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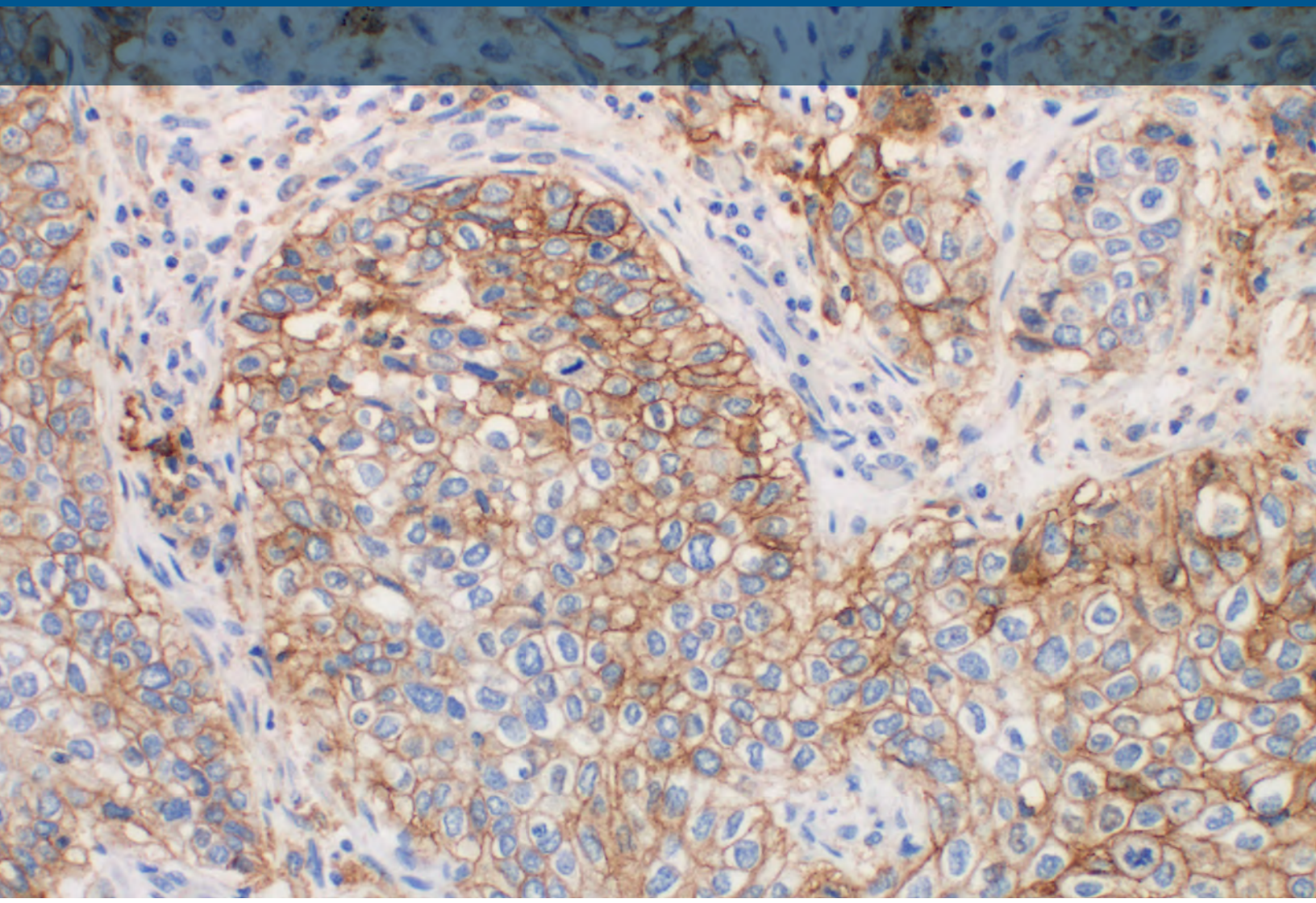
PD-L1 IHC 28-8 pharmDx Interpretation Manual — NSCLC

IVD for in vitro diagnostic use



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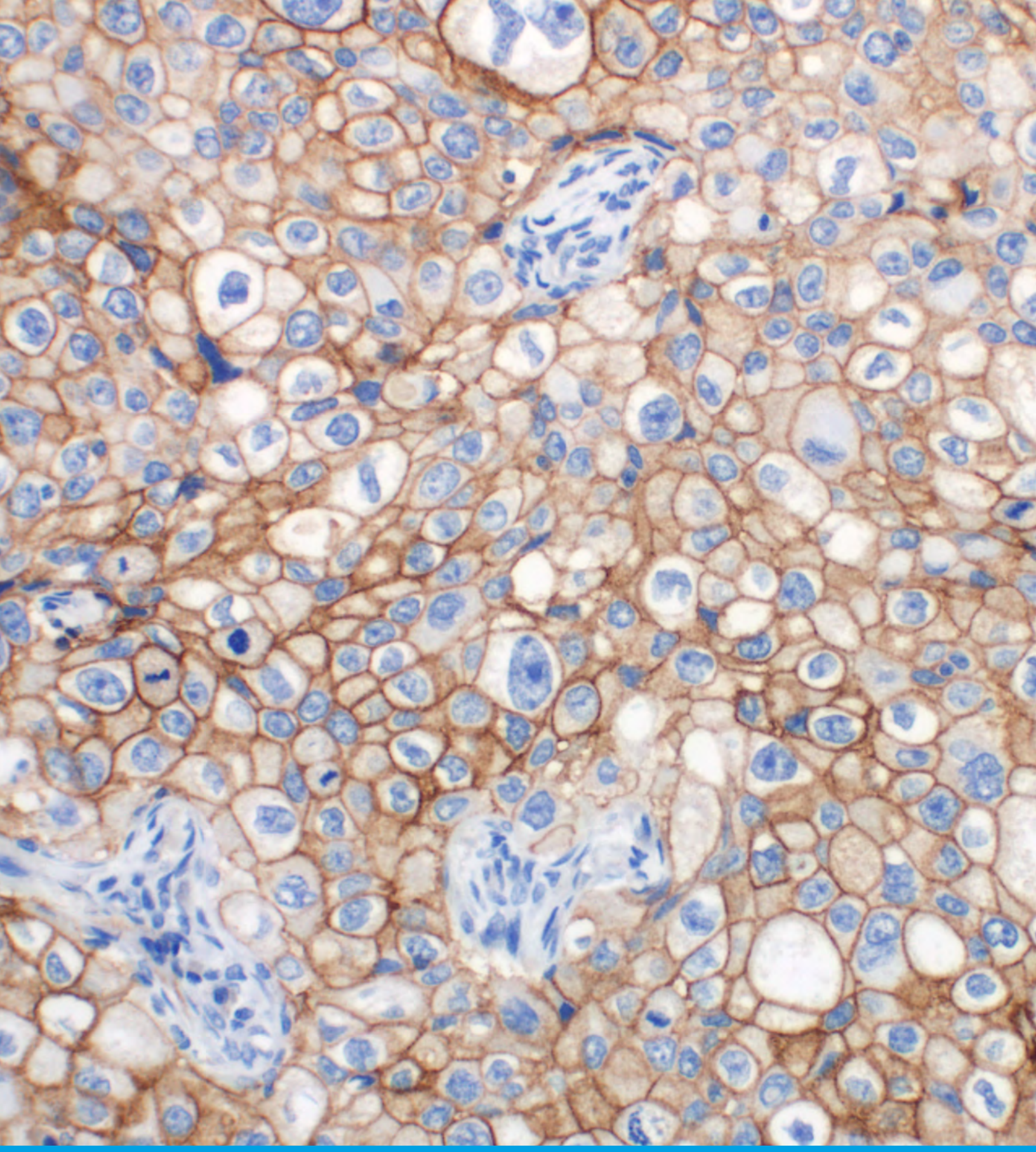


PD-L1 IHC 28-8 pharmDx
Interpretation Manual - NSCLC

Introduction

PD-L1 IHC 28-8 pharmDx Intended Use

For In Vitro Diagnostic Use

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

PD-L1 protein expression in NSCLC and non-squamous non-small cell lung cancer (nsNSCLC) 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
NSCLC	≥ 1% tumor cell expression	PD-L1 IHC 28-8 pharmDx is indicated as an aid in identifying early stage NSCLC patients for treatment with OPDIVO® (nivolumab) in combination with platinum-doublet chemotherapy.

Non-Companion Diagnostic Indication

Tumor Indication	PD-L1 Expression Clinical Cutoff	Intended Use
nsNSCLC	≥ 1%, ≥ 5%, ≥ 10% tumor cell expression	PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in non-squamous NSCLC (nsNSCLC) may be associated with enhanced survival from OPDIVO® (nivolumab).

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

Clinical study CA209816 investigated the clinical validity of PD-L1 IHC 28-8 pharmDx for the assessment of PD-L1 status in NSCLC patients treated with OPDIVO combined with platinum doublet chemotherapy as neoadjuvant treatment of resectable (stage IB, stage II, and stage IIIA) NSCLC.

Clinical study CA209057 investigated the clinical validity of PD-L1 IHC 28-8 pharmDx for the assessment of PD-L1 status in nsNSCLC patients treated with OPDIVO.

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

This PD-L1 IHC 28-8 pharmDx Interpretation Manual is provided as a tool to help guide pathologists and laboratory technicians to achieve correct and reproducible results. The goal of this manual is to familiarize you with the requirements for scoring NSCLC and nsNSCLC specimens stained with PD-L1 IHC 28-8 pharmDx.

A review of this PD-L1 IHC 28-8 pharmDx Interpretation Manual will provide a solid foundation for evaluating NSCLC and nsNSCLC 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. For more details regarding the country specific device and clinical data, please refer to instructions for use: Instructions For Use | Agilent

Photomicrographs of example cases are provided for reference.

OPDIVO is a trademark of Bristol Myers Squibb Company.

Acknowledgment

Photomicrographs

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

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

- Tissue samples supplied by BioIVT (Hicksville, NY, USA)
- The data and biospecimens used in this project were provided by US Biolab (Gaithersburg, MD, USA) with appropriate ethics approval and through Azenta Life Sciences
- The data and biospecimens used in this project were provided by Centre Hospitalier Universitaire (CHU) de Nice (Nice, France) with appropriate ethics approval and through Azenta Life Sciences
- Samples/tissue supplied by Conversant Biologics
- The data and biospecimens used in this project were provided by Sofia Bio LLC (New York, NY, USA) with appropriate ethics approval and through Azenta Life Sciences
- 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

Interpretation and Reporting

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 using a light microscope 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 pages 22 and 23.

PD-L1 Overview

The PD-1/PD-L1 Pathway Controls the Immune Response in Normal Tissue

Programmed death-ligand 1 (PD-L1) is a transmembrane protein that binds to the programmed death-1 receptor (PD-1) during immune system modulation. The PD-1 receptor is typically expressed on cytotoxic T-cells and other immune cells, while the PD-L1 ligand is typically expressed on normal cells. Normal cells use the PD-1/PD-L1 interaction as a mechanism of protection against immune recognition by inhibiting the action of T-cells (Figure a). Inactivation of cytotoxic T-cells downregulates the immune response such that the inactive T-cell is exhausted, ceases to divide, and might eventually die by programmed cell death, or apoptosis.

The Tumor Escapes Detection by Utilizing the PD-1/PD-L1 Pathway

Many tumor cells are able to upregulate the expression of PD-L1 as a mechanism to evade the body's natural immune response. Activated T-cells recognize the PD-L1 marker on the tumor cell, similar to that of a normal cell, and PD-L1 signaling renders the T-cell inactive (Figure b). The tumor cell escapes the immune cycle, continues to avoid detection for elimination and is able to proliferate.

Anti-PD-1 Therapy Enables the Immune Response Against Tumors

Anti-PD-1 therapy works by blocking the PD-1/PD-L1 interaction between tumor cells and activated T-cells, helping to prevent immunosuppression, thereby enabling cytotoxic T-cells to actively remove tumor cells (Figure c).

PD-L1 IHC 28-8 pharmDx Detects PD-L1 in NSCLC and nsNSCLC

PD-L1 upregulation in NSCLC is a biomarker for response to anti-PD-1 therapy. PD-L1 IHC 28-8 pharmDx was the only PD-L1 assay used in the OPDIVO (nivolumab) clinical trials (CheckMate-816 and CheckMate-057) to evaluate the relationship between PD-L1 expression and clinical efficacy.

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

Limiting damage to healthy tissue

Inactivation of T-cells limits damage to healthy tissue.

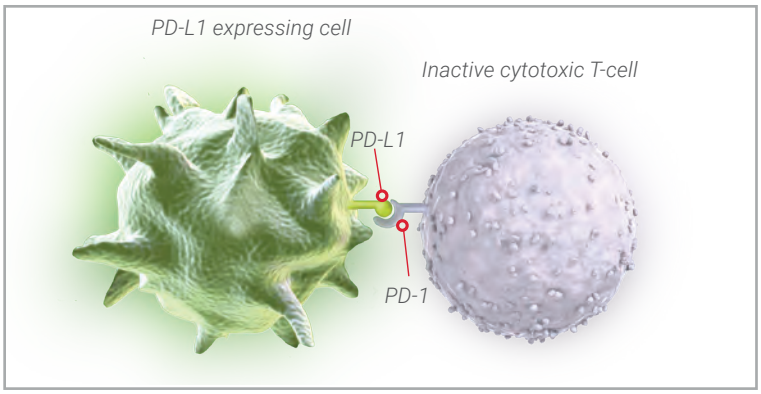


Figure a.

The tumor escapes detection

Inactivation of T-cells reduces tumor cell killing.

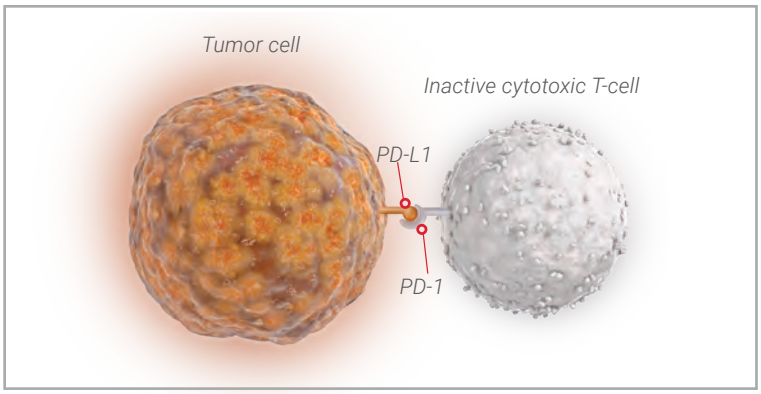


Figure b.

Immuno-oncology therapies harness the immune response to fight tumors

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

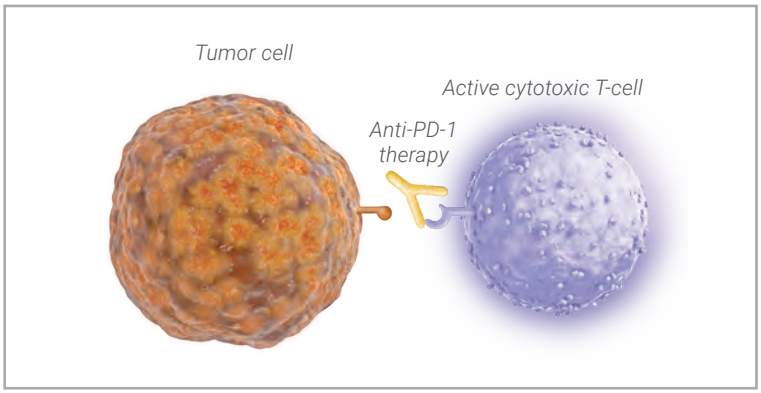


Figure c.

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 (Figure 1a). 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 (Figure 1b). 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

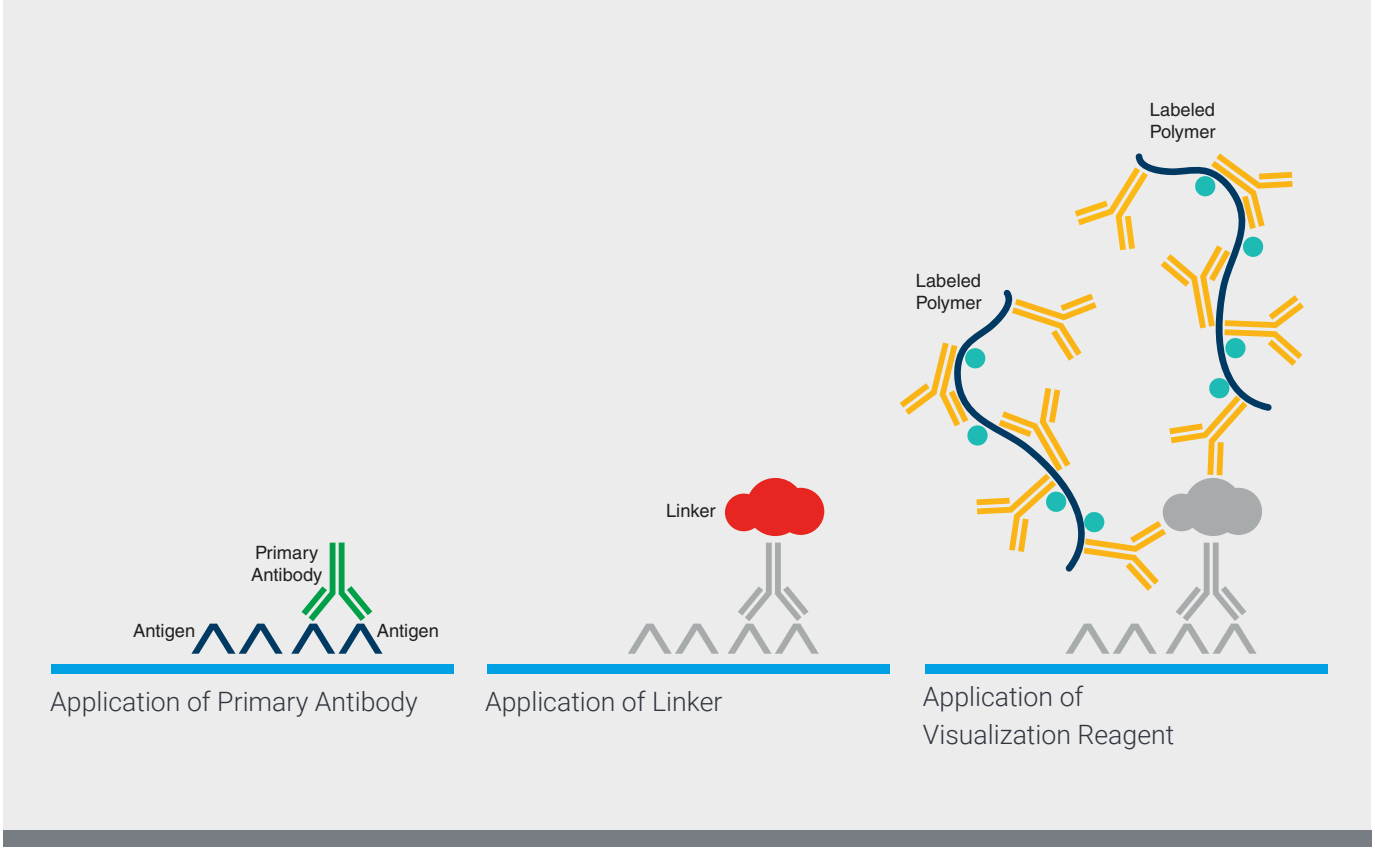


Figure 1a. PD-L1 IHC 28-8 pharmDx staining procedure.

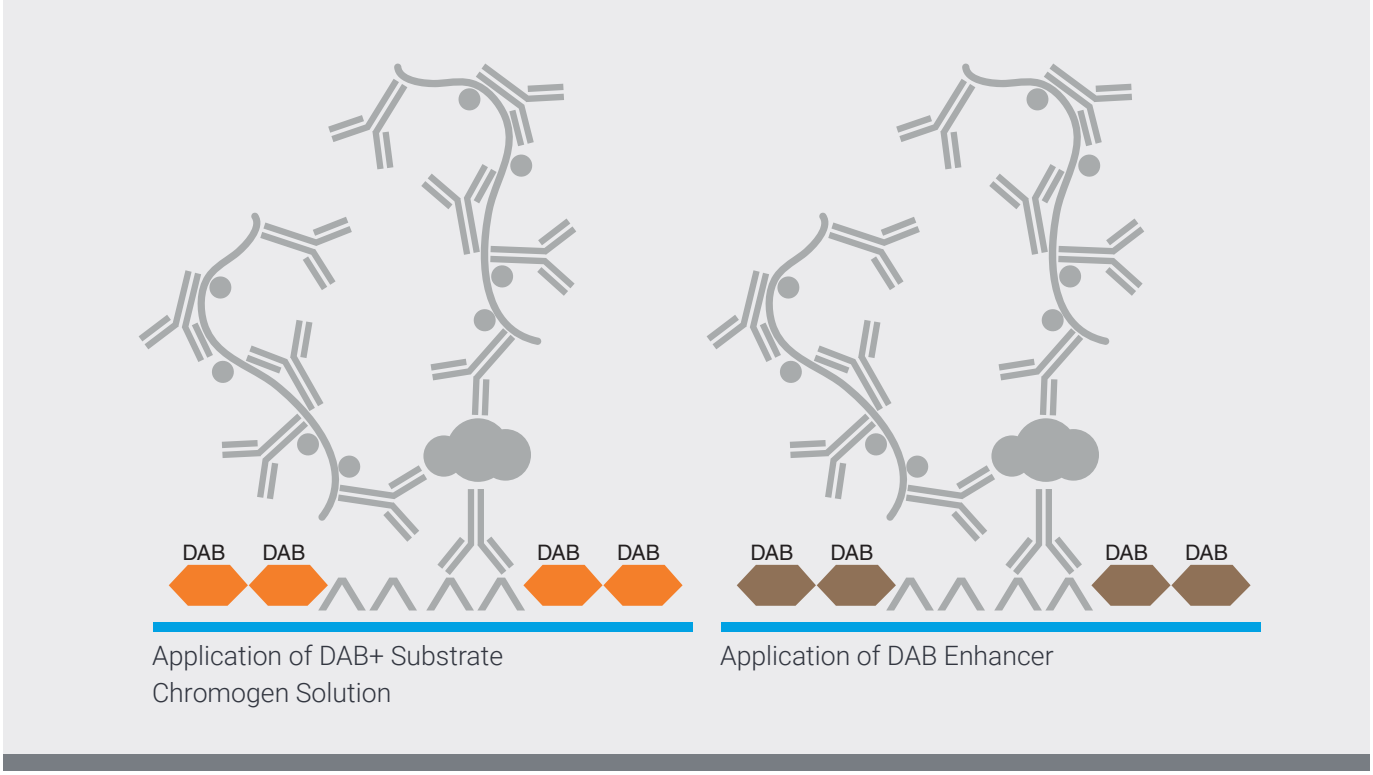


Figure 1b. PD-L1 IHC 28-8 pharmDx staining procedure.

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

All PD-L1 IHC 28-8 pharmDx reagents are to be performed on 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, 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 **except for squamous NSCLC** stored at room temperature up to 25 °C. Squamous NSCLC tissue sections stored at room temperature up to 25 °C should be stained within 2 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.

Controls should be biopsy/surgical specimens fixed, processed, and embedded as soon as possible in the same manner as the patient sample(s). 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. 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 to aid in detection of subtle changes in assay sensitivity. The ideal negative control tissue should demonstrate no staining of immune cells. However, prevalence of PD-L1 expression of immune cells is high, therefore a few staining immune cells is acceptable. Alternatively, negative portions of the positive control tissue may serve as the negative control tissue, but 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 (50x)

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 de-ionized water; the pH of 1x EnVision FLEX Target Retrieval Solution must be 6.1 ± 0.2. Do not modify the pH of 1x EnVision FLEX Target Retrieval Solution after preparation under any circumstance. If a problem is suspected with the EnVision FLEX Target Retrieval Solution pH, please refer to 'Troubleshooting' Section 18 of the IFU for more information. One 30 mL bottle of EnVision FLEX Target Retrieval Solution, Low pH (50x), diluted 1:50 will provide 1.5 L of 1x reagent, sufficient to fill 1 PT Link tank, which will treat up to 24 slides per use. Discard 1x EnVision FLEX Target Retrieval Solution after 3 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 1x EnVision FLEX Wash Buffer for the wash steps by diluting EnVision FLEX Wash Buffer (20x) 1:20 using distilled or de-ionized 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 mL of DAB+ Substrate Buffer and mix. Prepared Substrate-Chromogen is stable for 5 days if stored in the dark at 2–8 °C. Mix the Substrate-Chromogen Solution thoroughly prior to use. Any precipitate developing in the solution does not affect staining quality.

- **When using an entire bottle of DAB+ Substrate Buffer, add 9 drops of DAB+ Chromogen.** Although the 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 above. 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 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 EnVision FLEX Target Retrieval Solution, Low pH, 1x 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 (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.
- Leave Autostainer rack with slides in room temperature EnVision FLEX Wash Buffer for 5 minutes.

Staining and Counterstaining

- Place the Autostainer rack with slides on the Autostainer Link 48. Ensure slides remain wet with buffer while loading and prior to initiating the run. Dried tissue sections may display increased nonspecific staining.
- Select the PD-L1 IHC 28-8 pharmDx protocol. The instrument performs the staining and counterstaining procedures by applying the appropriate reagent, monitoring the incubation time, and rinsing slides between reagents. Counterstaining using EnVision FLEX Hematoxylin (Code K8008), for 7 minutes, is included in the staining protocol. Do not allow slides to dry prior to mounting.

Mounting

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

PD-L1 IHC 28-8 pharmDx Technical Checklist

Customer Name / Institution: _____

Name and Title: _____

Autostainer Link 48 Serial Number: _____ Software Version: _____

		Yes	No
1.	Regular preventive maintenance is performed on the Autostainer Link 48 and PT Link?	☐	☐
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 10% 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.	Non-squamous NSCLC 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.	Squamous NSCLC specimens are stained within 4 months of sectioning when stored in the dark at 2–8 °C or within 2 months of sectioning when stored in dark at room temperature up to 25 °C?	☐	☐
11.	EnVision FLEX Target Retrieval Solution, Low pH is prepared properly (working solution pH 6.1±0.2) ?	☐	☐
12.	EnVision FLEX Wash Buffer is prepared properly?	☐	☐
13.	DAB+ Substrate-Chromogen Solution is prepared properly?	☐	☐
14.	The Deparaffinization, Rehydration, and Target Retrieval 3-in-1 procedure is followed, using PT Link?	☐	☐
15.	Slides remain wet with buffer while loading and prior to initiating run on Autostainer Link 48?	☐	☐
16.	The PD-L1 IHC 28-8 pharmDx protocol is selected on Autostainer Link 48?	☐	☐
17.	Slides are counterstained with EnVision FLEX Hematoxylin?	☐	☐
18.	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 22 and 23, for an example of a pathology report form for PD-L1 IHC 28-8 pharmDx.

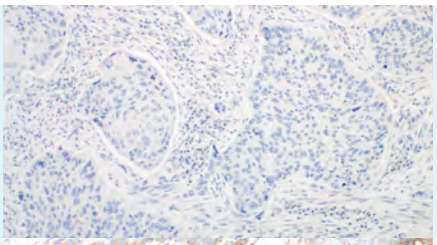
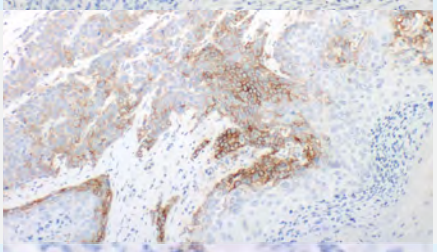
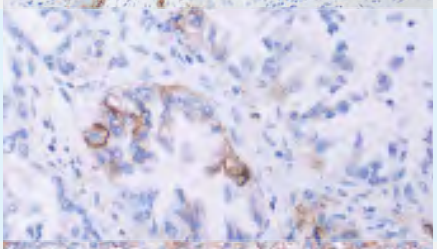
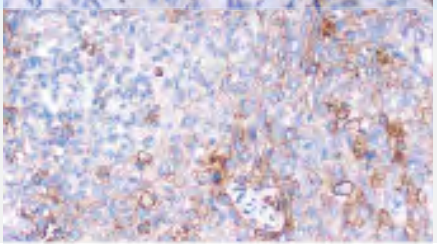
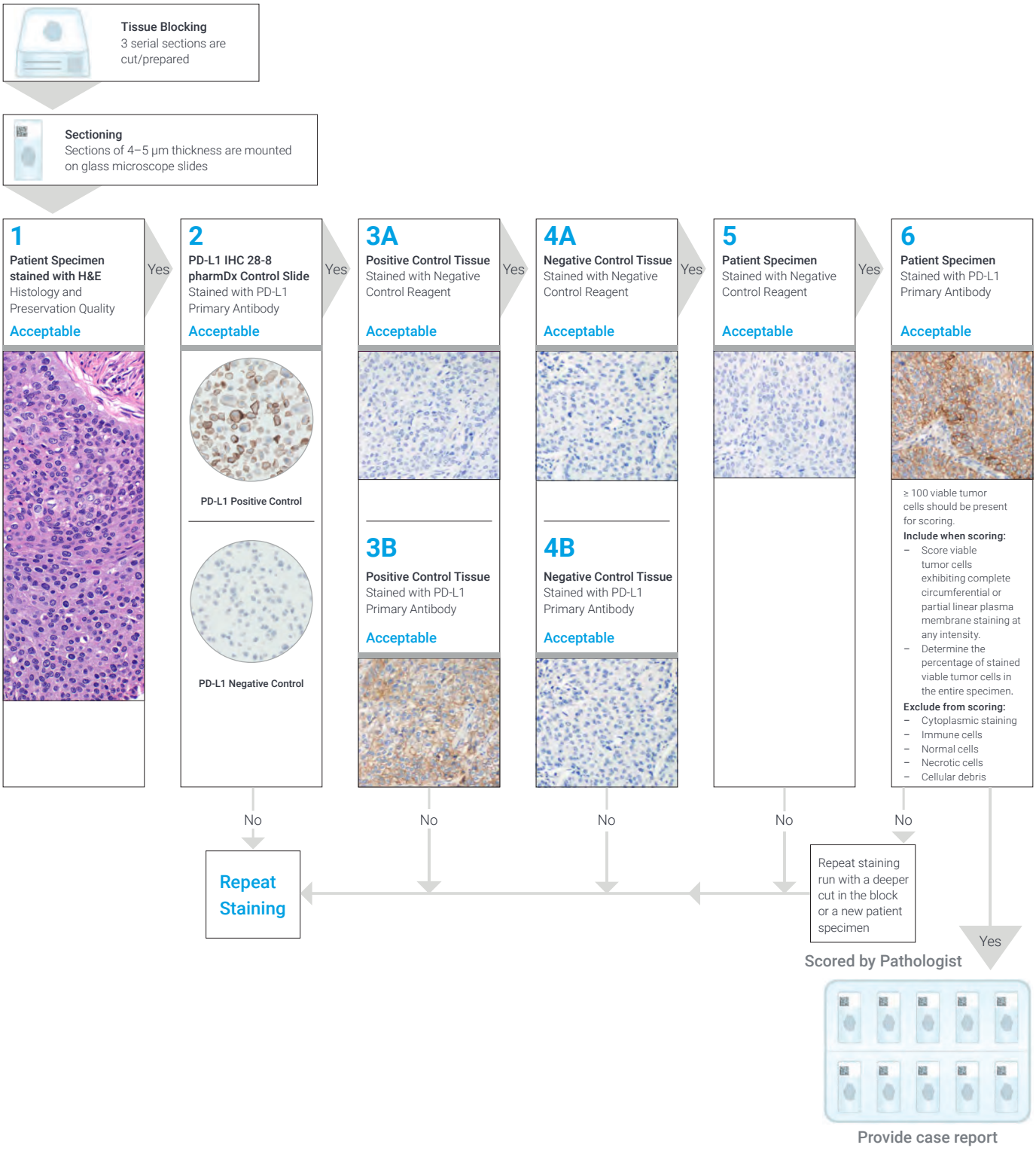
Staining Pattern	Examples of NSCLC and nsNSCLC	Examples of result reporting
< 1% of the viable tumor cells exhibit complete circumferential or partial linear plasma membrane staining at any intensity.		PD-L1 expression < 1%
≥ 1% of the viable tumor cells exhibit complete circumferential or partial linear plasma membrane staining at any intensity.		PD-L1 expression ≥ 1%
≥ 5% of the viable tumor cells exhibit complete circumferential or partial linear plasma membrane staining at any intensity.		PD-L1 expression ≥ 5% (nsNSCLC only)
≥ 10% of the viable tumor cells exhibit complete circumferential or partial linear plasma membrane staining at any intensity.		PD-L1 expression ≥ 10% (nsNSCLC only)

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

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

The following flow of slide review is recommended when conducting interpretation of PD-L1 IHC 28-8 pharmDx.



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

PD-L1 IHC 28-8 pharmDx evaluation should 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 Negative Control Reagent. Lastly, examine the patient specimen stained with Primary Antibody to assess the staining of viable tumor cells.

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

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

Patient Specimen Stained with H&E

A 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.

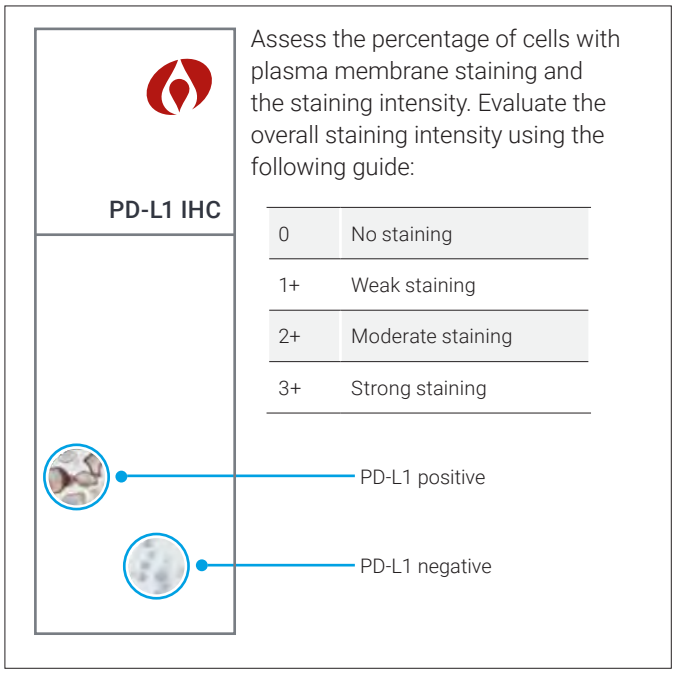


Figure 4. Each Control Slide contains sections of cell pellets with positive and negative PD-L1 expression.

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
- Nonspecific staining < 1+ intensity

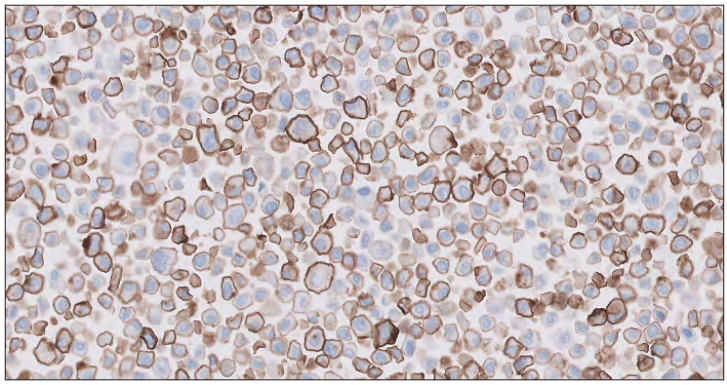


Figure 5. Acceptable Positive PD-L1 Control.

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

- No specific staining
- Nonspecific 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.

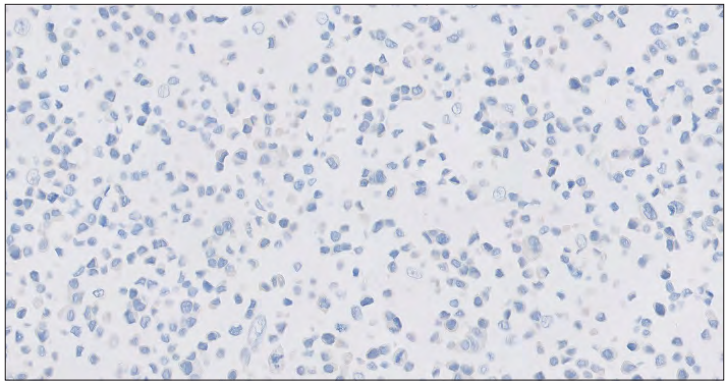


Figure 6. Acceptable Negative PD-L1 Control.

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 nonspecific 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 nonspecific 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 nonspecific staining should be ≤ 1+ intensity. If any staining is not satisfactory, results with the patient specimen should be considered invalid.

The NCR indicates nonspecific 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 nonspecific staining of the patient specimen stained with NCR. A minimum of 100 viable tumor cells should be present in the PD-L1 stained patient slide for 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 nonmalignant cells, necrotic cells, and cellular debris. Nonspecific 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. Nonmalignant cells and immune cells (e.g., infiltrating lymphocytes or macrophages) may also stain with PD-L1 but must be excluded.
3. Record the PD-L1 % tumor cell expression level. 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 % tumor cell expression
- Use higher magnifications to confirm cell types and areas absent of staining
- Be careful not to overlook weak 1+ staining, which can be missed at 4x and 10x
- Disregard cytoplasmic staining
- Necrotic tissue may stain but should be excluded
- Exclude any nonmalignant cells and immune cells

Indeterminate Specimen

The tumor cell membrane has been hampered for reasons attributed to the biology of the tumor tissue sample rather than improper sample preparation. For example, high cytoplasmic staining of the tumor cells can hamper scoring of the membrane staining. An additional cut section or section from another block of the same patient may be required for PD-L1 IHC 28-8 pharmDx evaluation.

Non-evaluable Specimens

The specimen should be considered non-evaluable if there are fewer than 100 viable tumor cells. A different section from the same block or another block from the same patient may be required to present sufficient viable tumor cells to support 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 nonmalignant 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).

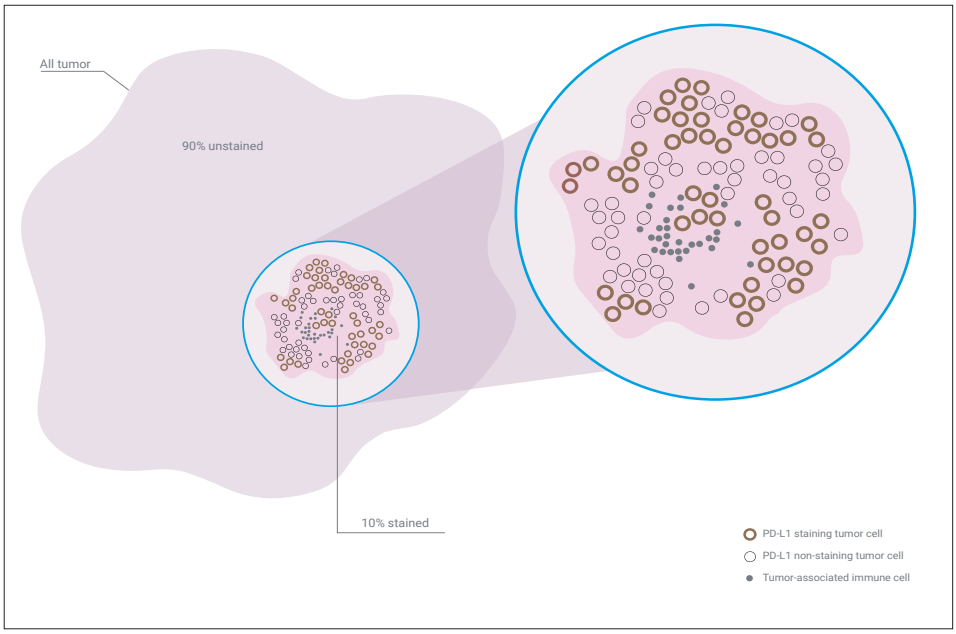


Figure 7. Example of tumor with small PD-L1 staining area.

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

Method 1

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

Method 2

$$\frac{50\% \times 10\%}{100} = 5\%\ \text{PD-L1 expression}$$

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 nonmalignant 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:

$$\frac{(80\% + 30\% + 50\% + 100\%)}{4 \text{ quadrants}} = 65\% \text{ PD-L1 expression}$$

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

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

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:

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

PD-L1 Results: PD-L1 IHC 28-8 pharmDx is indicated as an aid in identifying resectable (stage IB, stage II, and stage IIIA) NSCLC patients for treatment with OPDIVO (nivolumab) in combination with platinum-doublet chemotherapy (CA209816).

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

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

Pathologist's comments: _____

OPDIVO is a trademark of Bristol-Myers Squibb Company.

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

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

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:

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

PD-L1 Results: PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in nsNSCLC may be associated with enhanced survival from OPDIVO (CA209057).

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

☐ PD-L1 expression is ≥ 1%	☐ PD-L1 expression is < 1%
☐ PD-L1 expression is ≥ 5%	☐ PD-L1 expression is < 5%
☐ PD-L1 expression is ≥ 10%	☐ PD-L1 expression is < 10%

Pathologist's comments: _____

OPDIVO is a trademark of Bristol-Myers Squibb Company.

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

Positive Control Specimen

An example of NSCLC 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.

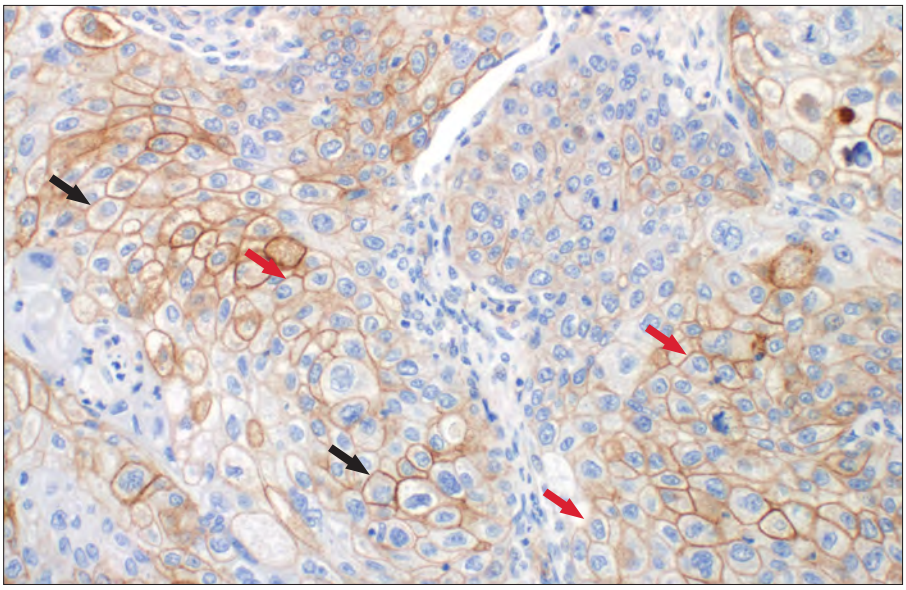


Figure 9. 20x magnification.

Distinguishing Tumor Cells from Immune Cells

NSCLC specimen showing strong staining of intratumoral associated immune cells (**red arrows**), while the tumor cells are negative (**black arrows**) for PD-L1 positivity.

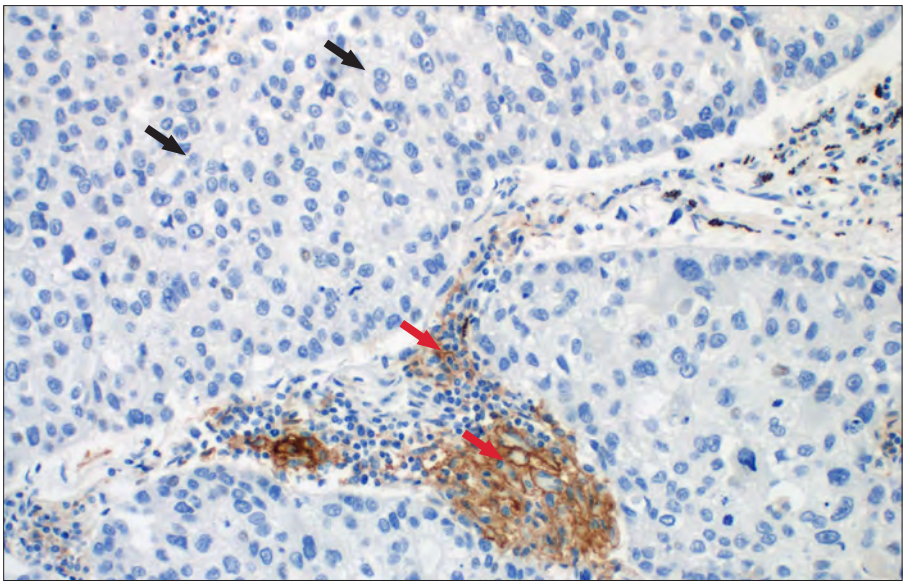


Figure 10. 20x magnification.

Distinguishing Tumor Cells from Immune Cells

Anthracotic pigment is an accumulation of carbon from inhaled smoke or coal dust. This example shows anthracotic pigment, small granular black spots, located within the immune cells (**red arrows**) which are helpful to distinguish from tumor cells. Anthracotic pigment is not found within tumor cells, which are PD-L1 negative (**black arrow**).

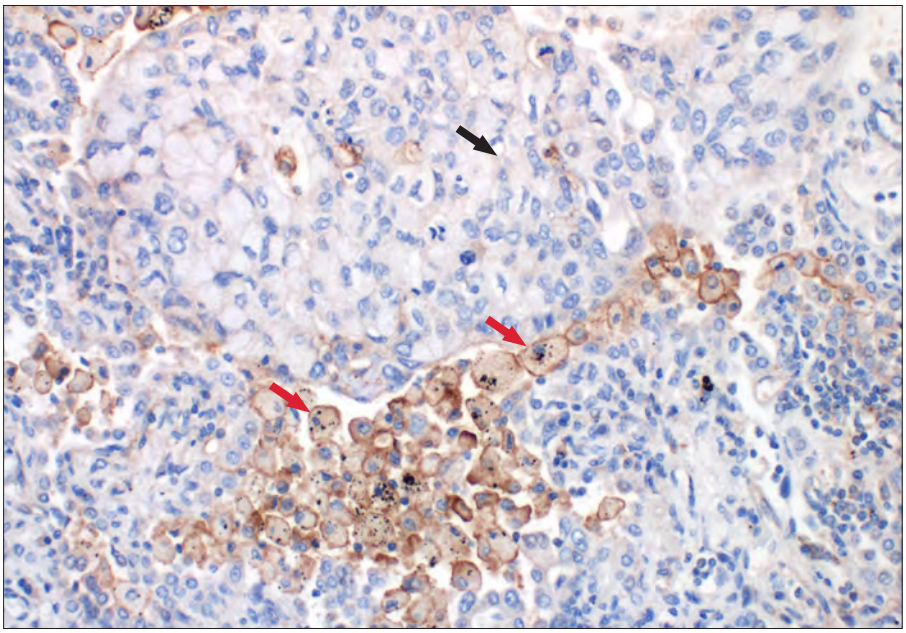


Figure 11. 20x magnification.

Distinguishing Tumor Cells from Immune Cells

NSCLC 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 % tumor cell expression.

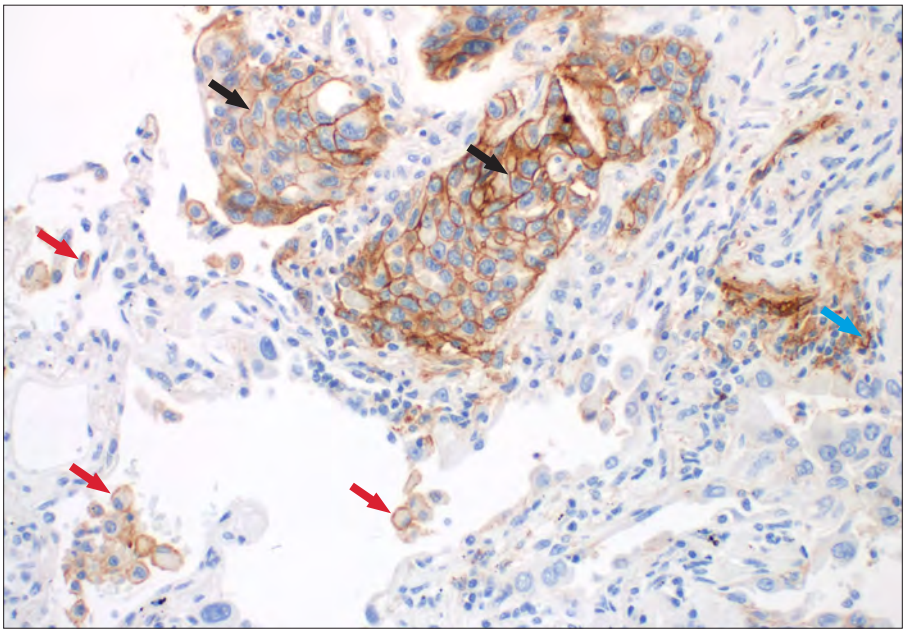


Figure 12. 20x magnification.

Cytoplasmic Staining

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

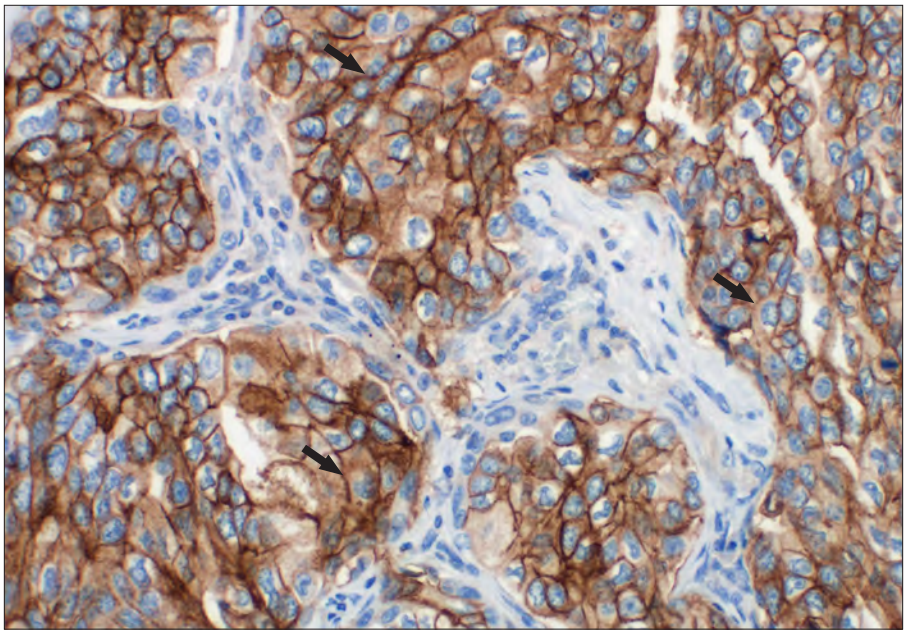


Figure 13. 20x magnification.

Granular Staining

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

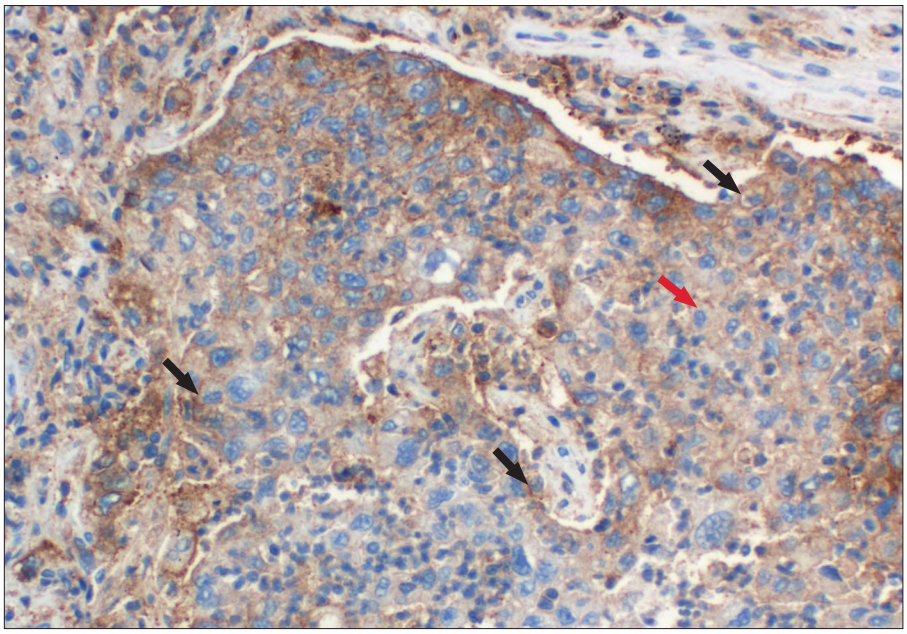


Figure 14. 20x magnification.

PD-L1 IHC 28-8 pharmDx NSCLC 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.

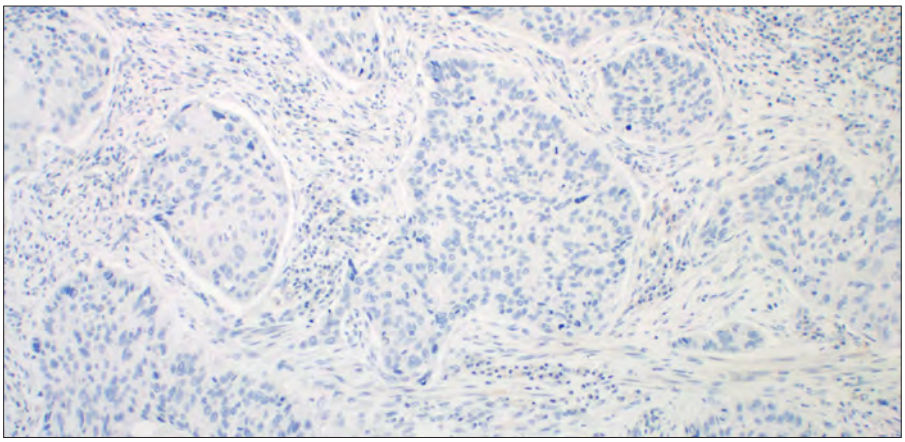


Figure 15b. 20x magnification.

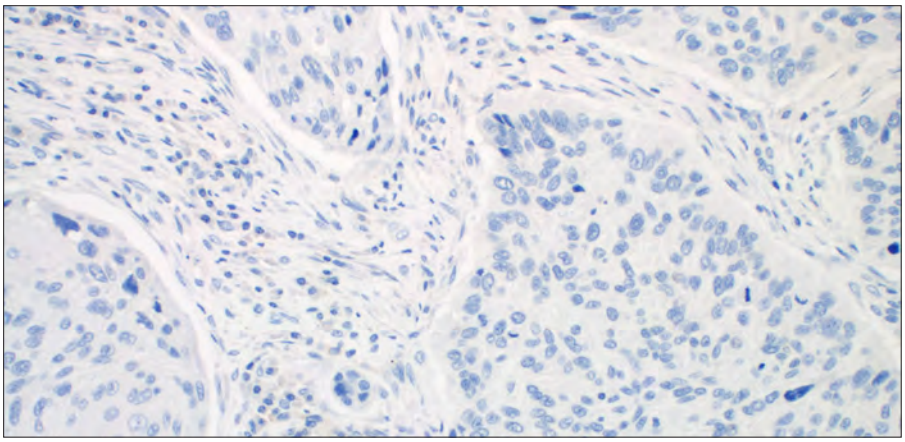
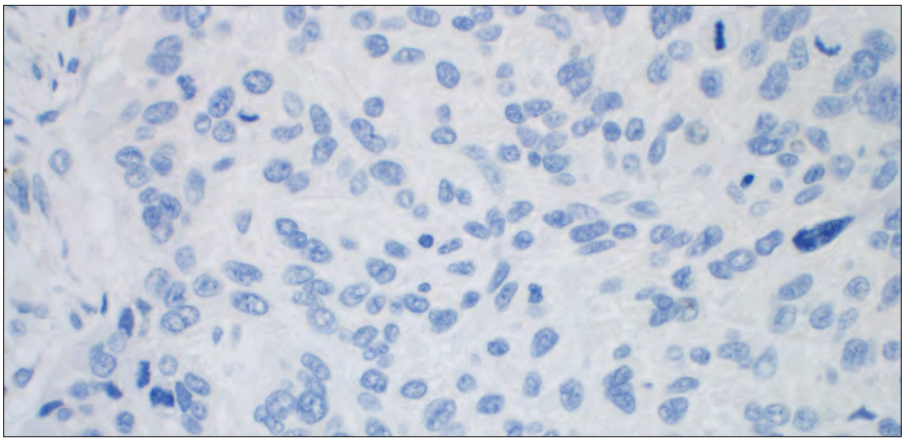


Figure 15c. 40x magnification.



Case 2: PD-L1 expression < 1%

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

Figure 16a. 10x magnification.

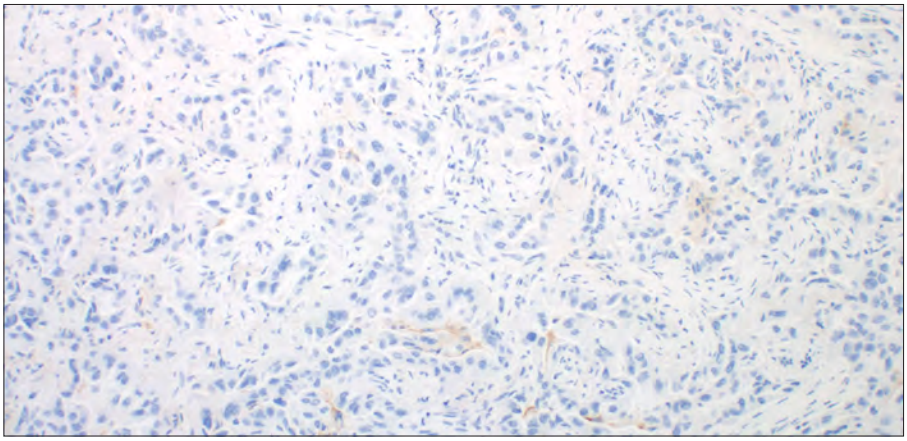


Figure 16b. 20x magnification.

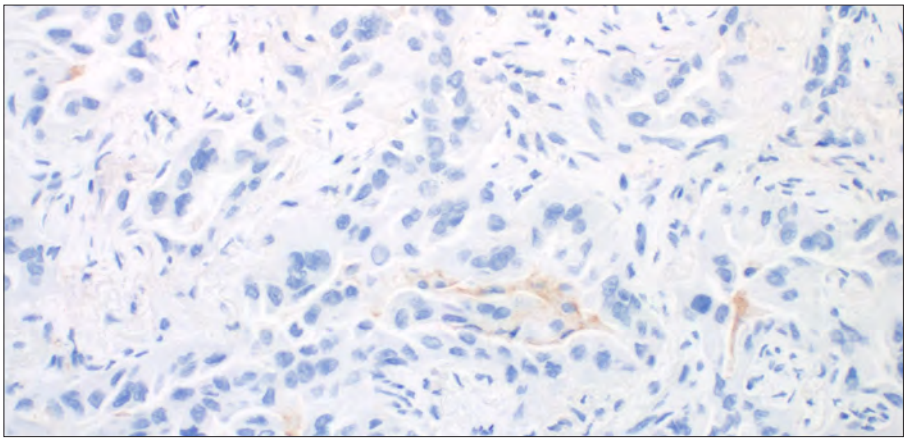
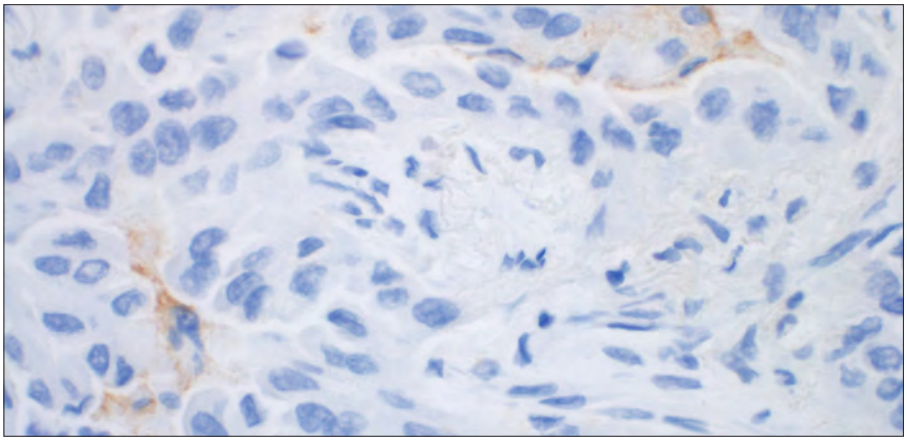


Figure 16c. 40x magnification.



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.

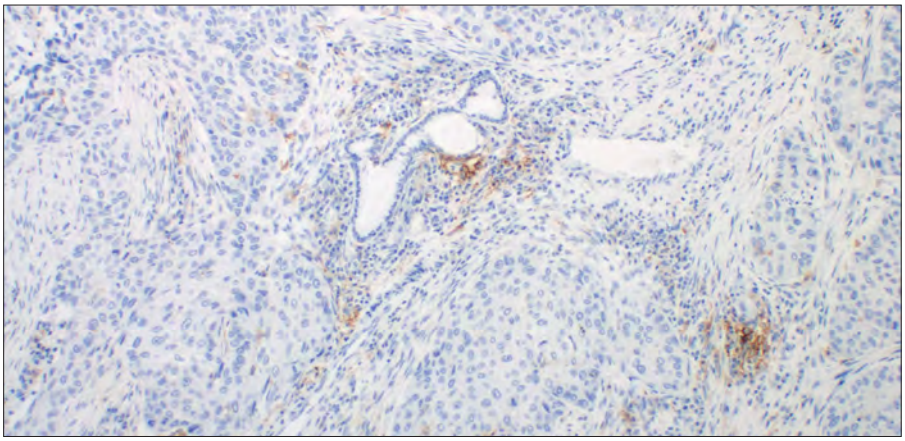


Figure 17b. 20x magnification.

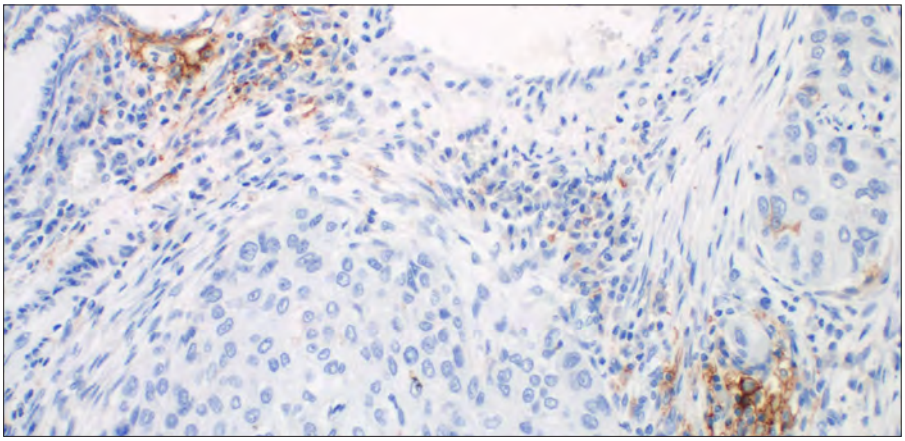
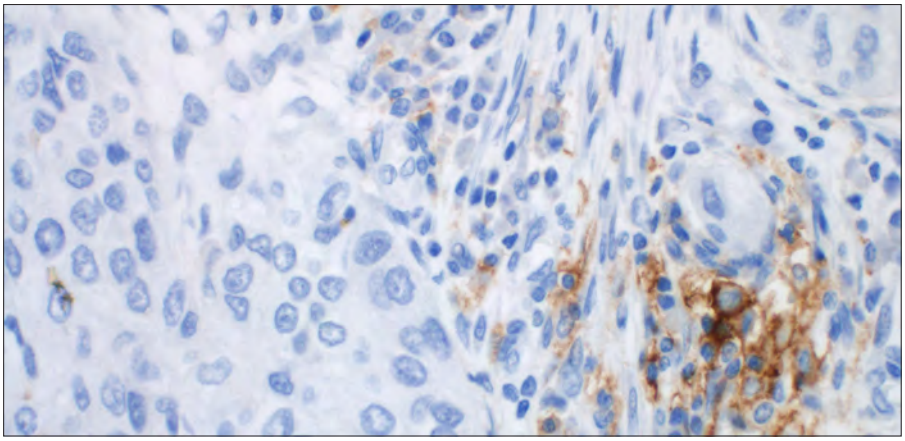


Figure 17c. 40x magnification.



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.

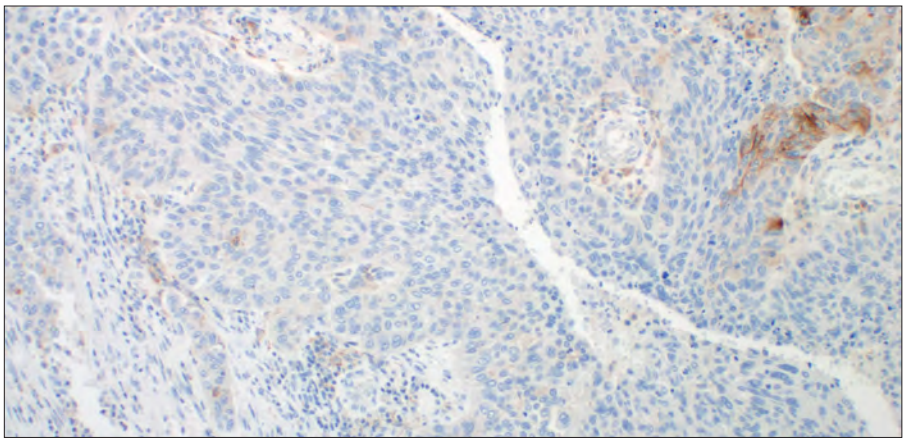


Figure 18b. 20x magnification.

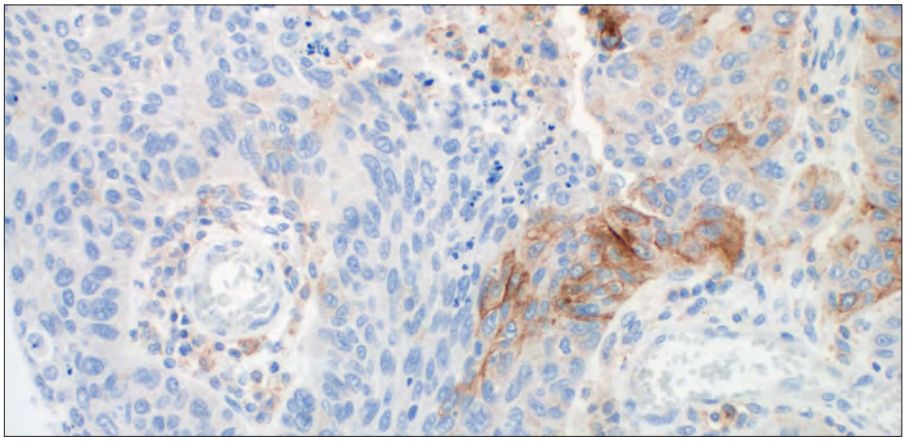
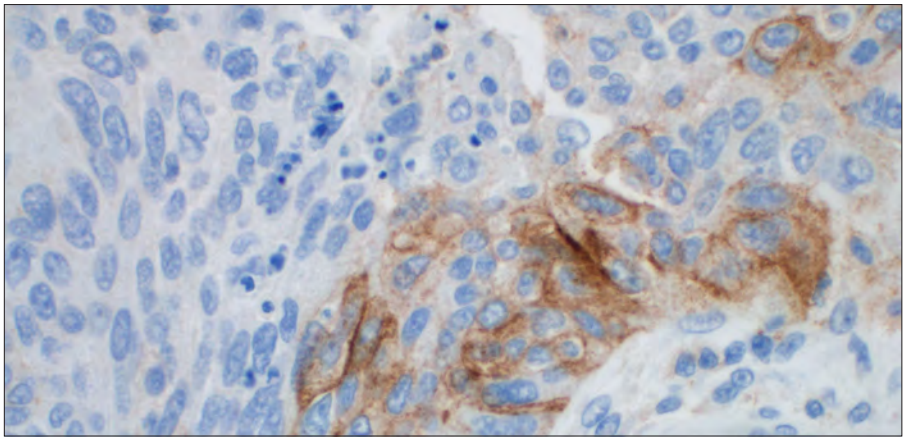


Figure 18c. 40x magnification.



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.

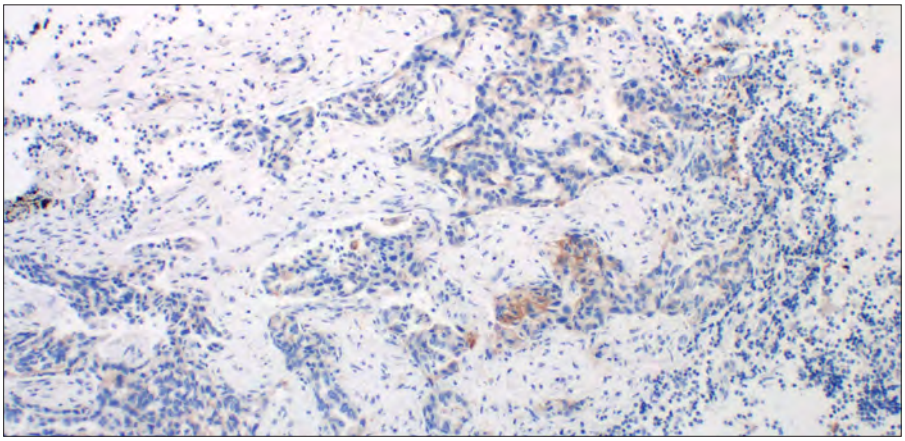


Figure 19b. 20x magnification.

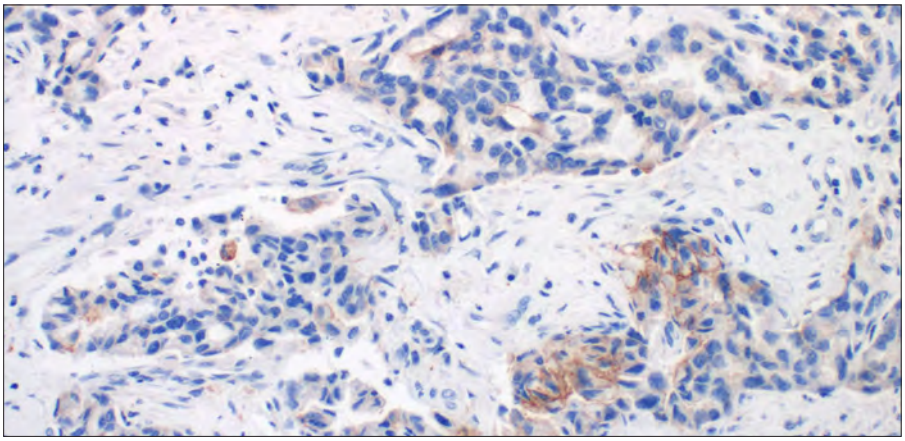
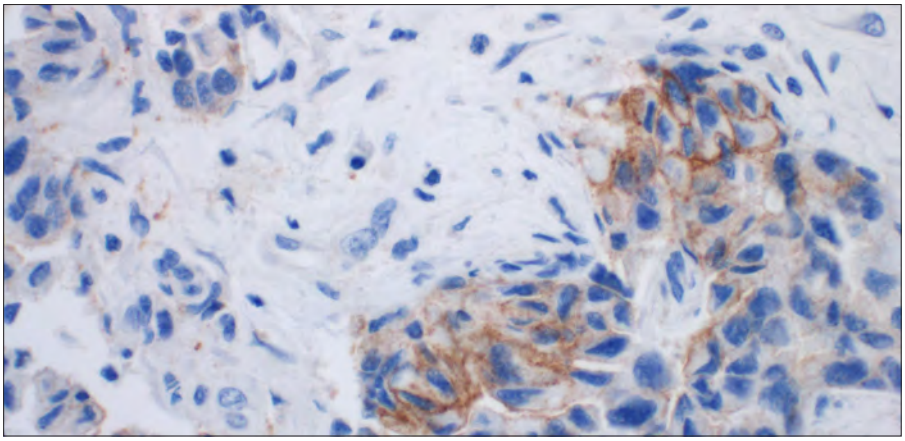


Figure 19c. 40x magnification.



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.

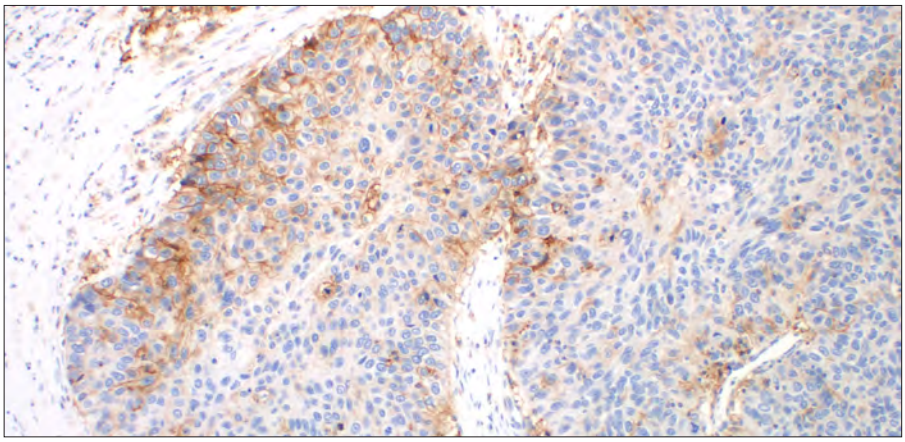


Figure 20b. 20x magnification.

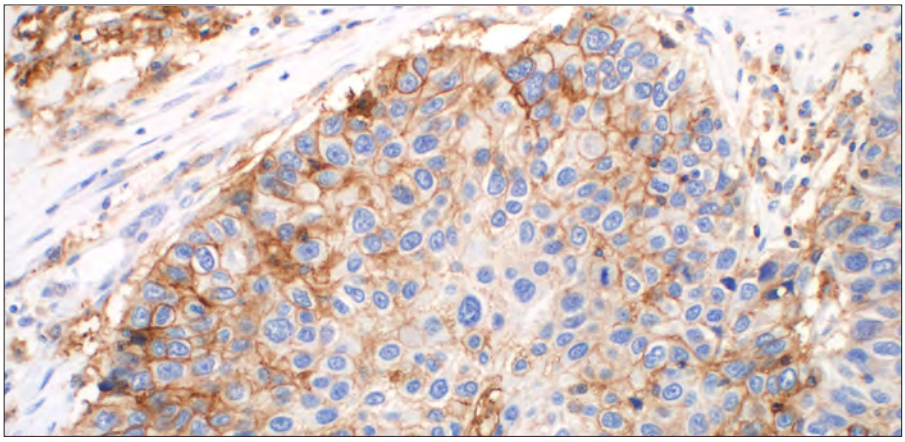
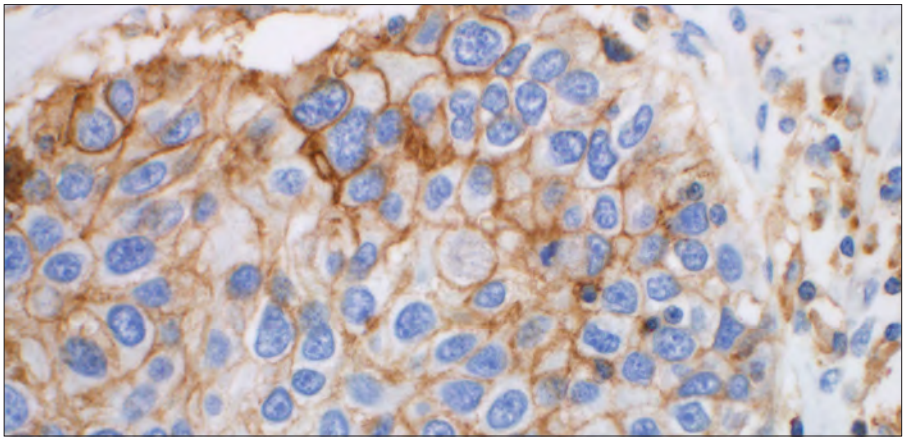


Figure 20c. 40x magnification.



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.

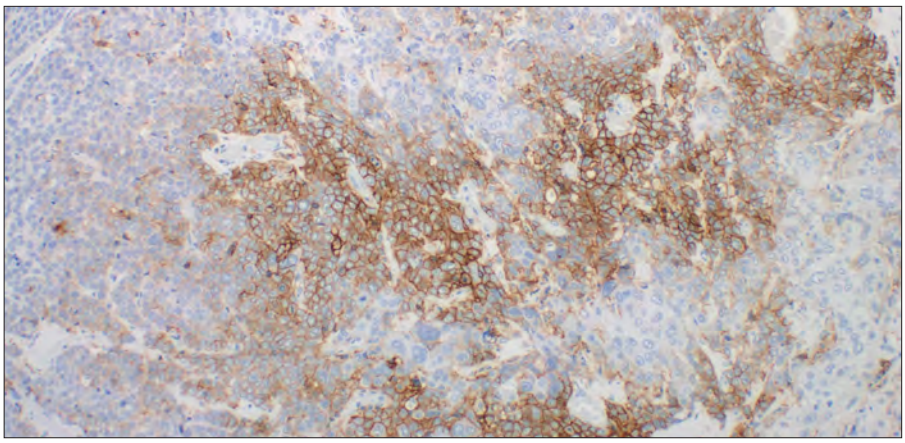


Figure 21b. 20x magnification.

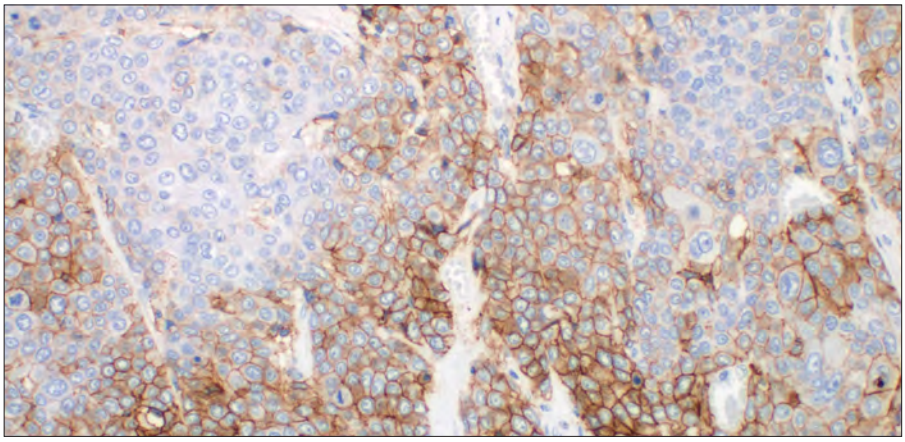
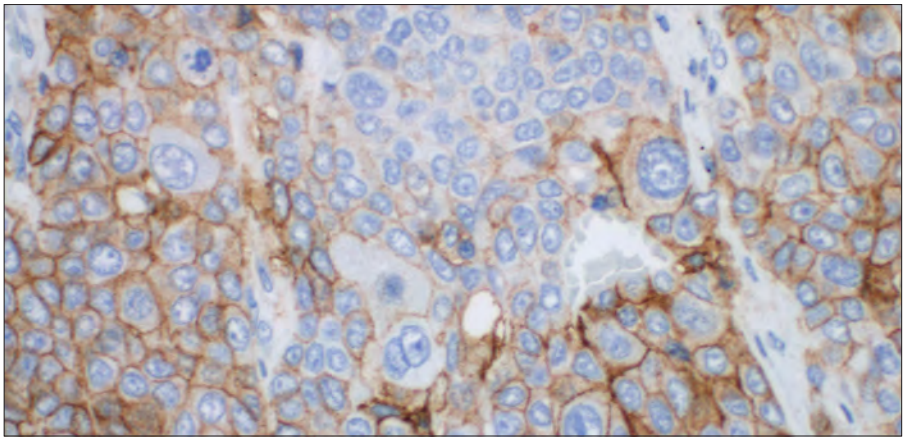


Figure 21c. 40x magnification.



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.

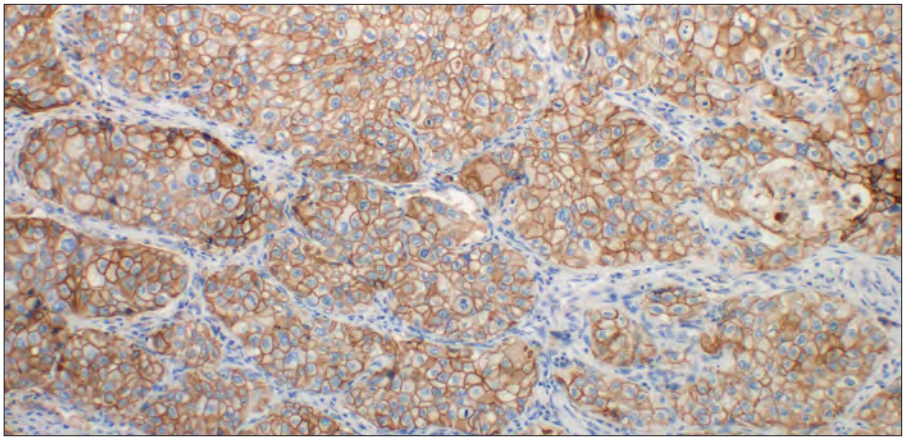


Figure 22b. 20x magnification.

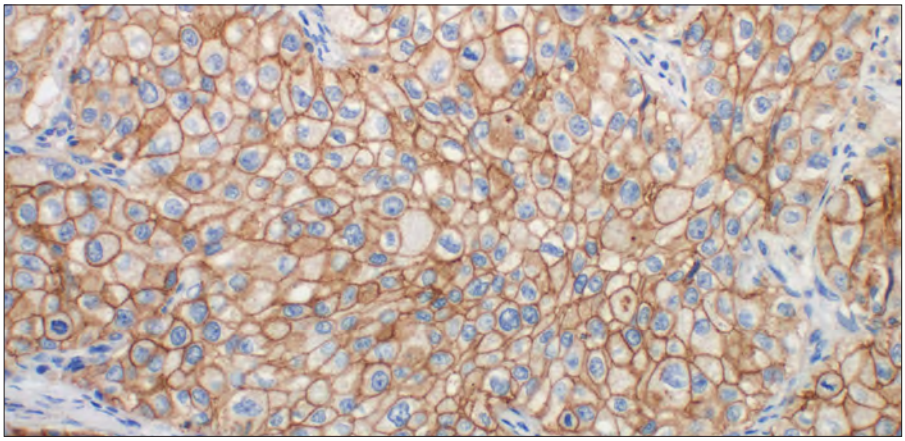
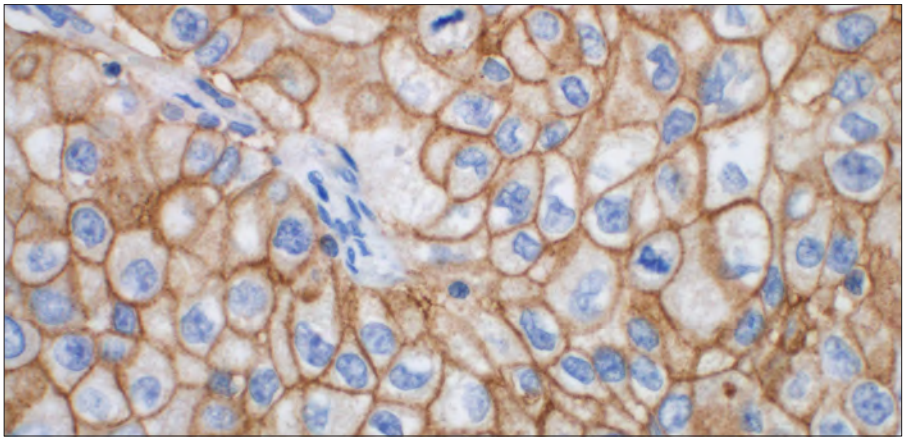


Figure 22c. 40x magnification.



Challenging Cases for NSCLC 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 % tumor cell expression.

Figure 23a. 10x magnification.

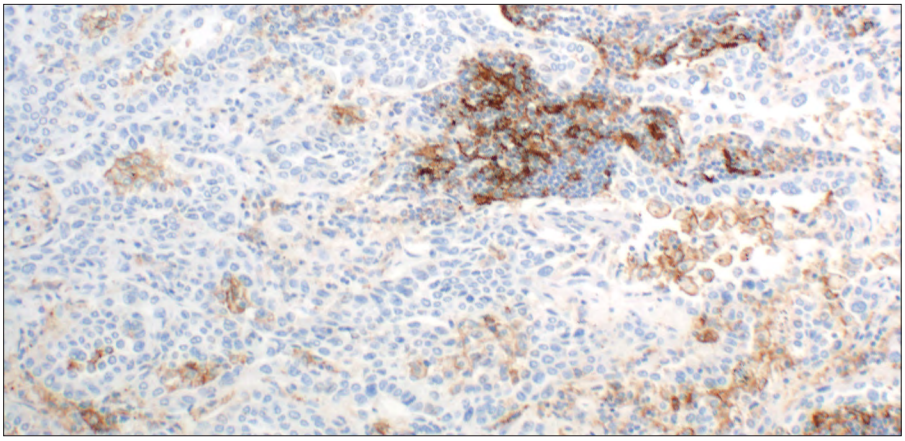


Figure 23b. 20x magnification.

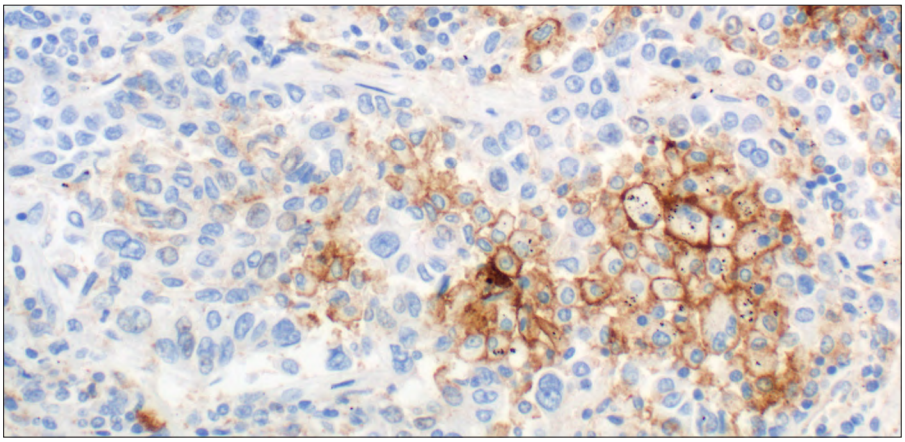
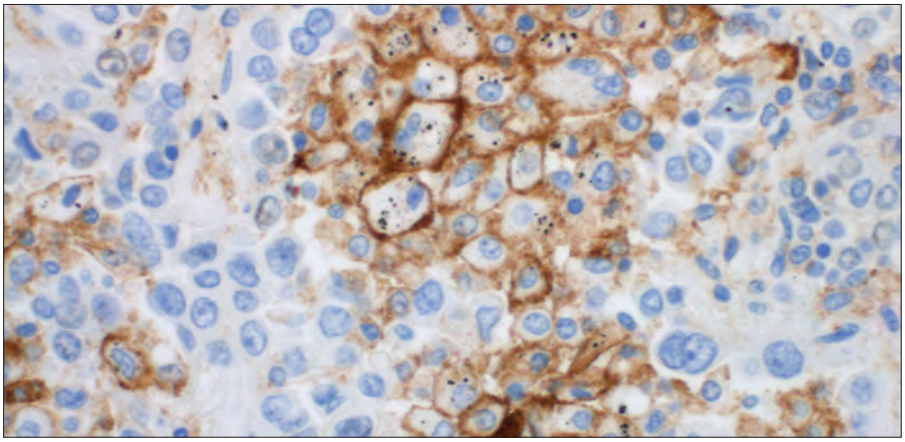


Figure 23c. 40x magnification.



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.

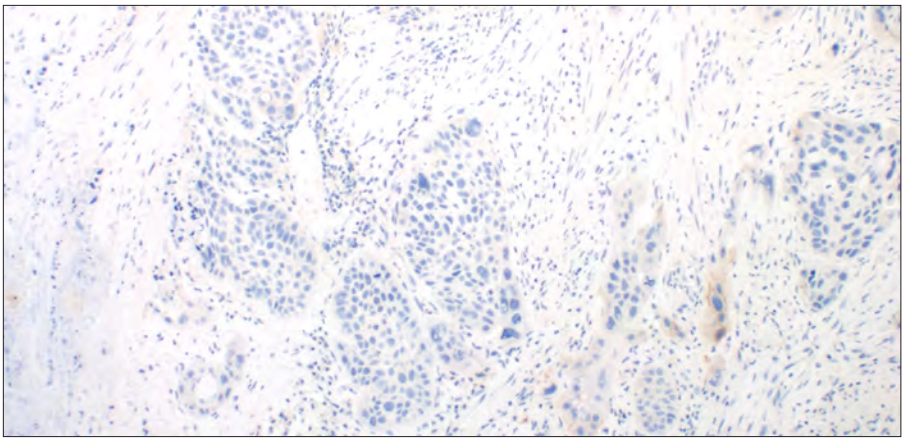


Figure 24b. 20x magnification.

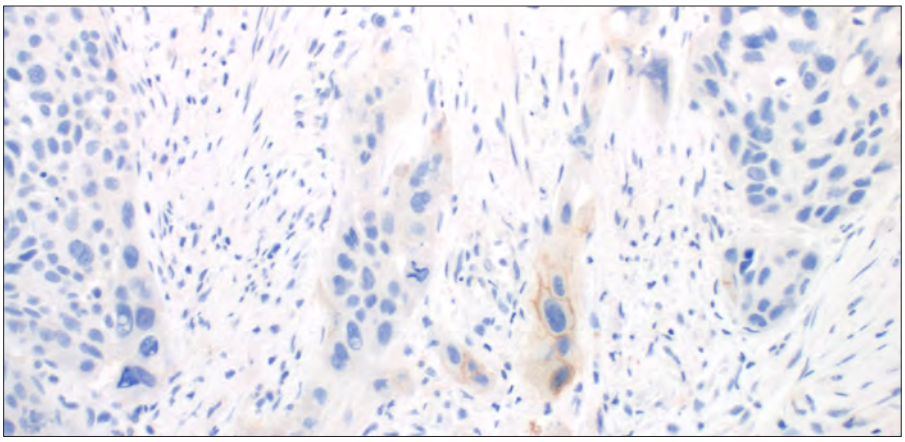
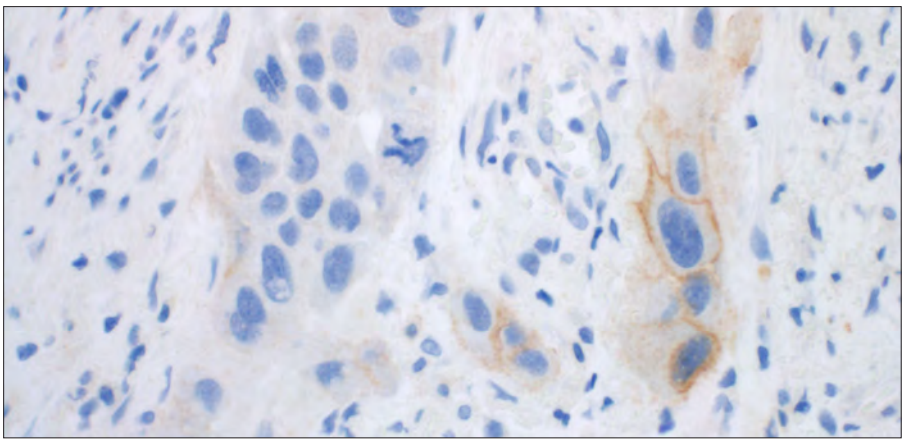


Figure 24c. 40x magnification.



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.

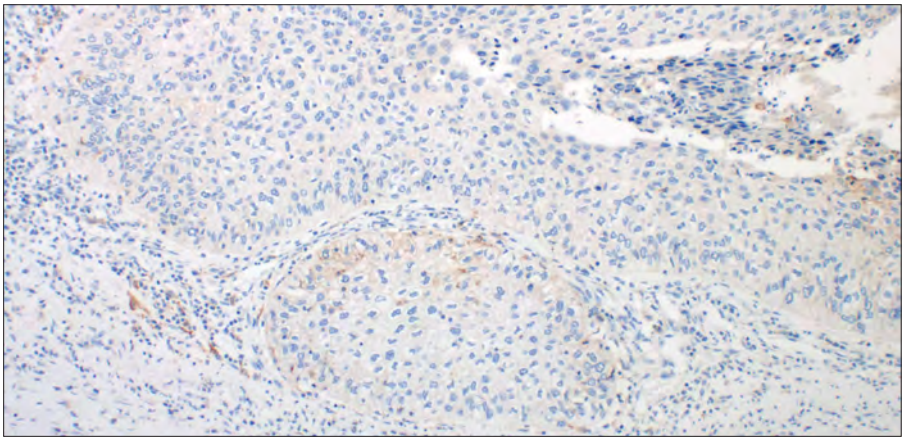


Figure 25b. 20x magnification.

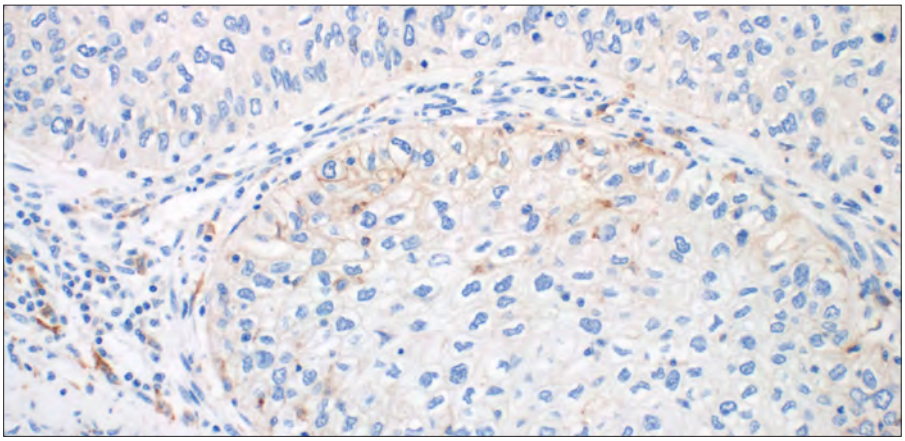
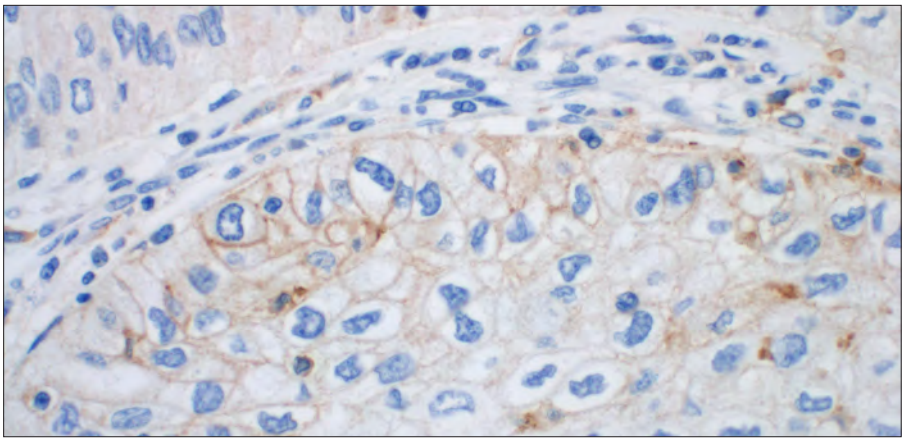


Figure 25c. 40x magnification.



Case 4: PD-L1 expression ≥ 1%

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

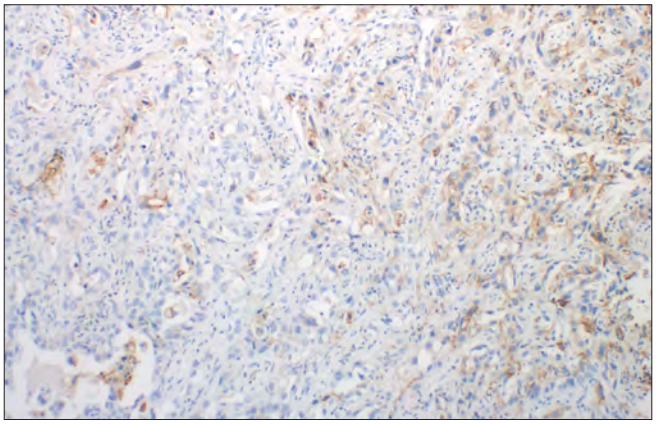


Figure 26a. 10x magnification.

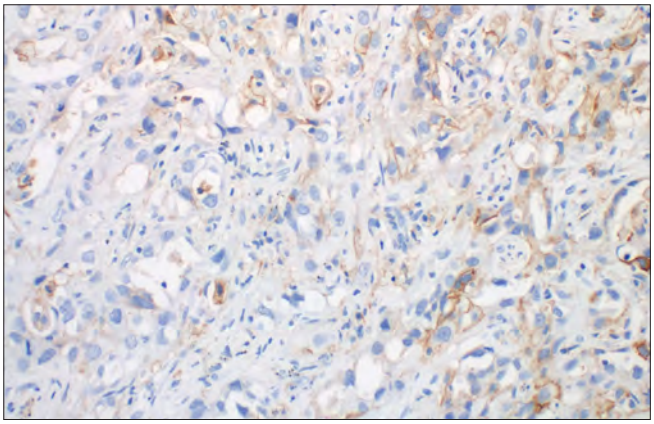


Figure 26b. 20x magnification.

Reactive fibroblasts (**red arrow**) may be mistaken as tumor cells (**black arrow**). Confirmation of cells types must be confirmed in the context of the H&E slide as shown. Reactive fibroblasts can express nonspecific staining but must be excluded from scoring. PD-L1 positive and negative staining tumor cells are observed and considered when determining the percent of PD-L1 expression in the tumor cells.

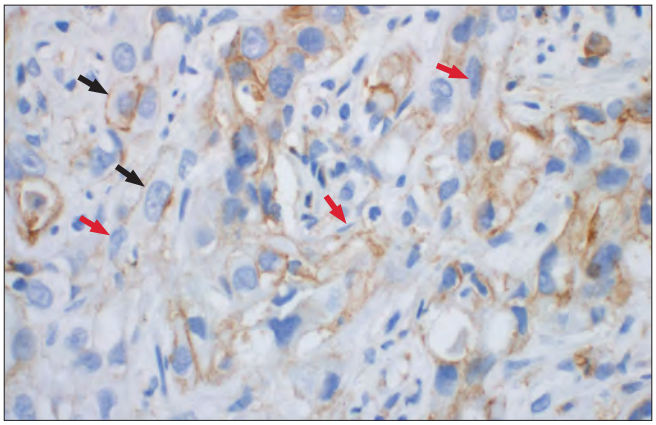


Figure 26c. 40x magnification.

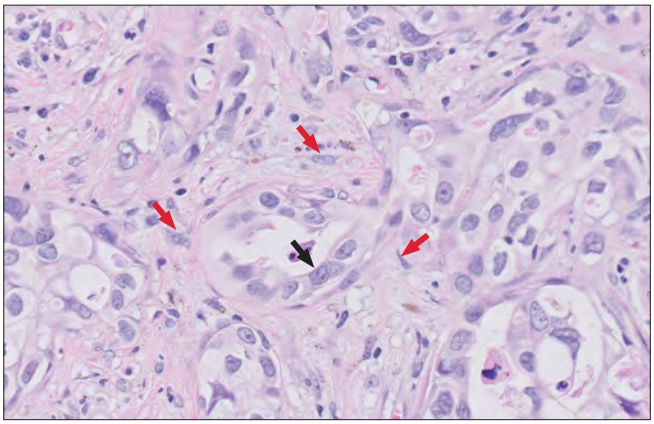


Figure 26d. 40x magnification.

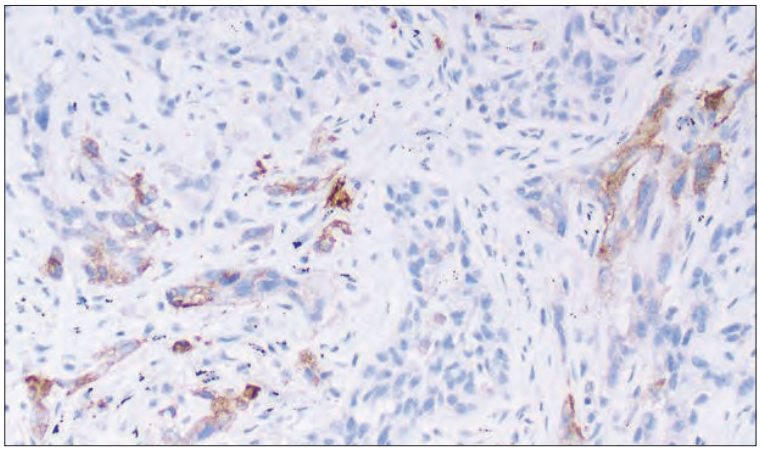
Additional Images for Assessing Non-Companion Cutoffs

nsNSCLC may be evaluated at the ≥ 5% and ≥ 10% cutoffs. Immunostaining examples of nsNSCLC cases are provided below showing PD-L1 % tumor cell expression around the ≥ 5% and ≥ 10% cutoffs.

PD-L1 expression ≥ 5%

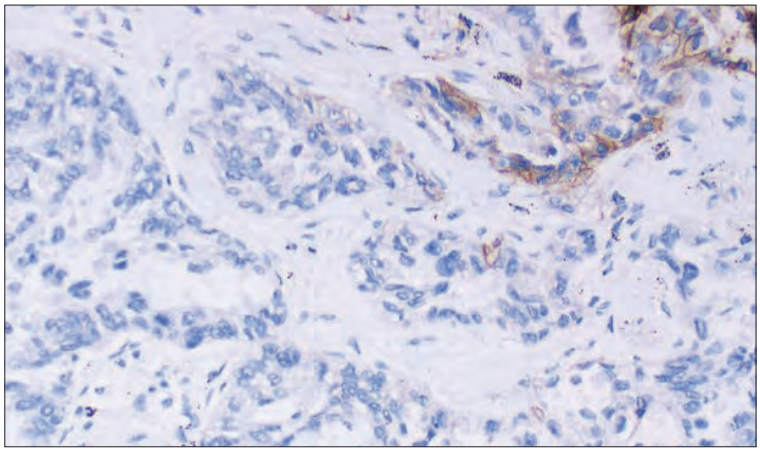
Case 1

Figure 27. 20x magnification.



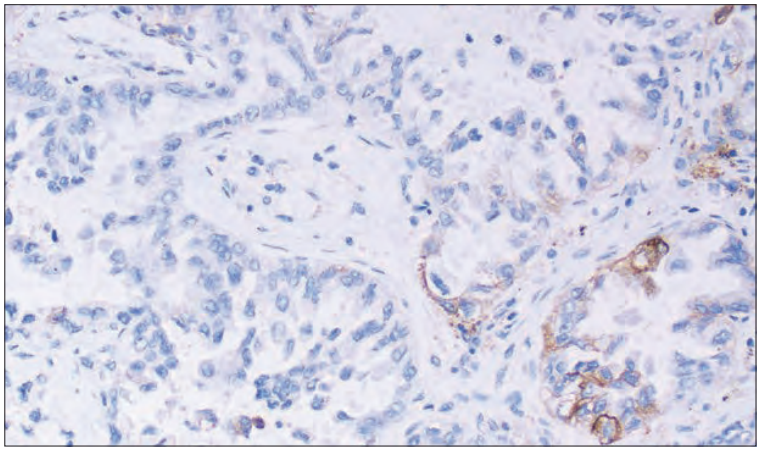
Case 2

Figure 28. 20x magnification.



Case 3

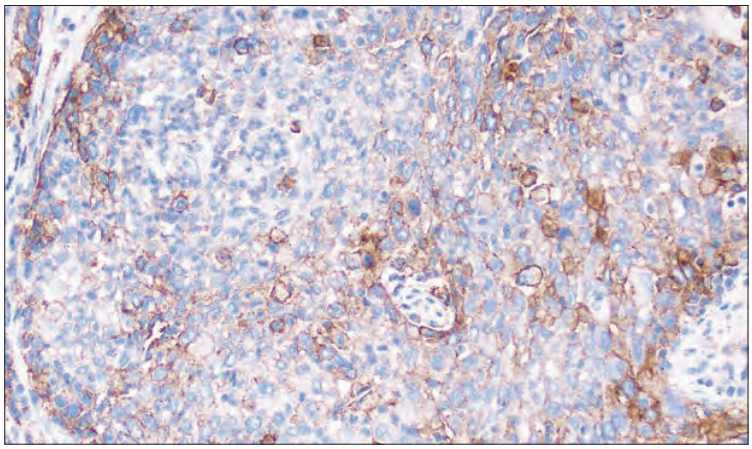
Figure 29. 20x magnification.



PD-L1 expression ≥ 10%

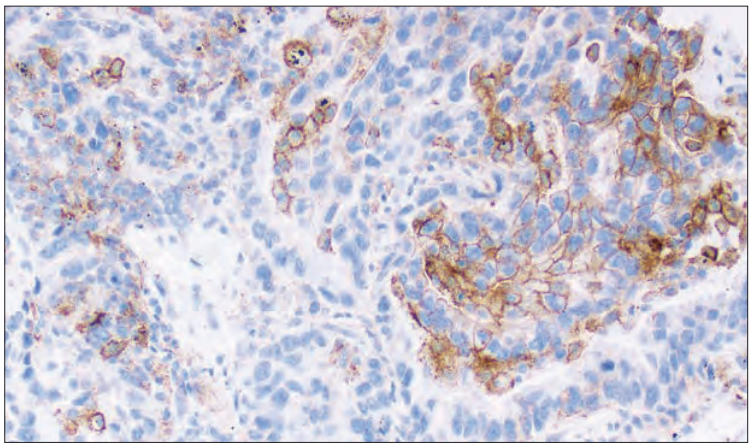
Case 1

Figure 30. 20x magnification.



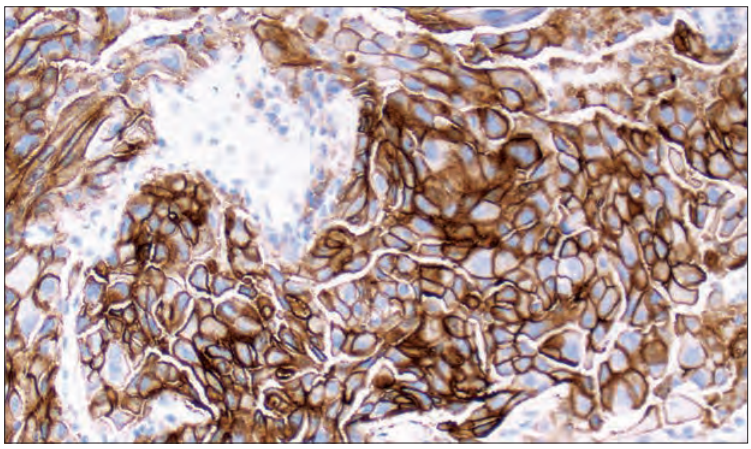
Case 2

Figure 31. 20x magnification.



Case 3

Figure 32. 20x magnification.



Artifacts

Nonspecific Staining

Nonspecific staining is defined as staining that is not related to primary antibody-antigen interaction and is influenced by factors such as reagent trapping, cartilage staining, DAB deposits, tissue folds, and edge drying. Other notable 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 nonspecific staining. If nonspecific staining interferes with interpretation of specific staining, the slide may be considered indeterminant.

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

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

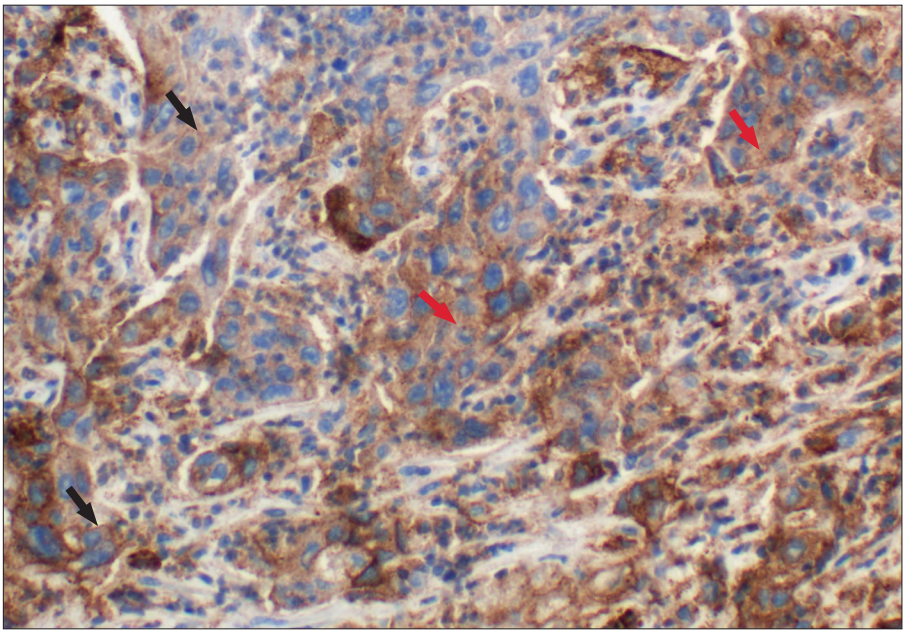


Figure 33. 20x magnification.

Necrosis

Necrotic tissue may show nonspecific 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 nonspecific staining and should not be included in scoring percent positivity of the tumor. Care should be taken to the only include viable tumor cells for scoring and not necrotic regions.

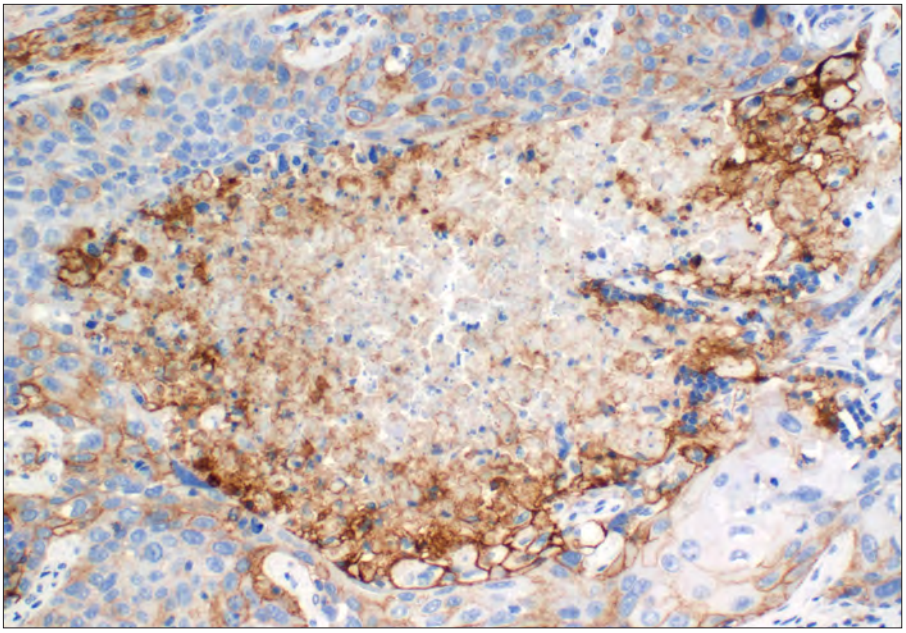


Figure 34. 20x magnification.

Troubleshooting Guide for PD-L1 IHC 28-8 pharmDx

Problem	Probable Cause	Suggested Action
1. No staining of control or specimen slides	1a. Programming error 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 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 pretreatment procedure was correctly performed
	3b. Inappropriate wash buffer used	3b. Use only EnVision FLEX Wash Buffer, Code K8007
4. Excessive nonspecific staining of slides	4a. Paraffin incompletely removed	4a. Verify that the 3-in-1 pretreatment 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. Nonspecific 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 recalibration, retest 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 de-ionized 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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