixing of wash buffer	4e. Ensure wash buffer is properly mixed
5. Tissue detached from slides	5a. Use of incorrect microscope slides	5a. Use FLEX IHC Microscope Slides (Code K8020), or Superfrost Plus charged slides
	5b. Inadequate preparation of specimens	5b. Cut sections should be placed in a 58 ± 2 °C oven for 1 hour prior to staining
6. Excessively strong specific staining	6a. Inappropriate fixation method used	6a. Ensure that only approved fixatives and fixation methods are used
	6b. Inappropriate wash buffer used	6b. Use only EnVision FLEX Wash Buffer, Code K8007
7. 1x EnVision FLEX Target Retrieval Solution is cloudy in appearance when heated	7. When heated the 1x EnVision FLEX Target Retrieval Solution turns cloudy in appearance	7. This is normal and does not influence staining

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

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Published in Europe, April 13, 2022
29489EN-EU D70803 2022APR06

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