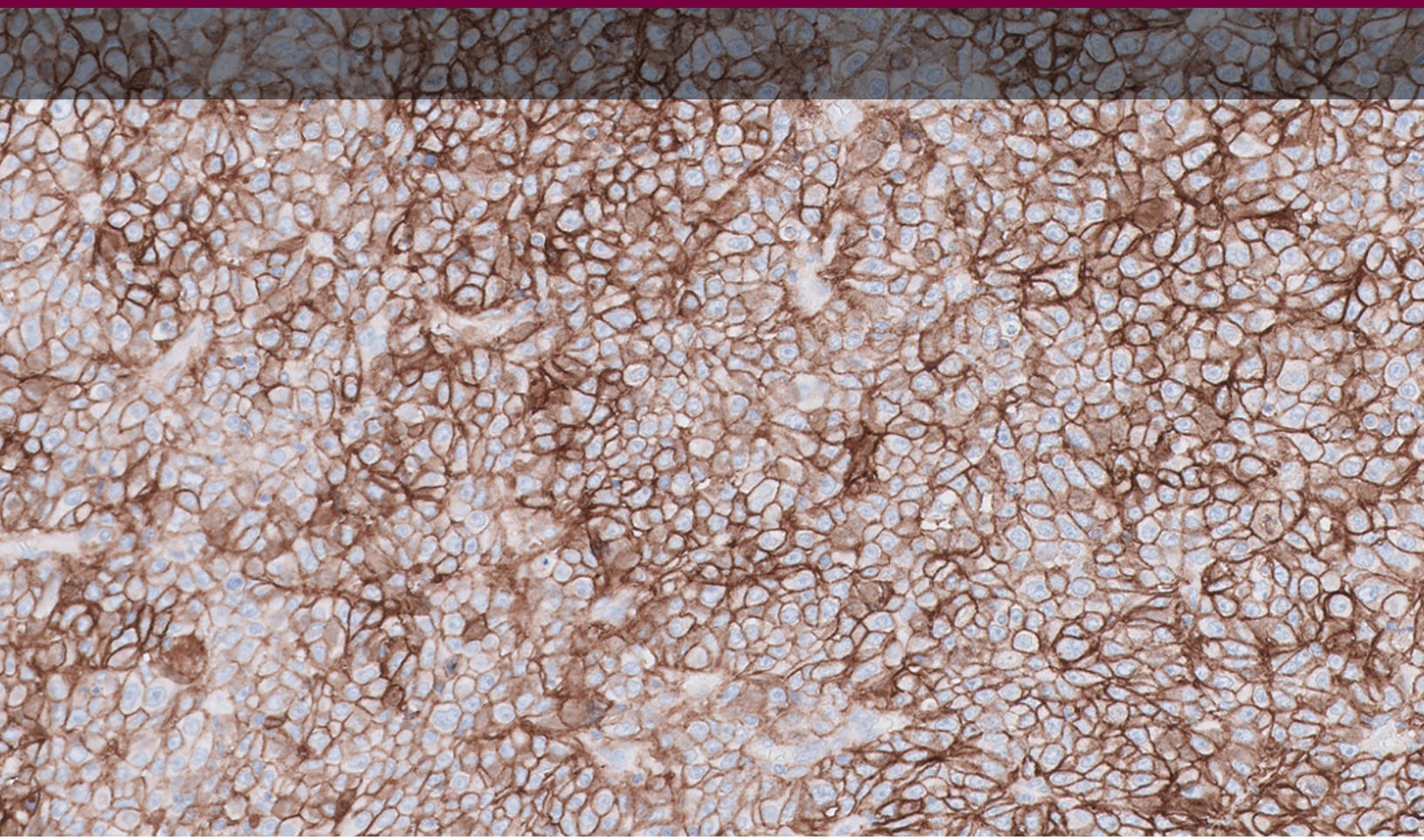
Agilent Dako

PD-L1 IHC 22C3 pharmDx Interpretation Manual – Triple-Negative Breast Cancer (TNBC)

FDA-approved for in vitro diagnostic use

Rx only



Agilent

Trusted Answers

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

Table of Contents

Intended Use

For in vitro diagnostic use.

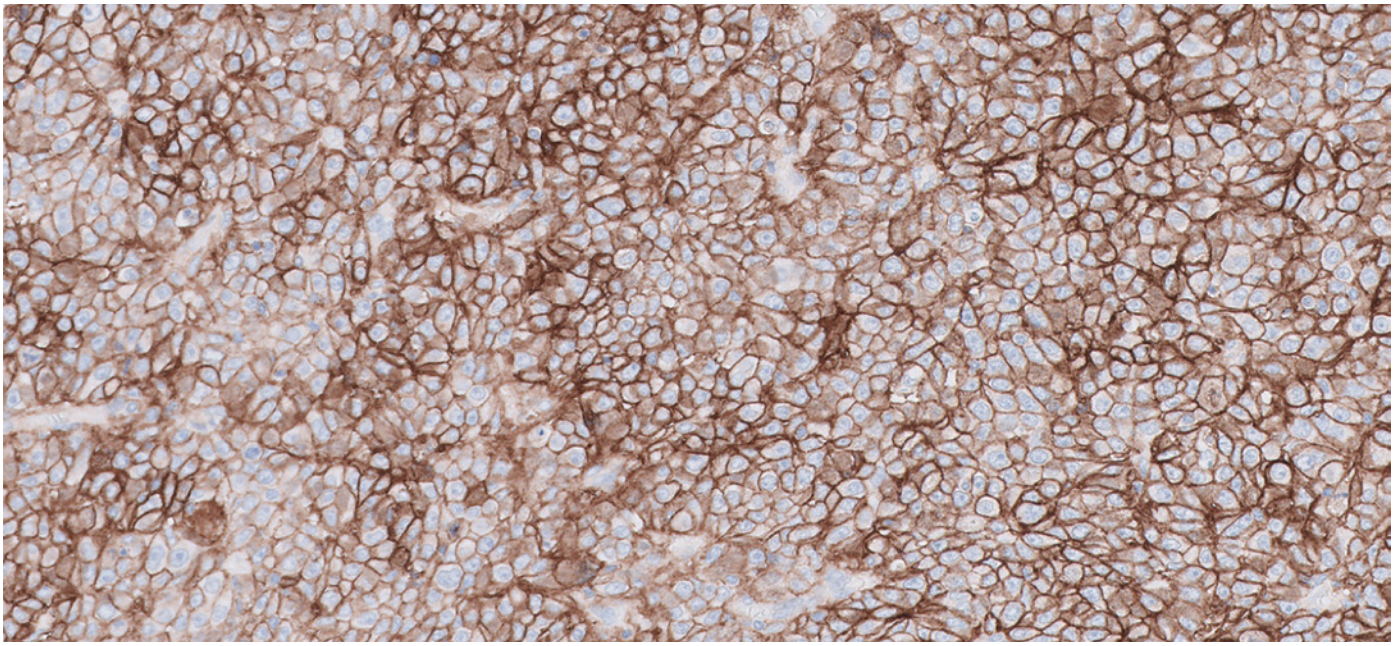
PD-L1 IHC 22C3 pharmDx is a qualitative immunohistochemical assay using monoclonal mouse anti-PD-L1, Clone 22C3 intended for use in the detection of PD-L1 protein in formalin-fixed, paraffin-embedded (FFPE) triple-negative breast cancer (TNBC) tissue using EnVision FLEX visualization system on Autostainer Link 48.

Triple-Negative Breast Cancer (TNBC)

PD-L1 protein expression in TNBC is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. The specimen should be considered to have PD-L1 expression if CPS ≥ 10.

PD-L1 IHC 22C3 pharmDx is indicated as an aid in identifying TNBC patients for treatment with KEYTRUDA® (pembrolizumab).

For descriptions of the intended use in other indications, please refer to the current version of the Instructions for Use (IFU) for PD-L1 IHC 22C3 pharmDx, Code SK006.



Introduction

PD-L1 IHC 22C3 pharmDx is the only companion diagnostic FDA-approved as an aid in identifying patients with triple-negative breast cancer (TNBC) for treatment with KEYTRUDA® (pembrolizumab). This Interpretation Manual is provided as a tool to help guide pathologists and laboratory personnel in achieving correct and reproducible results in assessing PD-L1 expression in formalin-fixed, paraffin-embedded (FFPE) TNBC specimens. PD-L1 expression evaluation may be used to identify patients for treatment with KEYTRUDA.

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

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

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

- CPS < 10
- CPS ≥ 10

PD-L1 expression levels are used to inform patient eligibility for treatment with KEYTRUDA. For more details on staining and interpretation, please refer to the current version of the IFU for PD-L1 IHC 22C3 pharmDx, Code SK006.

Assay Interpretation

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

Reporting Results

To help understand what information should be reported to the treating physician, please refer to the Reporting Results section of this manual on page 32.

Photomicrographs

The included photomicrographs are of TNBC unless otherwise noted.

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

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

Source of materials is Conversant Biologics.

Tissue samples were supplied by BioIVT Asterand®

The data and biospecimens used in this project were provided by Tumorgenetika, Budapest, Hungary, Hospice Civils de Lyon, Lyon, France, and by Centre Antoine Lacassagne, Nice, France with appropriate ethics approval and through Trans-Hit Biomarkers Inc.

Tissue samples supplied by BioIVT (Hicksville, NY, USA).

Data and biospecimens used in this project were provided by US Biolab (Gaithersburg, MD, USA) and IOM Ricera (Viagrande, Italy) with appropriate ethics approval and through Azenta Life Sciences.

PD-L1 Overview

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

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

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

Many tumor cells are able to upregulate the expression of PD-L1 as a mechanism to evade the body's natural immune response. Activated T-cells recognize the PD-L1 marker on the tumor cell, similar to that of a normal cell, and PD-L1 signaling renders the T-cell inactive (Figure 2). The tumor cell escapes the immune cycle, continues to avoid detection for elimination, and is able to proliferate.

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

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

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

PD-L1 upregulation in TNBC is a biomarker for response to anti-PD-1 therapy. PD-L1 IHC 22C3 pharmDx was the only PD-L1 assay used in the KEYTRUDA® (pembrolizumab) clinical trial (KEYNOTE-355) to evaluate the relationship between PD-L1 expression and clinical efficacy. KEYTRUDA is a humanized monoclonal PD-1-blocking antibody.

The PD-1/PD-L1 Pathway

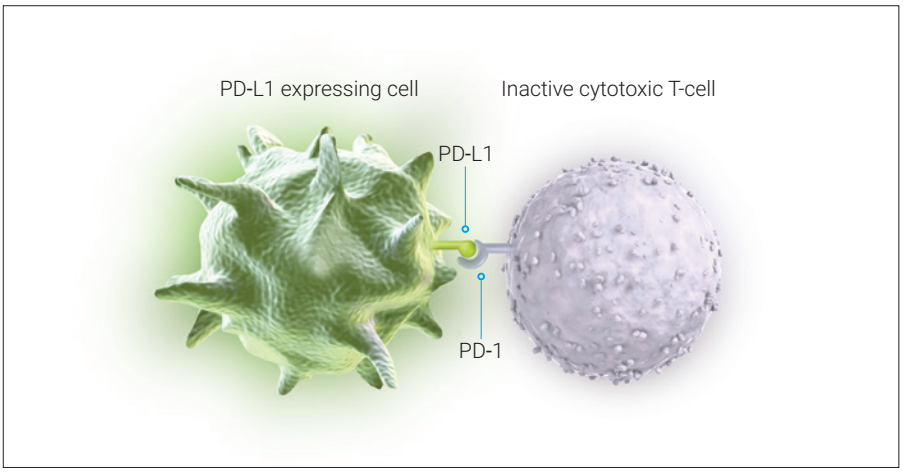


Figure 1: Inactivation of T-cells limits damage to normal tissue.

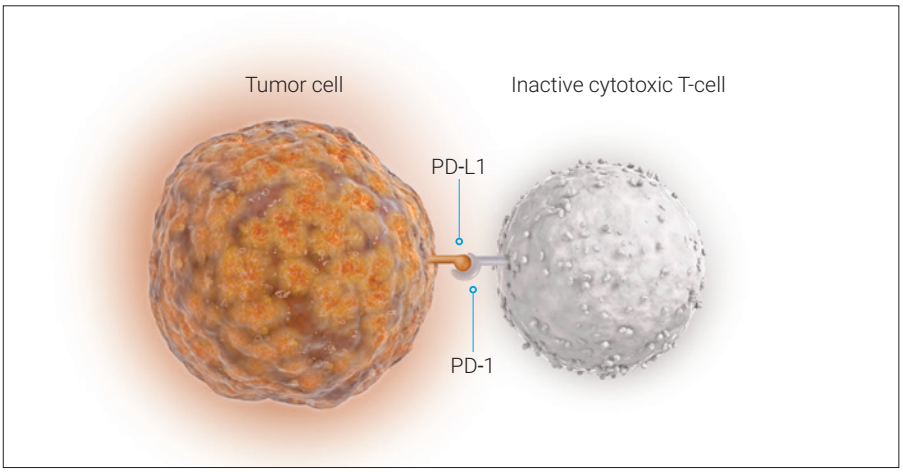


Figure 2: Inactivation of T-cells reduces tumor cell death and elimination.

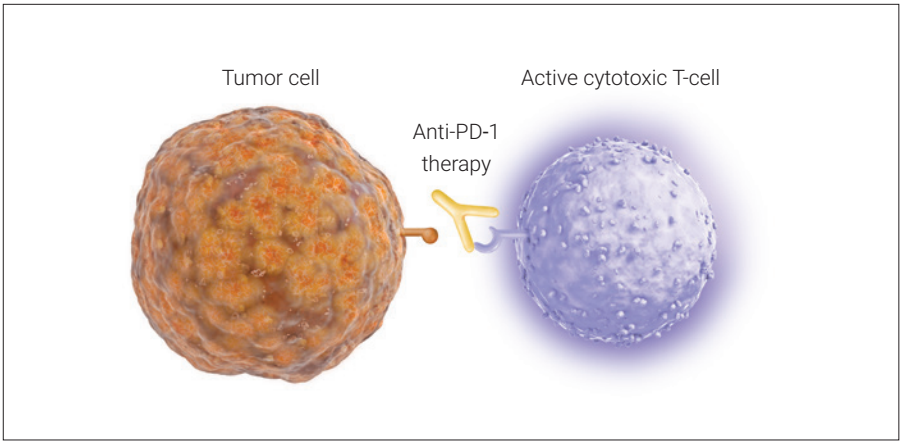


Figure 3: Blocking the PD-1/PD-L1 interaction helps to enable active T-cells and tumor cell death and elimination.

PD-L1 IHC 22C3 pharmDx Overview

What is PD-L1 IHC 22C3 pharmDx?

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

Components of PD-L1 IHC 22C3 pharmDx

PD-L1 IHC 22C3 pharmDx contains optimized reagents to perform an IHC staining procedure using a linker and a chromogen enhancement reagent (Figure 4). Deparaffinization, rehydration, and target retrieval is performed using a 3-in-1 procedure on PT Link. Following peroxidase block, specimens are incubated with the monoclonal mouse primary antibody to PD-L1 or the Negative Control Reagent. Specimens are then incubated with a Mouse LINKER, followed by incubation with a ready-to-use Visualization Reagent consisting of secondary antibody molecules and horseradish peroxidase molecules coupled to a dextran polymer backbone.

The enzymatic conversion of the subsequently added chromogen results in precipitation of a visible reaction product at the site of the antigen. The color of the chromogenic reaction is modified by a chromogen enhancement reagent. The specimen may then be counterstained and coverslipped. Results are interpreted using a light microscope.

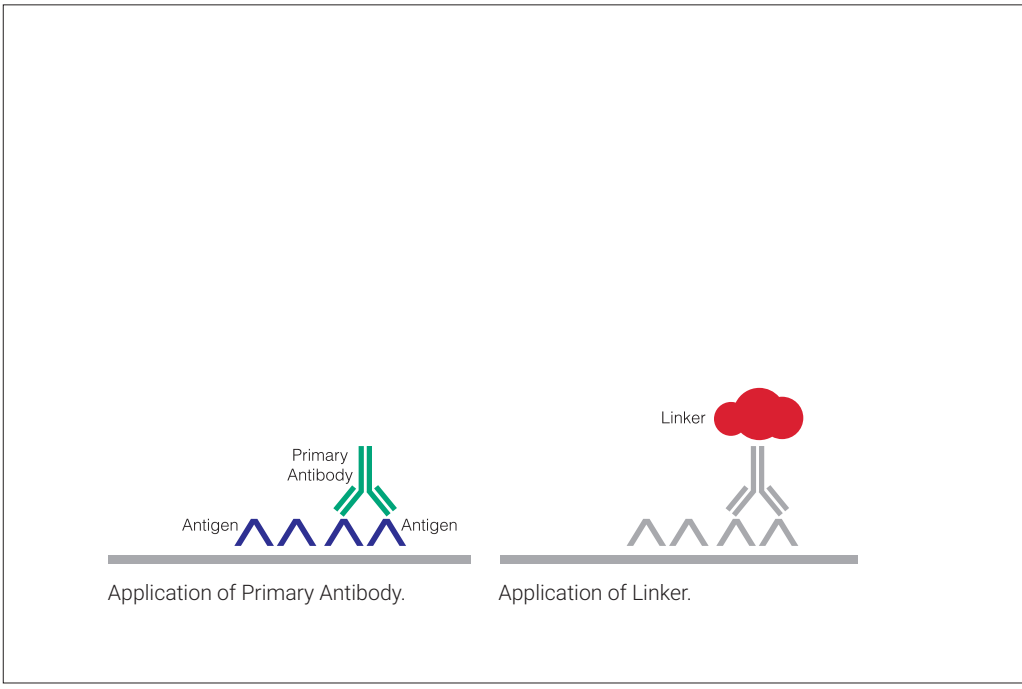


Figure 4: PD-L1 IHC 22C3 pharmDx staining procedure.

Kit Configuration (SK006)

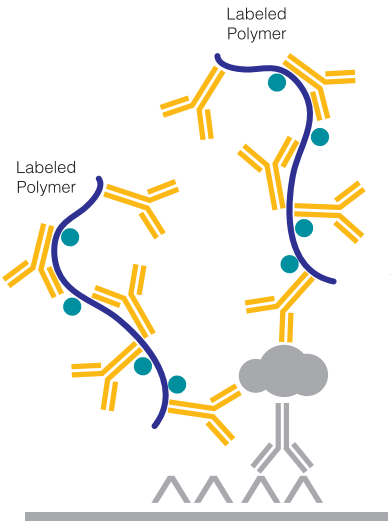
Figure 5: PD-L1 IHC 22C3 pharmDx components.

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

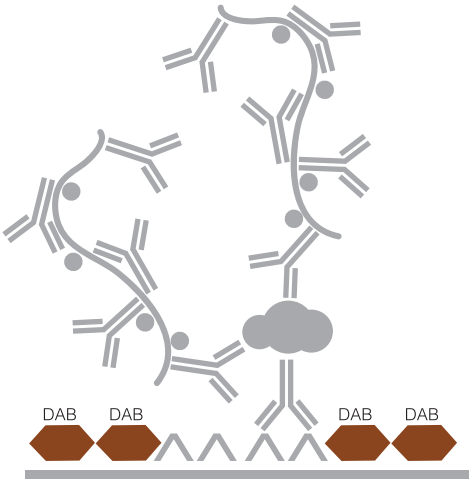
1. EnVision FLEX Target Retrieval Solution, Low pH, (50×)
2. Peroxidase-blocking Reagent
3. Primary Antibody: Monoclonal Mouse Anti-PD-L1, Clone 22C3
4. Negative Control Reagent
5. Mouse LINKER
6. Visualization Reagent-HRP
7. DAB+ Substrate Buffer
8. DAB+ Chromogen
9. DAB Enhancer
10. PD-L1 IHC 22C3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

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

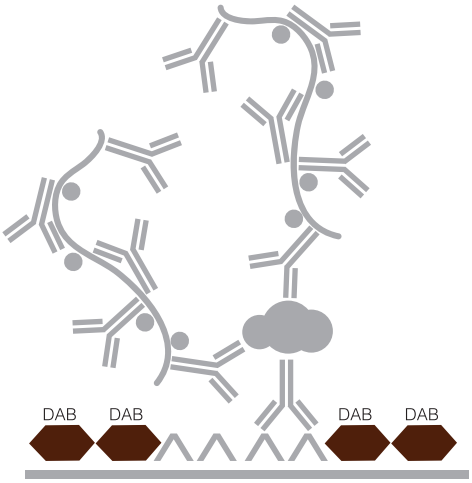
** Dr. AF Gazdar and Dr. JD Minna at NIH are acknowledged for their contribution in developing NCI-H226 (ATCC Number: CRL-5826™)*



Application of Visualization Reagent.



Application of DAB+ Substrate Chromogen Solution.



Application of DAB Enhancer.

Technical Considerations

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

Specimen Preparation

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

Lab-Supplied Control Tissue

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

Refer to the 'Recommended Order of Slide Evaluation' table in the PD-L1 IHC 22C3 pharmDx IFU for additional information on appropriate control tissue.

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

Optional Additional Lab-Supplied Control: Tonsil Tissue

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

Tissue Processing

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

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

PD-L1 IHC 22C3 pharmDx Staining Procedure

The PD-L1 IHC 22C3 pharmDx reagents and instructions have been designed for optimal performance. Further dilution of the reagents, alteration of incubation times, temperatures, or materials may give erroneous results. All of the required steps and incubation times for staining are pre-programmed in the DakoLink software.

Reagent Storage

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

Reagent Preparation

Equilibrate all components to room temperature (20–25 °C) prior to immunostaining. Do not use after the expiration date printed on the outside of the package.

EnVision FLEX Target Retrieval Solution, Low pH

Dilute EnVision FLEX Target Retrieval Solution, Low pH, (50×) 1:50 using distilled or deionized water (reagent-quality water). One 30 mL bottle of concentrate provides 1.5 L of working solution, which is sufficient to fill one PT Link tank. Discard 1× EnVision FLEX Target Retrieval Solution, Low pH after 3 uses or 5 days after dilution.

EnVision FLEX Wash Buffer

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

DAB+ Substrate-Chromogen Solution

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

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

Controls to Assess Staining Quality

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

- One PD-L1 IHC 22C3 pharmDx Control Cell Line Slide stained with the primary antibody
- Positive and negative lab-supplied control tissues stained with the primary antibody and Negative Control Reagent
- Serial section of each patient specimen stained with the Negative Control Reagent

Deparaffinization, Rehydration, and Target Retrieval

Use PT Link to perform a Deparaffinization, Rehydration, and Target Retrieval 3-in-1 procedure:

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

Staining and Counterstaining

- Place the Autostainer racks with slides one rack at a time on the Autostainer Link 48
- Prior to initiating the staining procedure on the Autostainer Link 48, 1x EnVision FLEX Wash Buffer should be manually applied to the slides using a wash bottle for prevention of tissue drying. 1x EnVision FLEX Wash Buffer should not be applied directly on the tissue section but applied sufficiently to the slide so that the tissue section is amply covered. Slides should remain wet prior to the initiation of the staining procedure. Do not allow tissue sections to dry after deparaffinization, rehydration and target retrieval (3-in-1) procedure (specimen pre-treatment) or at any time during the staining procedure. Dried tissue sections may display increased nonspecific staining (including nuclear staining).
- Ensure that Autostainer slide racks are level prior to initiation of the IHC staining procedure. Level Autostainer slide racks are required for staining. Unlevel and/or warped Autostainer slide racks can result in uneven reagent distribution and improper pooling on the glass away from the specimen areas, which increases the risk for tissue drying and may lead to the appearance of nonspecific staining on the Primary Antibody and/or NCR-stained slides. Perform level testing using dry untreated slides for each slide position in all Autostainer slide racks every 3 months or whenever the Autostainer Link 48 is moved or adjusted on the counter. Discard Autostainer slide racks that fail level testing in any slide position and/or have undergone ≥ 175 PT Link pre-treatment cycles
- Ensure that the Autostainer Link 48 lid is properly closed to prevent reagent evaporation during the staining procedure
- Select the PD-L1 IHC 22C3 pharmDx protocol. The instrument performs the staining and counterstaining procedures by applying the appropriate reagent, monitoring the incubation time, and rinsing slides between reagents
- Counterstain slides using EnVision FLEX Hematoxylin, Code K8008

Mounting

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

Technical Checklist

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

Customer Name/Institution _____

Name and Title _____

Autostainer Link 48 Serial Number _____ Software Version _____

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

Additional observations or comments:

_____

_____

_____

Slide Evaluation

General Considerations

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

PD-L1 interpretation is best assessed by requesting 3 serial tissue sections (H&E, PD-L1 stain, and NCR stain) so that if the H&E is first assessed and is acceptable, the 2 remaining serial sections are likely to retain the same favorable tissue quality.

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

Specimen Adequacy

Confirm the Presence of at Least 100 Viable Tumor Cells

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

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

Instructions for Patient Specimens with Less Than 100 Viable Tumor Cells

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

Evaluating Controls

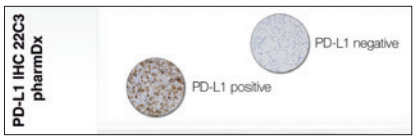


Figure 6: Each Control Cell Line Slide contains sections of cell pellets with positive and negative PD-L1 expression.

PD-L1 IHC 22C3 pharmDx Control Cell Line Slide

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

Evaluate the overall staining intensity using the following guide:

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

Positive Control Cell Pellet

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

- Cell membrane staining of ≥ 70% of cells
- ≥ 2+ average staining intensity of cells with membrane staining
- Nonspecific staining < 1+ intensity

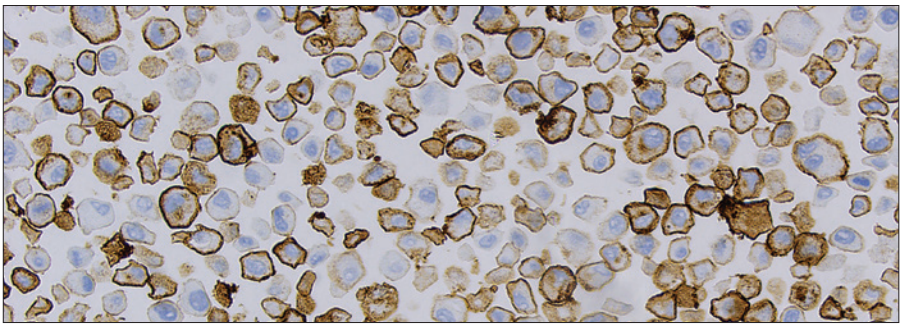


Figure 7: Positive cell pellet with acceptable staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20× magnification).

Negative Control Cell Pellet

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

- No cells with membrane staining*
- Nonspecific staining < 1+ intensity*

*Note that staining of a few cells in the MCF-7 cell pellet may occasionally be observed. The following acceptance criteria are applicable: the presence of ≤ 10 total cells with distinct cell membrane staining and/or nonspecific staining with ≥ 1+ intensity within the boundaries of the MCF-7 cell pellet are acceptable.

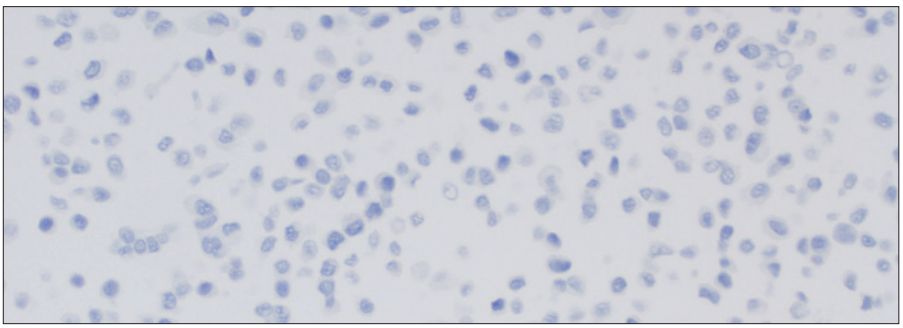


Figure 8: Negative cell pellet with no staining of PD-L1 IHC 22C3 pharmDx Control Cell Line Slide (20× magnification).

See the Control Cell Line (CCL) Appendix on page 76 for images of passing, borderline, and failing control cell line staining.

Positive and Negative Lab-Supplied Control Tissue

Examine the Positive Control Tissue Slides to verify that the fixation method and epitope retrieval process are effective. The Positive Control Tissue Slides should be stained with both PD-L1 primary antibody and Negative Control Reagent. The ideal positive control tissue provides a complete dynamic representation of weak-to-moderate staining of tumor cells and tumor-associated mononuclear inflammatory cells (MICs) (Figure 9). Known positive tissue controls should only be utilized for monitoring the correct performance of processed tissues and test reagents, not as an aid in formulating a specific diagnosis of patient samples. If staining of positive lab-supplied control tissue is not satisfactory, all results with the patient specimen should be considered invalid.

- Requirements for slide stained with PD-L1: Presence of brown cell membrane staining should be observed. Nonspecific staining, including nuclear staining, should be ≤ 1+
- Requirements for slide stained with Negative Control Reagent: No membrane staining. Nonspecific staining, including nuclear staining, should be ≤ 1+

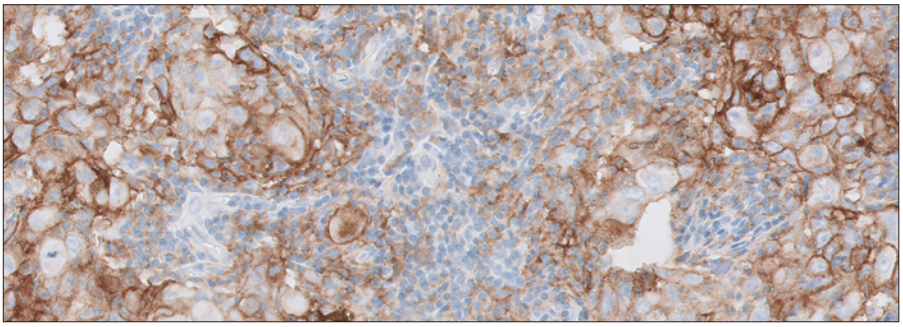


Figure 9: Ideal positive lab-supplied TNBC control tissue stained with PD-L1 primary antibody (20x magnification).

Examine the Negative Control Tissue Slides to verify the specificity of the labeling of the target antigen by the primary antibody. The Negative Control Tissue Slides (known to be PD-L1 negative) should be stained with both PD-L1 primary antibody and Negative Control Reagent. The ideal negative control tissue should demonstrate no staining of tumor cells and immune cells (Figure 10). However, because prevalence of PD-L1 expression on immune cells is high, a few staining immune cells are acceptable. The variety of different cell types present in most tissue sections offers internal negative control sites; this should be verified by the user.

If inappropriate staining occurs in the lab-supplied control tissues, results with the patient specimen should be considered invalid.

Note: As an alternative, negative portions of the Positive Control Tissue may serve as the Negative Control Tissue, but this should be verified by the user.

- Requirements for slide stained with PD-L1: No membrane staining in tumor cells. Nonspecific staining, including nuclear staining, should be ≤ 1+
- Requirements for slide stained with Negative Control Reagent: No membrane staining. Nonspecific staining, including nuclear staining, should be ≤ 1+

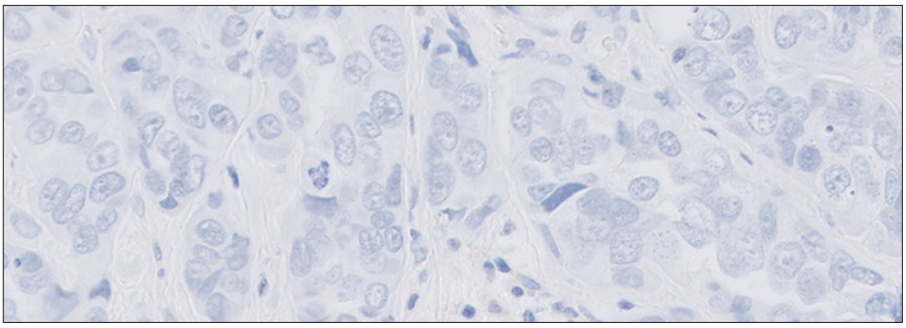


Figure 10: Ideal negative lab-supplied TNBC control tissue stained with the PD-L1 primary antibody demonstrating lack of staining of tumor cells and immune cells (20x magnification).

Optional Control Tissue

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

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

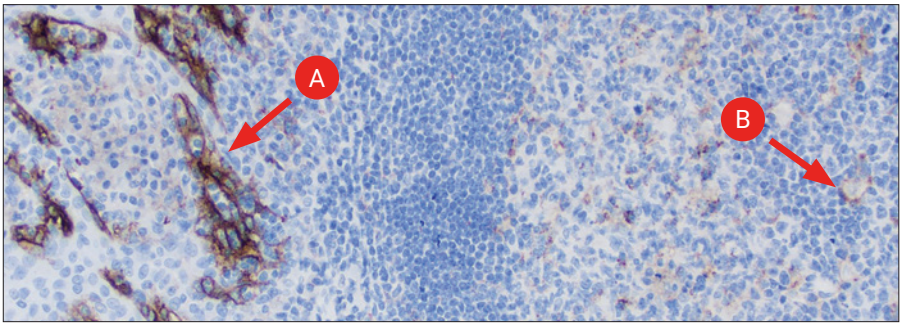


Figure 11: Tonsil stained with PD-L1 primary antibody exhibiting strong membrane staining in portions of the crypt epithelium (A) and weak-to-moderate membrane staining of follicular macrophages in the germinal centers (B) (10× magnification).

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

Negative Control Reagent (NCR)

Examine the patient slide stained with the NCR to identify nonspecific staining, including nuclear staining, that may interfere with interpreting the PD-L1 stained slide, making the specimen non-evaluable. Satisfactory performance is indicated by the absence of staining. Nonspecific staining, including nuclear staining, should be ≤ 1+ (Figure 12).

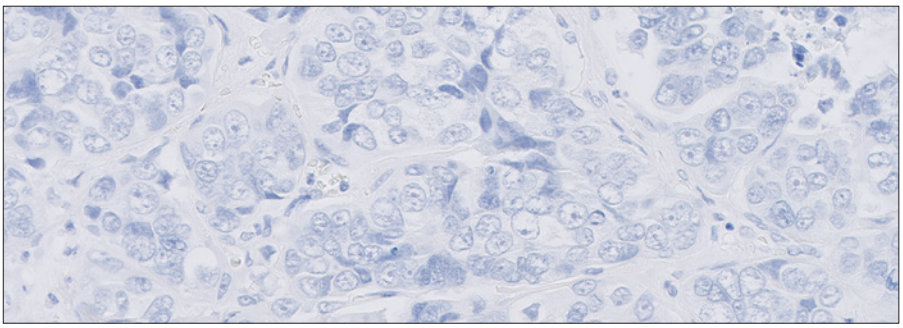


Figure 12: TNBC tissue specimen stained with NCR (20× magnification).

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

Slide Evaluation Flowchart

Figure 13: Recommended order of slide evaluation.

Combined Positive Score

Definition of Combined Positive Score (CPS)

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

CPS is defined accordingly:

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

** Macrophages and histiocytes are considered the same cells*

CPS Numerator Inclusion and Exclusion Criteria

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

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

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

Determining Combined Positive Score

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

Table 1: CPS Numerator Inclusion/Exclusion Criteria

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

** In MICs, membrane and cytoplasmic staining are often indistinguishable due to a high nuclear to cytoplasmic ratio. Therefore, membrane and/or cytoplasmic staining of MICs are included in the score*
† Adjacent MICs are defined as being within the same 20× field as the tumor. However, MICs that are NOT directly associated with the response against the tumor should be excluded
‡ Macrophages and histiocytes are considered the same cells

Table 2: CPS Denominator Inclusion/Exclusion Criteria

Tissue Elements	Included in the Denominator	Excluded from the Denominator
Tumor Cells	All viable invasive tumor cells	– Nonviable tumor cells – Carcinoma in situ (DCIS and LCIS)
Immune Cells	Not included	All immune cells
Other Cells	Not included	– Benign cells – Stromal cells (including fibroblasts) – Necrotic cells and/or cellular debris

Suggested Methods

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

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

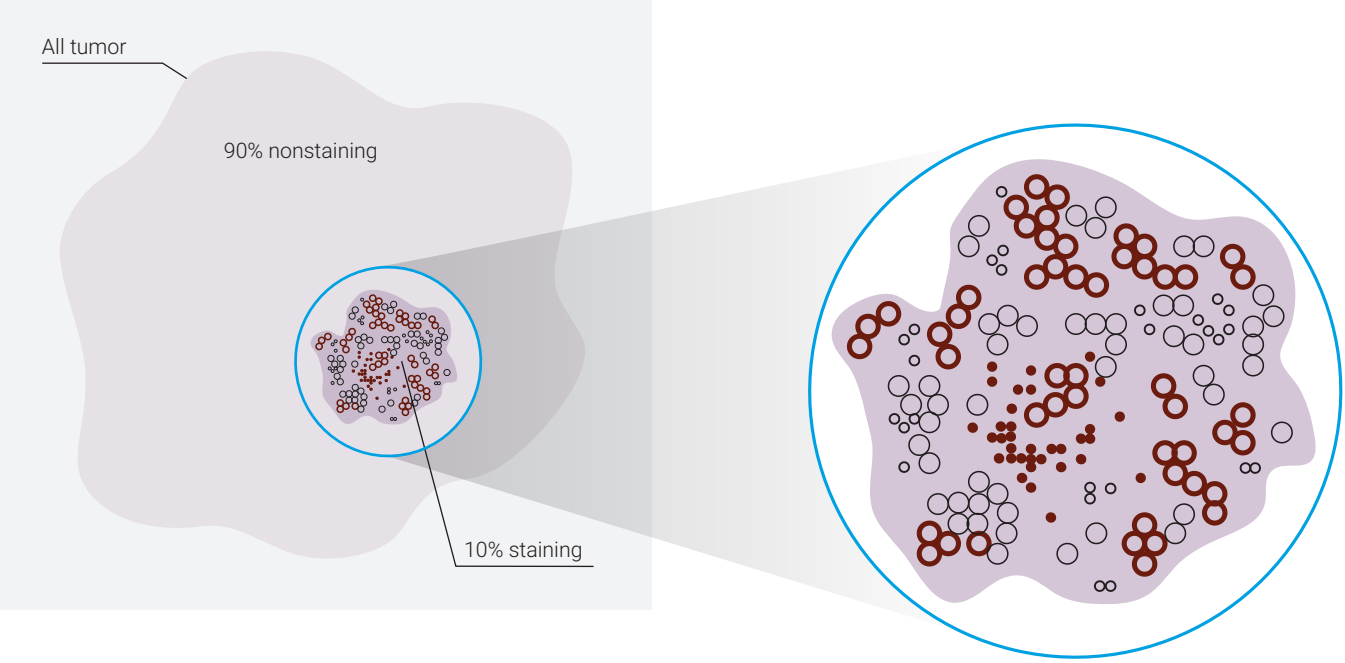
Example 1: Calculation of Combined Positive Score Based on a Small PD-L1 Staining Area

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

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

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

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



Calculate the Combined Positive Score of the entire tumor area:

Assessment:

CPS of area with staining:

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

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

Clinical Interpretation: CPS < 10

§ *Including tumor cells, lymphocytes, macrophages*

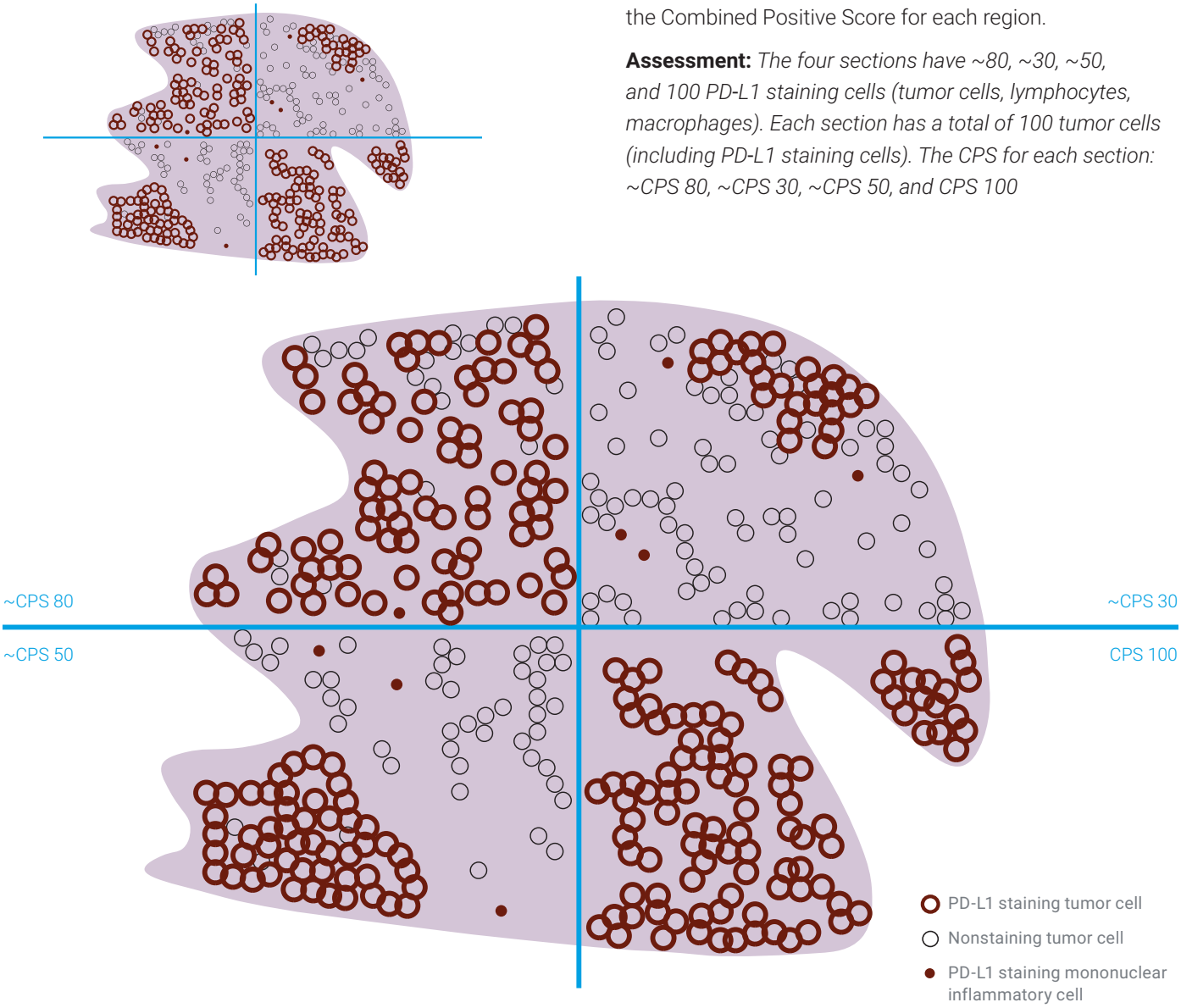
Figure 14: Example of tumor with small PD-L1 staining area.

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

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

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

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



Calculate the CPS of the entire tumor area:

Assessment:

(80+30+50+100/ 4) x100 ≈ CPS 65

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

Clinical Interpretation: CPS ≥ 10

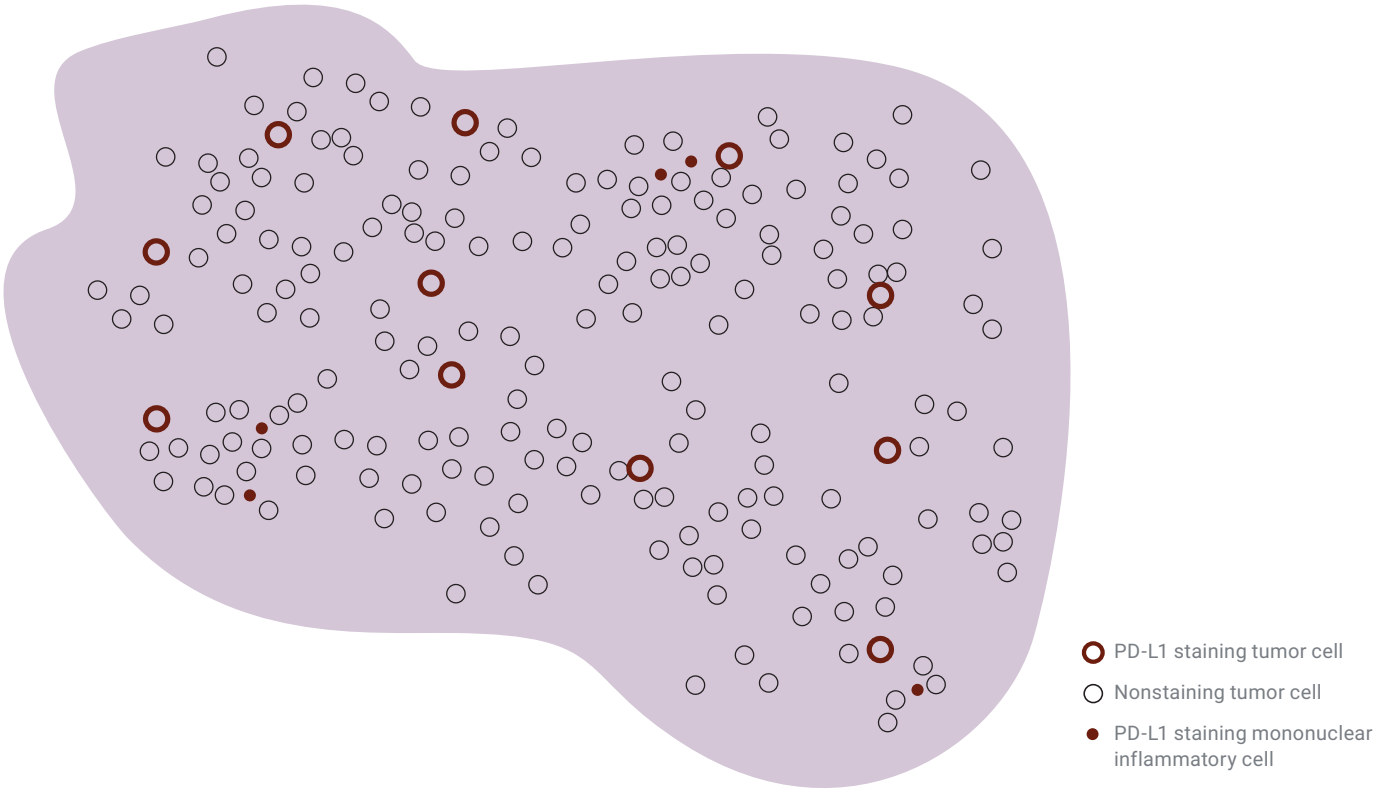
Figure 15: Example with heterogeneous PD-L1 staining area.

Example 3: Calculation of Combined Positive Score for a Near Cutoff Specimen

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

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

Assessment: *Tumor specimen has perceptible and convincing staining. There are 16 PD-L1 staining cells (tumor cells, lymphocytes, macrophages). There are approximately 200 viable tumor cells present in the entire specimen*



Calculate the CPS of the entire tumor area:

Assessment:

$$\text{CPS} = \frac{\text{\# PD-L1 staining cells*}}{\text{Total \# of viable tumor cells}} \times 100 = \frac{\text{16 PD-L1 staining cells}}{\text{200 tumor cells}} \times 100 = \text{CPS 8}$$

Clinical Interpretation: CPS < 10

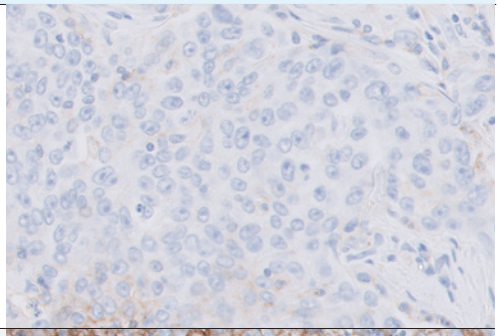
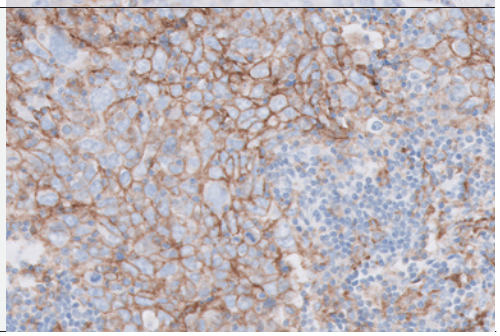
** Including tumor cells, lymphocytes, macrophages*

Figure 16: Example of near cutoff specimen.

Interpretation of CPS

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

Table 3: CPS and Corresponding PD-L1 Expression Levels

CPS	PD-L1 Expression Level	Image (20× magnification)
< 10	CPS is less than 10	
≥ 10	CPS is greater than or equal to 10	

Identifying Patients With TNBC for Treatment

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

Clinical Validation of PD-L1 IHC 22C3 pharmDx in Patients With TNBC

The clinical validity of PD-L1 IHC 22C3 pharmDx in evaluating PD-L1 expression in patients with TNBC is based on the KEYTRUDA KEYNOTE-355 study sponsored by Merck & Co. Specimens from patients with locally recurrent unresectable or metastatic TNBC, which had not been previously treated with chemotherapy, were tested for PD-L1 expression using PD-L1 IHC 22C3 pharmDx. In the KEYNOTE-355 clinical trial, 38% of enrolled patients had TNBC that expressed PD-L1 with a Combined Positive Score (CPS) of greater than or equal to 10 (CPS ≥ 10) (Table 4). Clinical efficacy of KEYTRUDA treatment in combination with paclitaxel, paclitaxel protein-bound, or gemcitabine and carboplatin is presented in the Clinical Performance Evaluation section on pages 89-90.

Table 4: PD-L1 Prevalence in Patients with Locally Recurrent Unresectable or Metastatic TNBC Enrolled in KEYNOTE-355

PD-L1 Expression	CPS < 10	CPS ≥ 10
Prevalence % (n)	62% (524)	38% (323)

PD-L1 IHC 22C3 pharmDx Testing Scheme

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

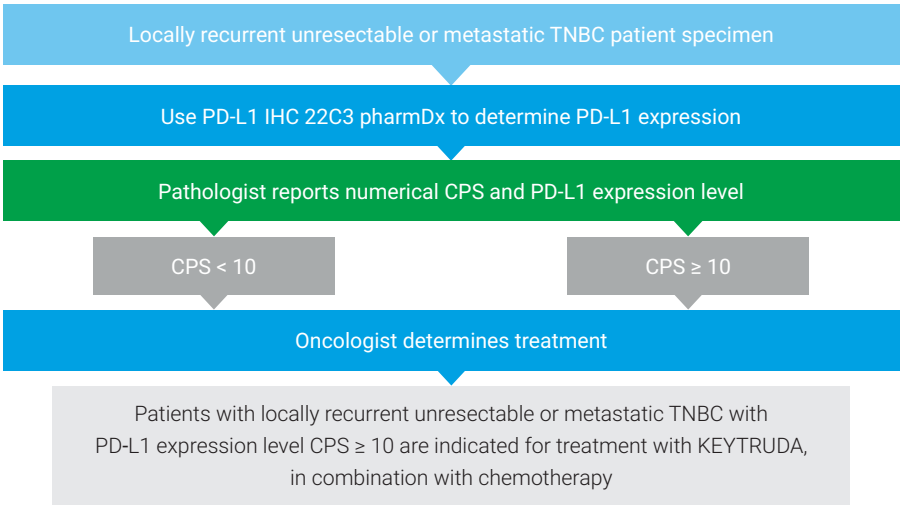


Figure 17: Testing scheme for PD-L1 IHC 22C3 pharmDx.

Reporting Results

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

PD-L1 IHC 22C3 pharmDx Summary of Sample Tested

Date of Run: _____

PD-L1 IHC 22C3 pharmDx Lot: _____

Staining Run Log ID: _____

Specimen ID: _____

Patient Identifiers: _____

Type of Service: IHC Stain With Manual Interpretation

Other: _____

PD-L1 Testing Results

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

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

PD-L1 IHC 22C3 pharmDx Result to Treating Physician

Combined Positive Score*: _____

CPS < 10: ☐ CPS ≥ 10: ☐

Comments to Treating Physician:

- KEYTRUDA® (pembrolizumab), in combination with chemotherapy, is indicated for the treatment of patients with locally recurrent unresectable or metastatic TNBC whose tumors express PD-L1 (CPS ≥ 10) as determined by an FDA-approved test. See KEYTRUDA prescribing information for details.
- * FDA has reviewed and approved this assay as a qualitative assay at a CPS ≥ 10 cutoff (positive test result) for the TNBC indication. The correlation between the raw CPS and clinical outcome has not been established, and has not been reviewed or approved by FDA.

Combined Positive Score Summary and Examples

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

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

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

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

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

This section will define and illustrate scoring inclusions and exclusions for accurate determination of Combined Positive Score. All images are TNBC, unless otherwise noted in the figure caption.

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

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

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

Tumor Cells

Linear Membrane Staining

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

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

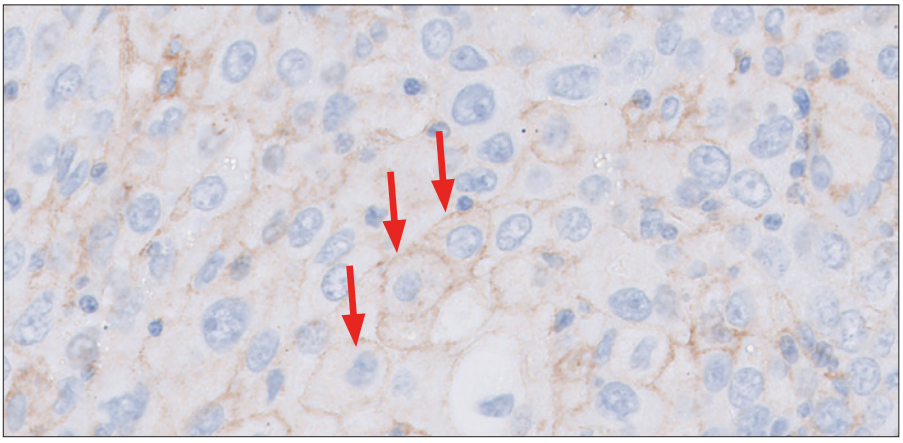


Figure 18a: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting 1+ linear membrane staining of tumor cells (arrows) (20× magnification).

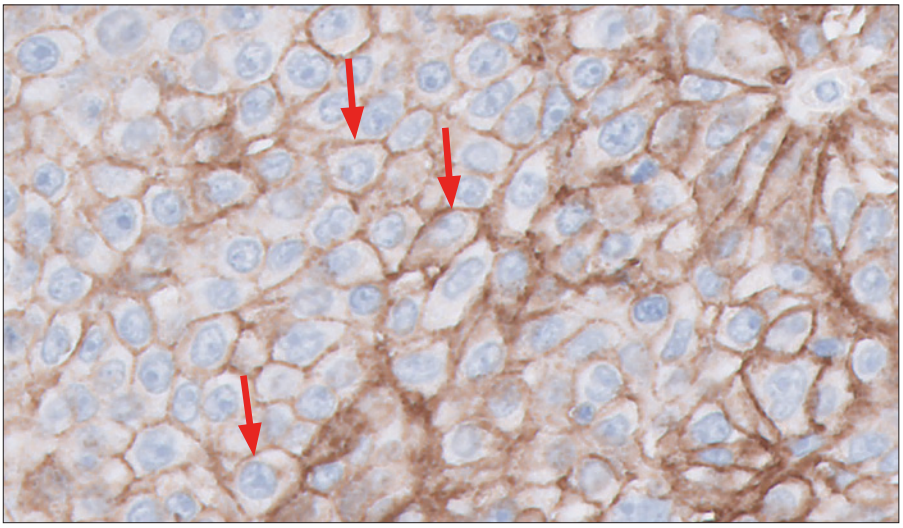


Figure 18b: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting 2+ linear membrane staining of tumor cells (arrows) (20× magnification).

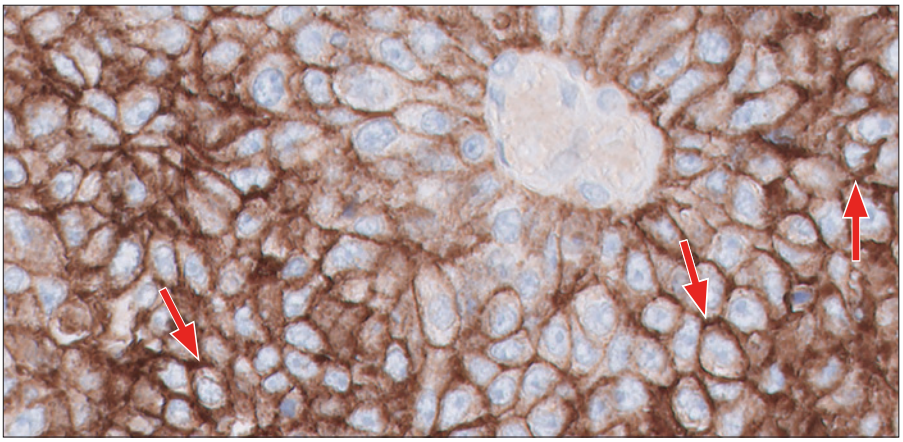


Figure 18c: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting 3+ linear membrane staining of tumor cells (arrows) (20× magnification).

Key Point

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

Partial Linear Membrane Staining

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

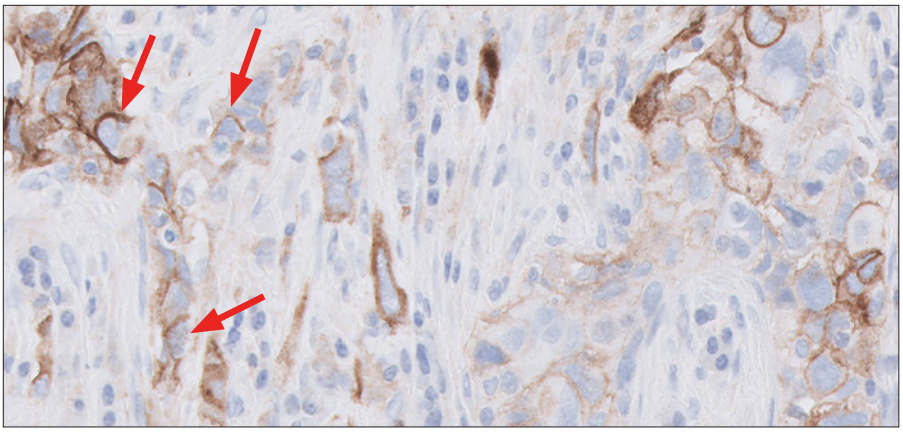


Figure 19: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting partial linear membrane staining of tumor cells (arrows) (20× magnification).

Key Point

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

Linear Membrane and Cytoplasmic Staining

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

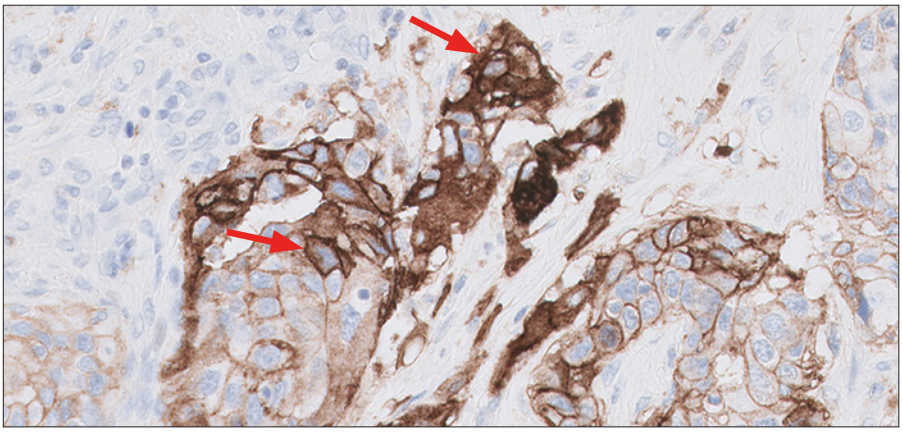


Figure 20a: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting linear membrane staining distinct from cytoplasmic staining (arrows) (20× magnification).

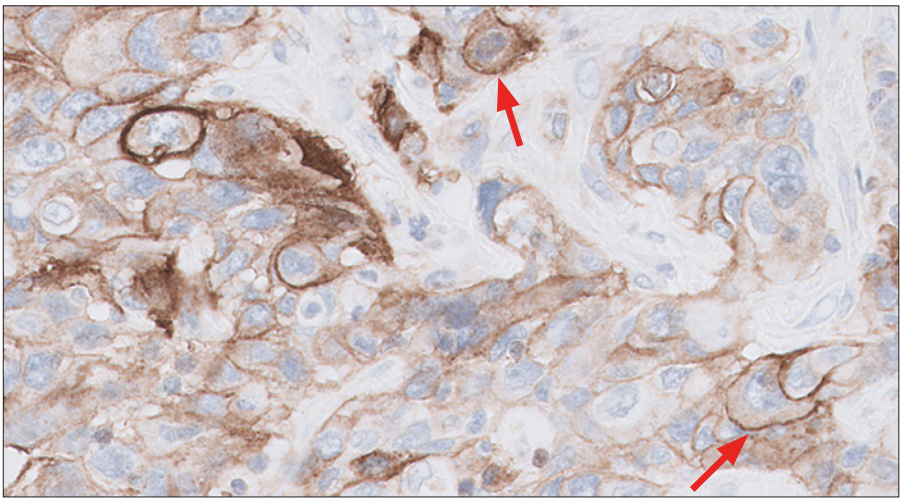


Figure 20b: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting linear membrane staining distinct from cytoplasmic staining (arrows) (20× magnification).

Key Point

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

Granular Staining

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

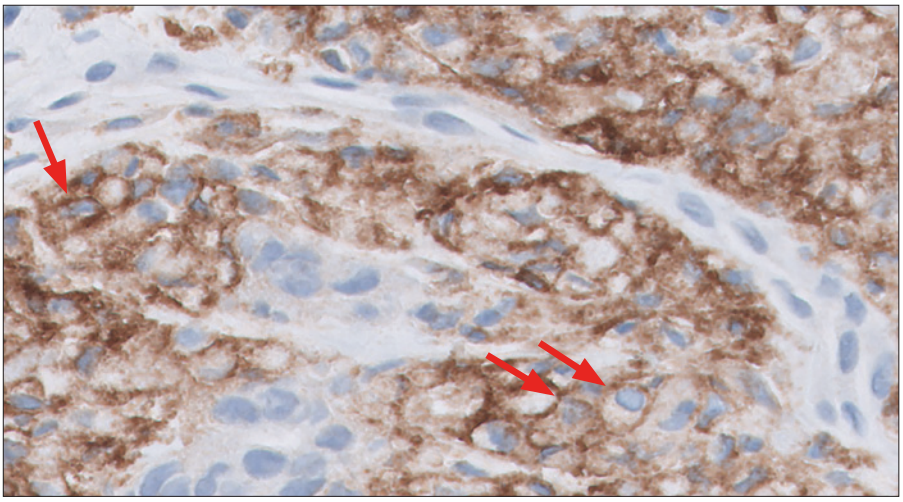


Figure 21a: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting granular linear membrane staining pattern (arrows) (20× magnification).

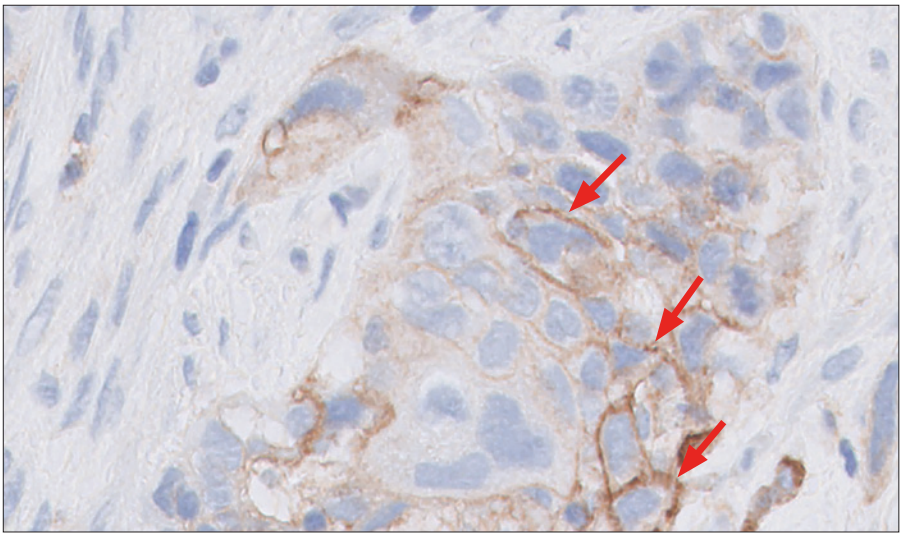


Figure 21b: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting granular linear membrane staining pattern (arrows) (20× magnification).

Key Point

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

Multinucleate Tumor Cells

Some tumor cells in TNBC may be multinucleate and each multinucleate tumor cell should be counted as one cell. The same rules should apply for inclusion in the numerator and denominator: all viable tumor cells should be included in the denominator and all tumor cells with partial or complete linear membrane staining should be included in the numerator.

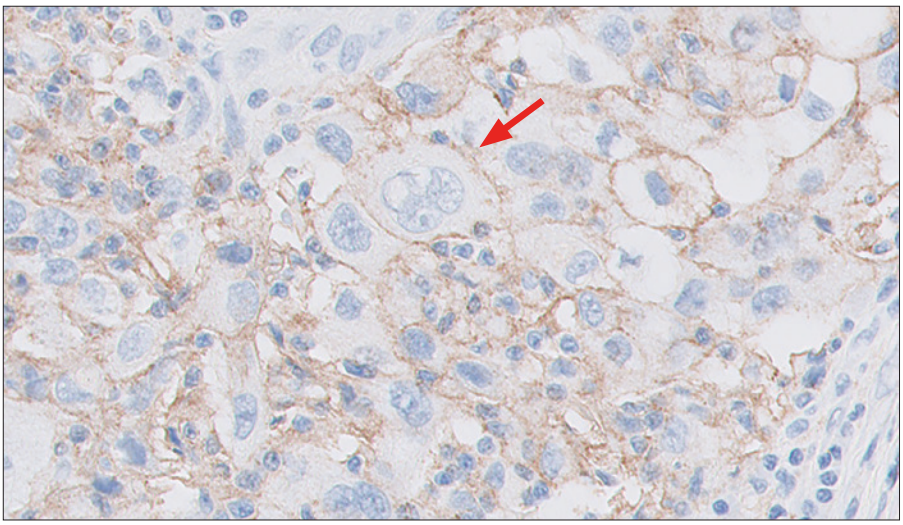


Figure 22: Multinucleate tumor cells (arrow) (20× magnification).

Key Point

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

Immune Cells

Tumor-associated Mononuclear Inflammatory Cells (MICs)

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

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

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

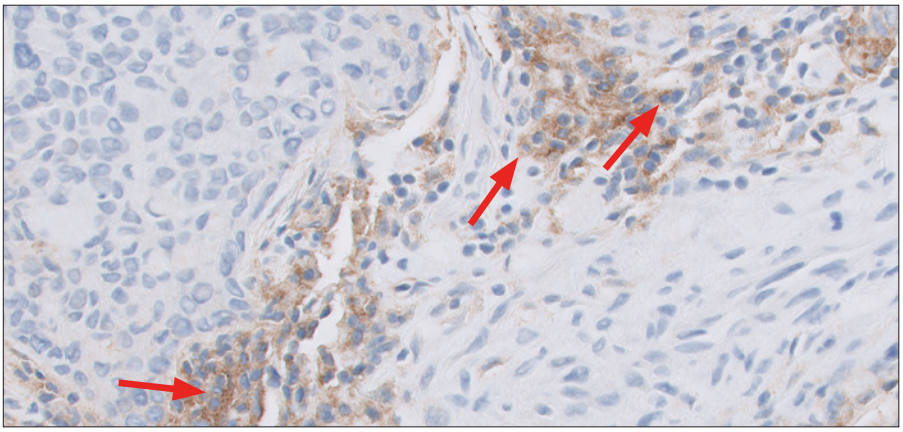


Figure 23a: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting staining of tumor-associated lymphocytes (arrows) (20× magnification).

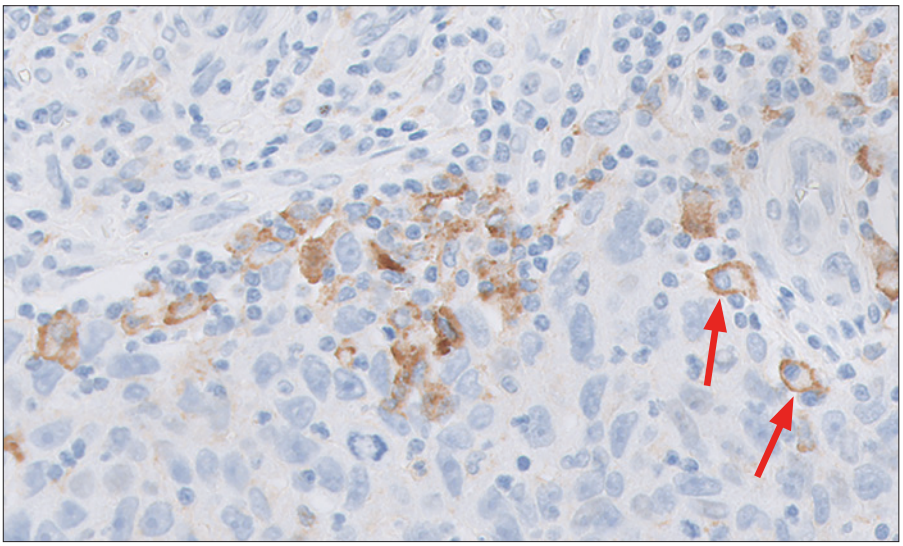


Figure 23b: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting staining of tumor-associated macrophages (arrows) (20× magnification).

Multinucleate Giant Cells

Multinucleate giant cells can be seen in TNBC and, if PD-L1 staining is present on these cells, each multinucleate giant cell should be counted as one cell and included in the numerator.

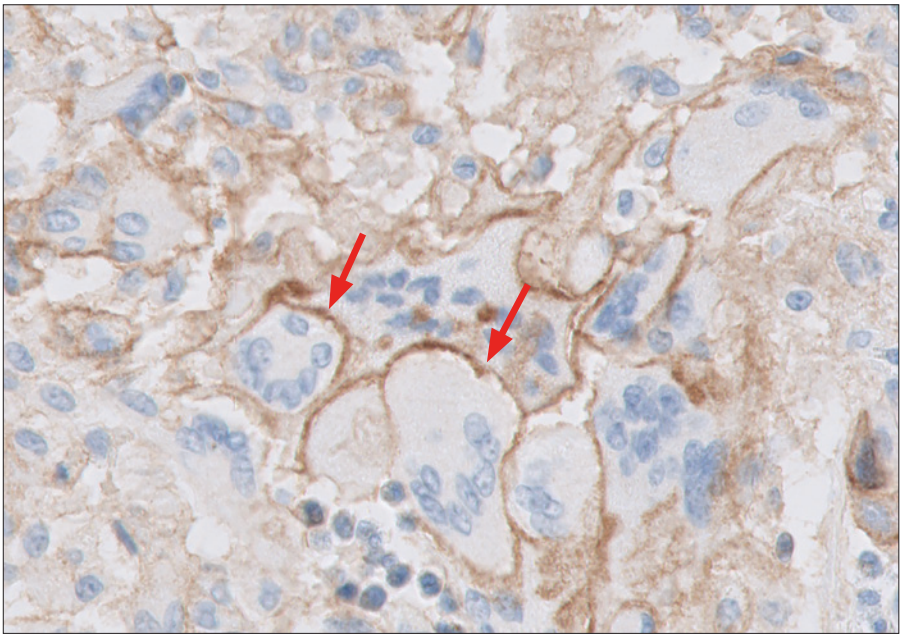


Figure 24: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting staining of multinucleate giant cells (arrows) (20× magnification).

Key Point

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

Indistinguishable Tumor and Immune Cells

Tumor cells and tumor-associated lymphocytes and macrophages may be indistinguishable from each other when examining the slide with PD-L1 IHC 22C3 pharmDx primary antibody staining due to small tumor cell size and staining characteristics. It is recommended to use the corresponding H&E slide to distinguish cell morphology. This is especially important when determining the denominator.

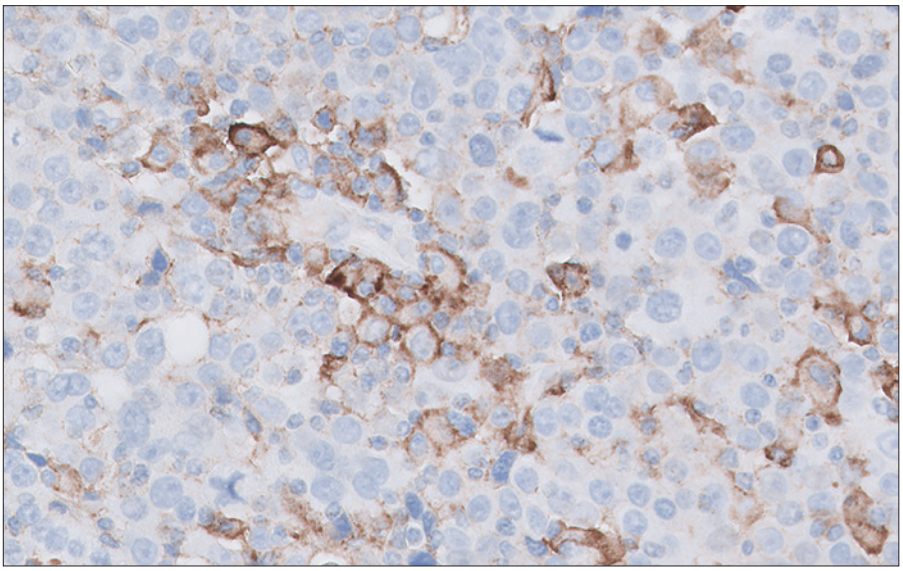


Figure 25a: Tumor cells and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other and exhibit PD-L1 IHC 22C3 pharmDx primary antibody staining (20× magnification).

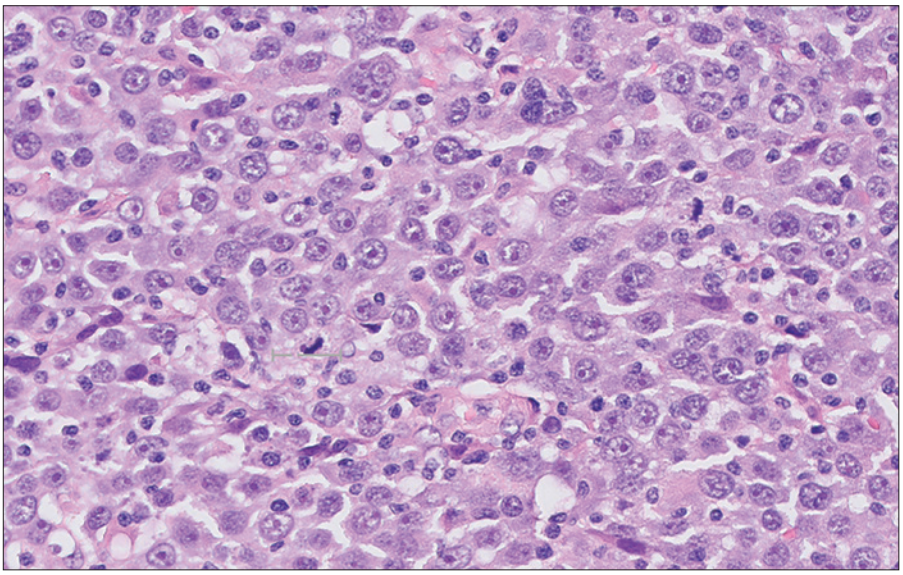


Figure 25b: Corresponding H&E to reference when tumor cells and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other (20× magnification).

Key Point

Utilize the H&E slide when it is challenging to distinguish tumor cells from immune cells

Immune Cell Inclusion/Exclusion: 20× Rule

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

Move the slide so that the tumor is in the approximate center of a 20× field. Immune cells surrounding the tumor in this field should be included in scoring. Immune cells outside of this field should be excluded from scoring as long as they do not surround neighboring tumor cells. In general, include PD-L1 staining MICs that are within 0.5 mm of the tumor cells. This rule may be applied to tumors within lymph nodes that contain PD-L1 staining MICs. See Figures 26a–26c for an example of determining which MICs are included in the CPS numerator.

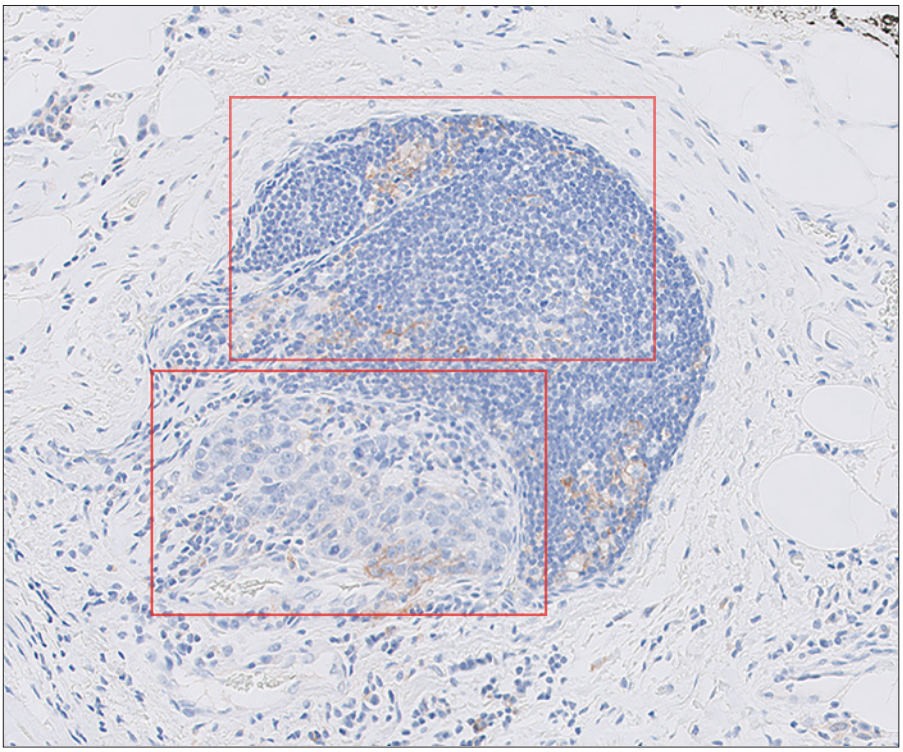


Figure 26a: At 10× magnification, two areas of PD-L1 staining mononuclear inflammatory cells are visible. To demonstrate which immune cells to include in the numerator, zoom in to 20× magnification on the boxed fields (10× magnification).

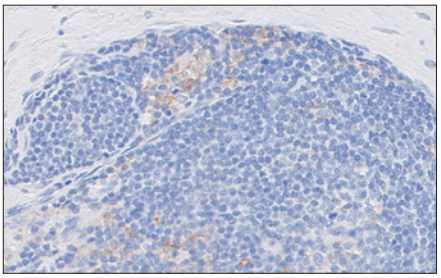


Figure 26b: Tumor cells are absent from this 20× field containing PD-L1 staining mononuclear inflammatory cells, thus none of these cells should be included in the numerator (20× magnification).

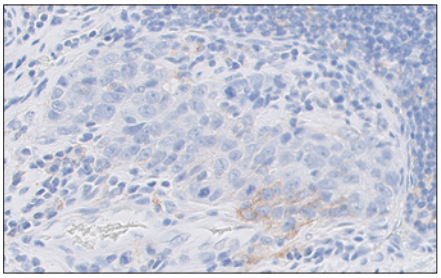


Figure 26c: When positioning the tumor cells in the approximate center of a 20× field, PD-L1 staining mononuclear inflammatory cells that are present within the same field should be included in the numerator (20× magnification).

Tumor Cell Size

TNBC includes different morphologies and tumor cell sizes that can impact the Combined Positive Score (CPS) by increasing or decreasing the total number of tumor cells that are included in the denominator. Well-differentiated squamous cell carcinoma may exhibit larger tumor cells with abundant keratinous cytoplasm, and will commonly have fewer cells per 20× field. Alternatively, a poorly-differentiated, basaloid pattern will commonly have a higher number of tumor cells per 20× field due to the smaller size and scant cytoplasm of the tumor cells. The more tumor cells included in the denominator, the greater the number of PD-L1 staining tumor cells, lymphocytes, and macrophages that are needed in the numerator to bring the overall score to CPS 10 or above. As a guideline, if tumor cells are 20 µm in diameter and fill a 20× field, there would be approximately 2500 tumor cells in that field.

Small Cell Size

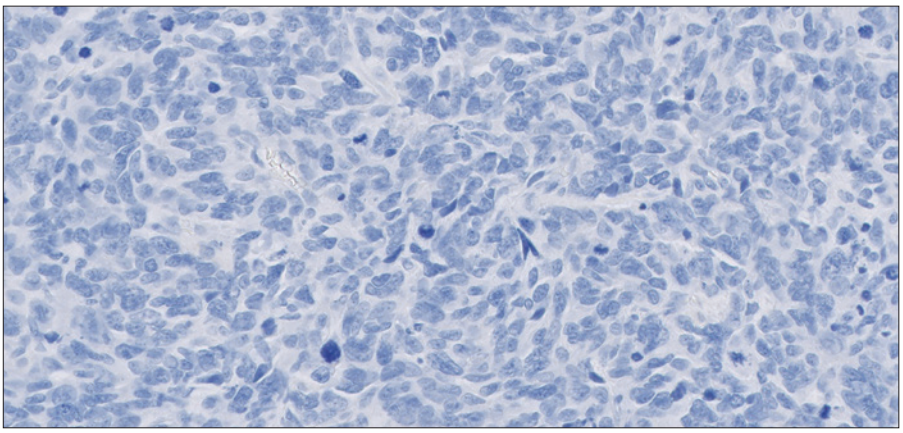


Figure 27: TNBC specimen with small tumor cells (20× magnification).

Medium Cell Size

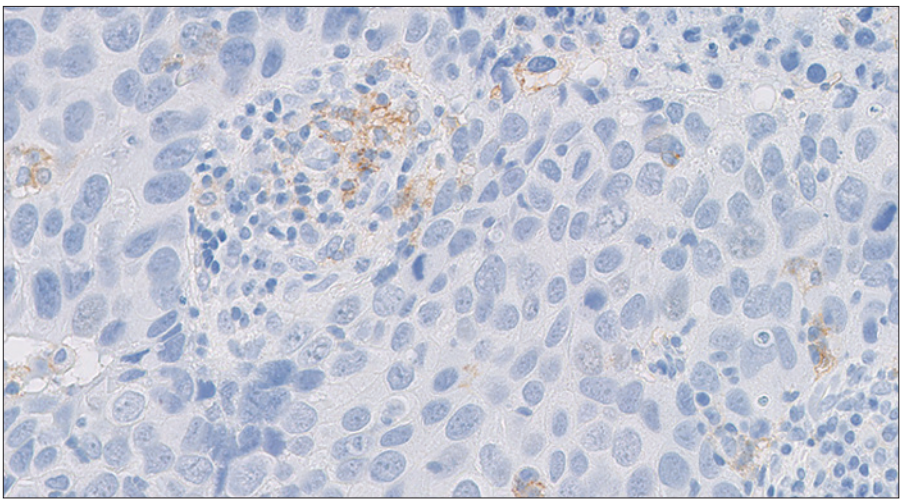


Figure 28: TNBC specimen with medium tumor cells (20× magnification).

Large Cell Size

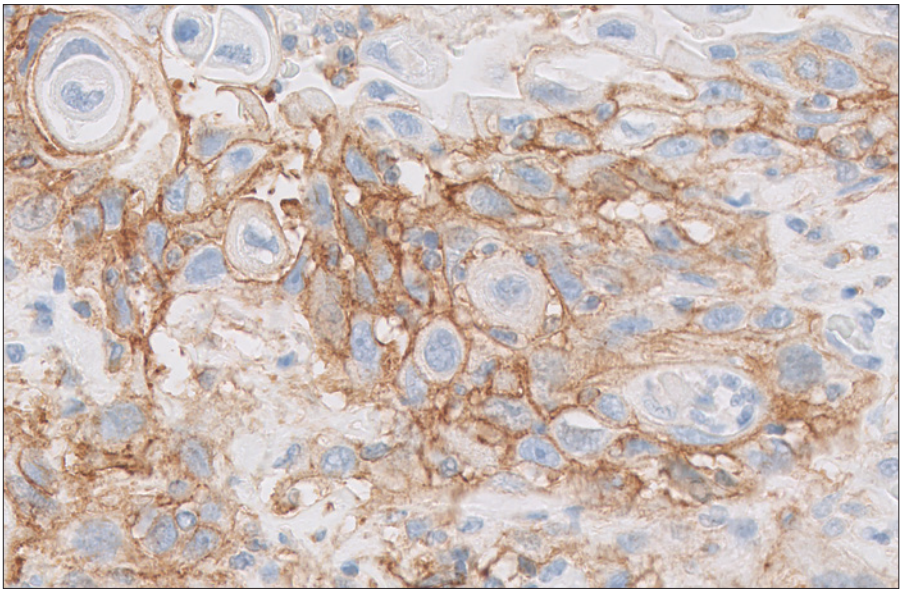


Figure 29: TNBC specimen with large tumor cells (20× magnification).

Key Point

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

Cells Excluded from CPS

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

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

Tumor Cells with Only Cytoplasmic Staining

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

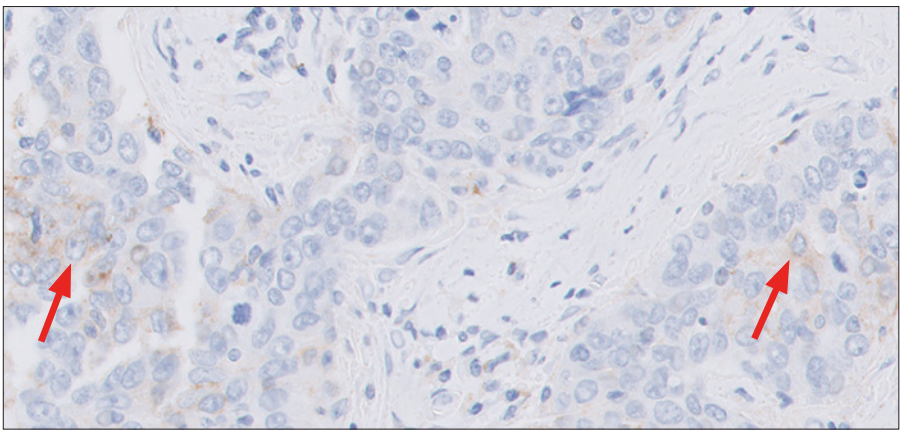


Figure 30: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting only cytoplasmic staining of tumor cells (arrows) (20× magnification).

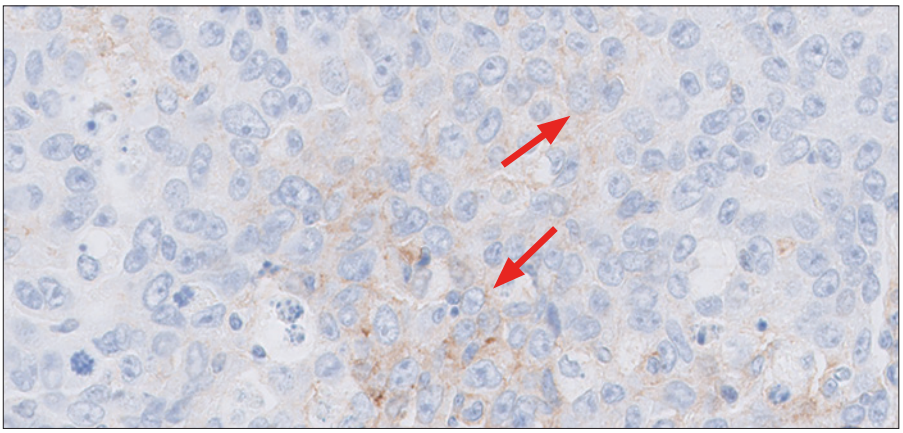


Figure 31: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting only cytoplasmic staining of tumor cells (arrows) (20× magnification).

Key Point

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

Carcinoma In Situ (CIS)

Carcinoma in situ (both ductal carcinoma in situ, DCIS, and lobular carcinoma in situ, LCIS) may be present in TNBC and should be excluded from both the numerator and denominator. Additionally, PD-L1 staining mononuclear inflammatory cells (MICs) that are associated with the CIS component(s) should be excluded from the numerator.

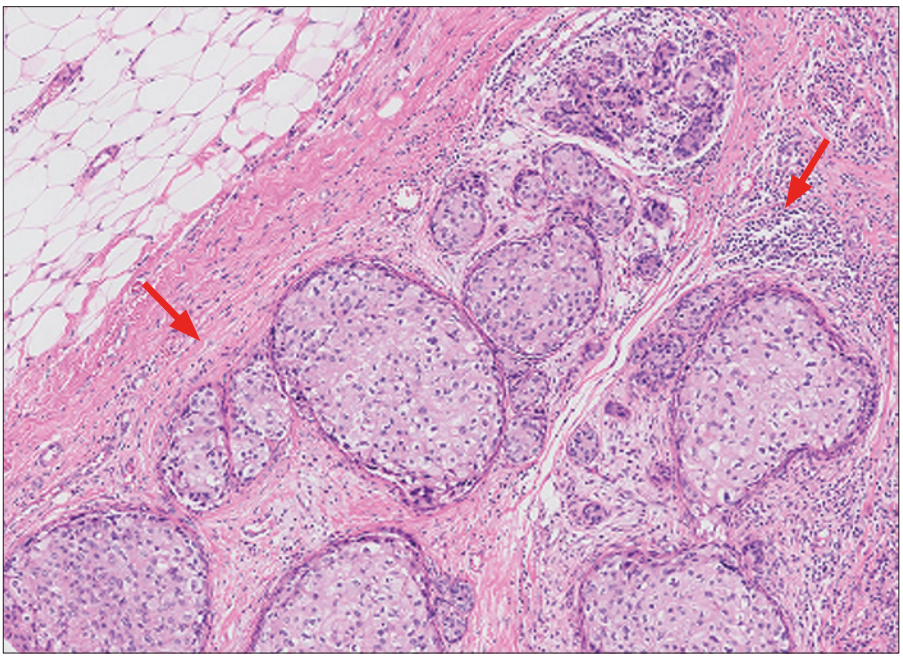


Figure 32a: Hematoxylin and eosin (H&E) section demonstrating ductal carcinoma in situ (DCIS) (arrows) (2× magnification).

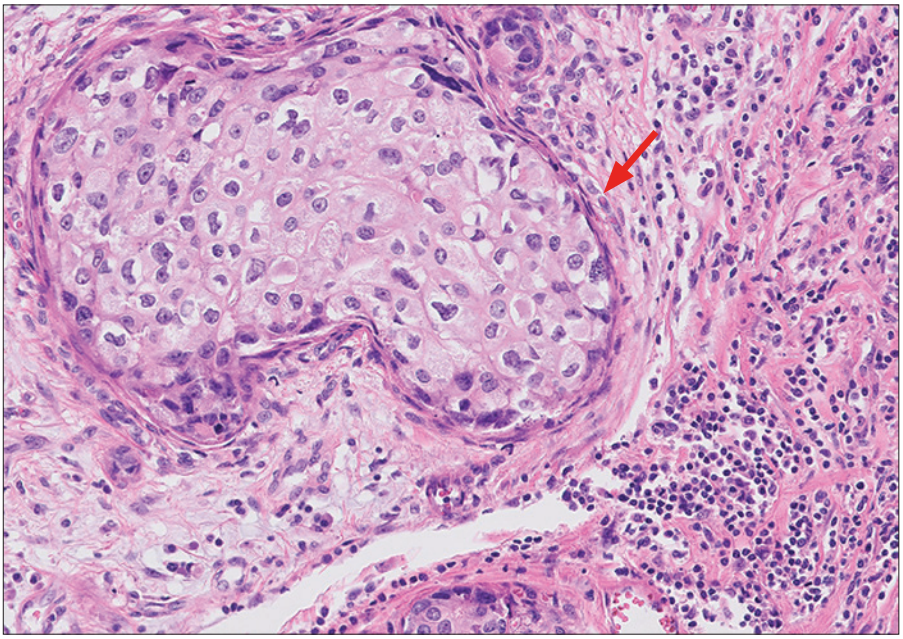


Figure 32b: Hematoxylin and eosin (H&E) section demonstrating ductal carcinoma in situ (DCIS) (arrow) (20× magnification).

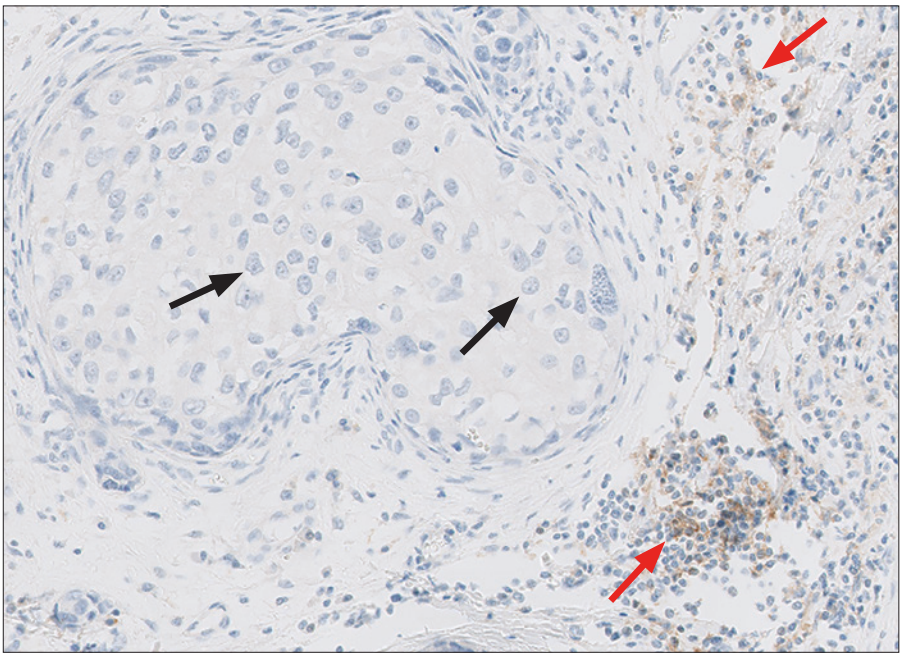


Figure 33: Any tumor cells that are part of the CIS component should be excluded from the numerator and denominator (black arrows). Any mononuclear inflammatory cells (MICs) (red arrows) associated with the DCIS component should be excluded from the numerator (20× magnification).

Key Point

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

Stromal Cells

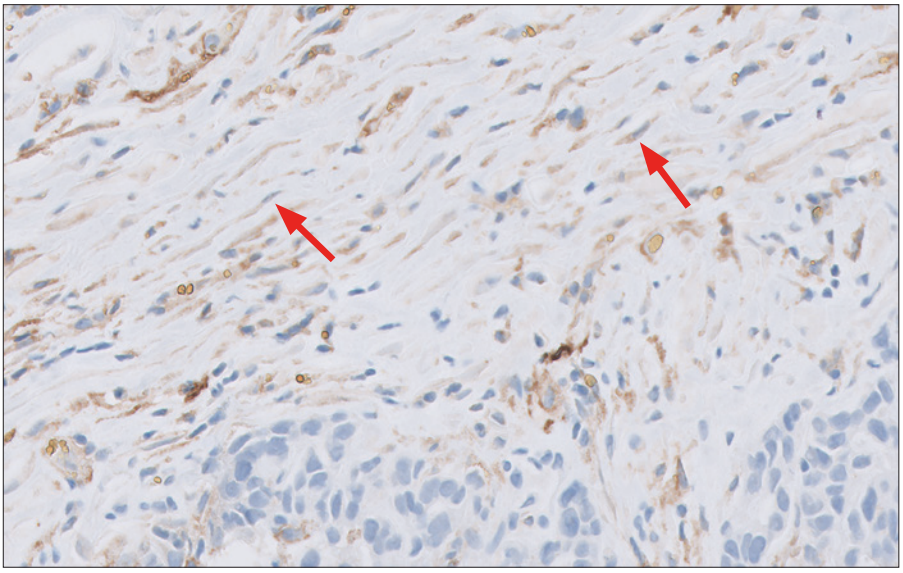


Figure 34: PD-L1 IHC 22C3 pharmDx primary antibody staining on stromal cells (arrows) (20× magnification).

Key Point

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

Other Immune Cells Excluded from CPS

Various types of immune cells can exhibit PD-L1 staining, but only tumor-associated lymphocytes and macrophages should be included in the CPS calculation. Refer to page 43 for the immune cell inclusion/exclusion 20× rule. PD-L1 staining neutrophils, eosinophils, and plasma cells should be excluded from the score.

Benign Cells

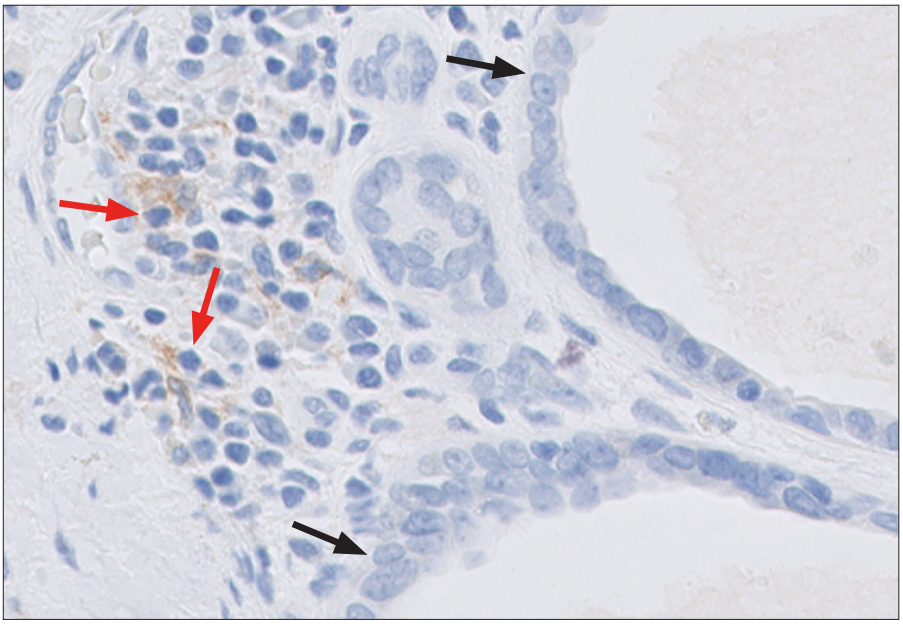


Figure 35: TNBC specimen exhibiting benign glands (black arrows) and associated PD-L1 IHC 22C3 pharmDx primary antibody staining mononuclear inflammatory cells (MICs) (red arrows) (20× magnification).

Key Point

Benign cells and MICs associated with the benign component may exhibit PD-L1 staining and should be excluded from the score

Neutrophils

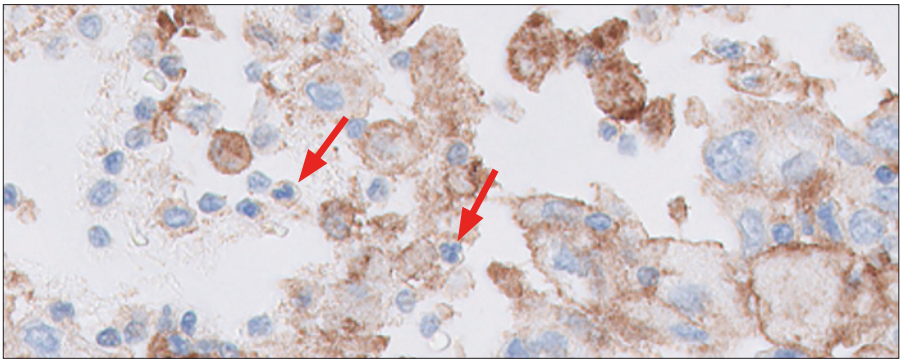


Figure 36: PD-L1 IHC 22C3 pharmDx primary antibody staining on neutrophils (20× magnification).

Plasma Cells

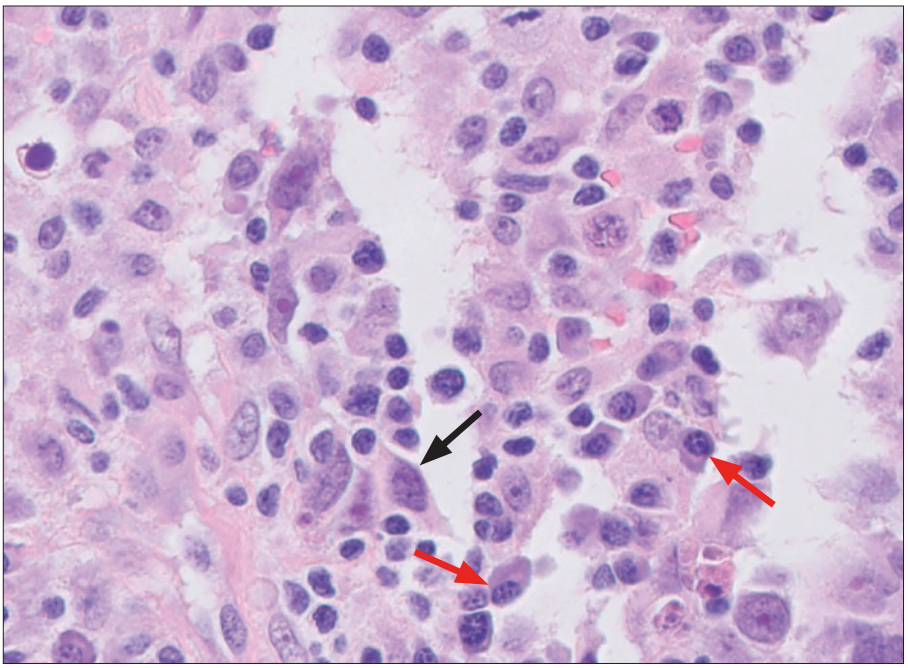


Figure 37a: TNBC specimen stained with H&E exhibiting staining of both tumor cells (black arrow) and plasma cells (red arrows) (20× magnification).

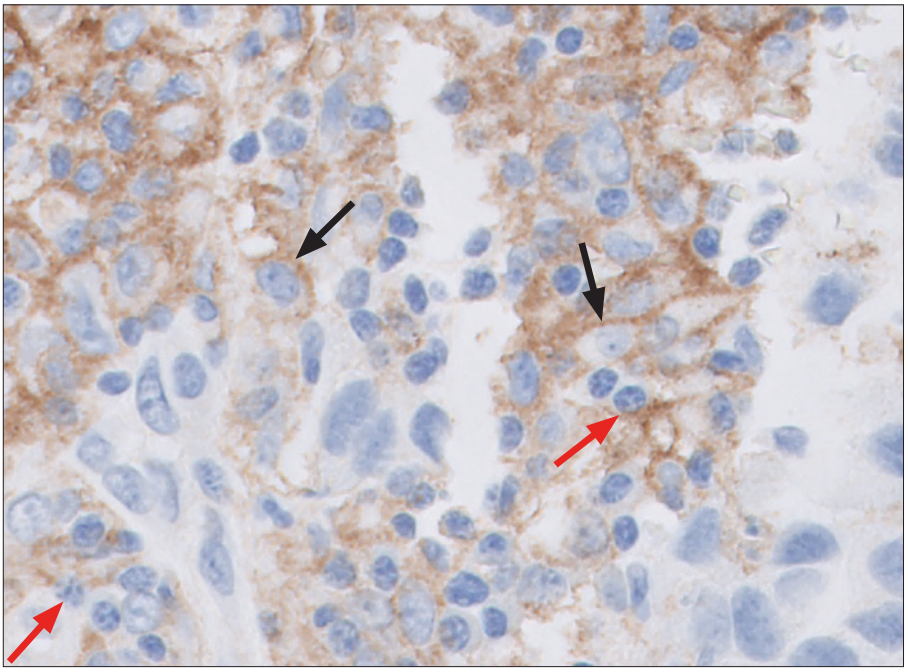


Figure 37b: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting staining of both tumor cells (black arrows) and plasma cells (red arrows) (20× magnification).

Key Point

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

Artifacts

The following pages provide examples of artifacts you may see when staining with PD-L1 IHC 22C3 pharmDx.

Specific Nonscorable Staining

Specific nonscorable staining is defined as chromogen-related staining due to the anti-PD-L1 antibody/PD-L1 antigen interaction that is present on cells or cellular compartments that are not included when scoring with CPS. It is specific staining because it is present on the PD-L1 slide and absent from the NCR slide; however, it is nonscorable because it is present on either cells (e.g., stromal cells, peripheral nerve, plasma cells, etc.) or cellular compartments (e.g., cytoplasm or nucleus of tumor cells) that are not included in the CPS algorithm. It is recommended that pathologists use their best clinical judgment to determine whether specific nonscorable staining present in the PD-L1 slide interferes with their evaluation of specific scorable PD-L1 expression to such an extent that a confident CPS score cannot be rendered. In cases where interference from specific nonscorable staining prevents confidence in scoring, the PD-L1 slide is considered non-evaluable. Note that even cases with high (> 1+ intensity) specific staining of nonscorable tissue components in the PD-L1 slide are considered evaluable if the pathologist can still provide a confident CPS score from the tissue.

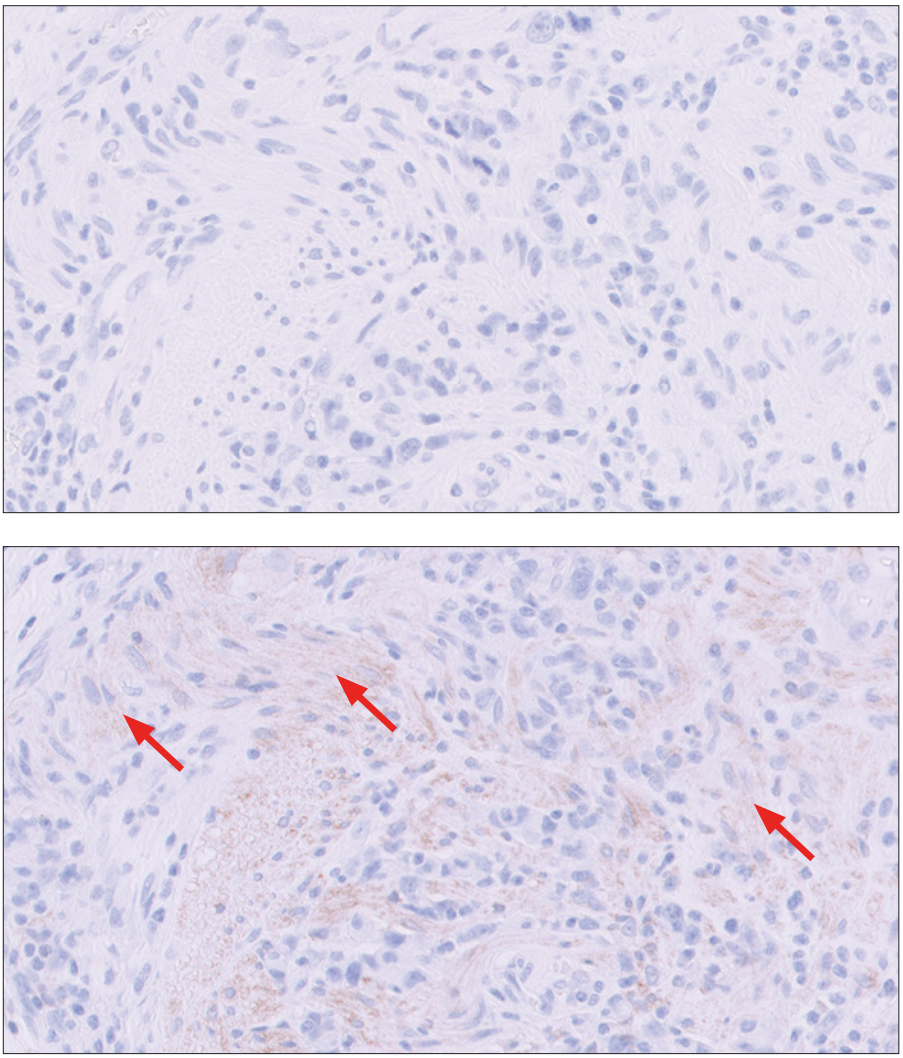


Figure 38a: Gastric or GEJ adenocarcinoma specimen exhibiting ≤1+ intensity specific nonscorable cytoplasmic staining of peripheral nerve cells (arrows). Absence of similar staining in the NCR-stained slide (top) indicates that this cytoplasmic staining in the PD-L1 slide (bottom) is specific, but normal cells such as peripheral nerve cells should be excluded from scoring (20x magnification).

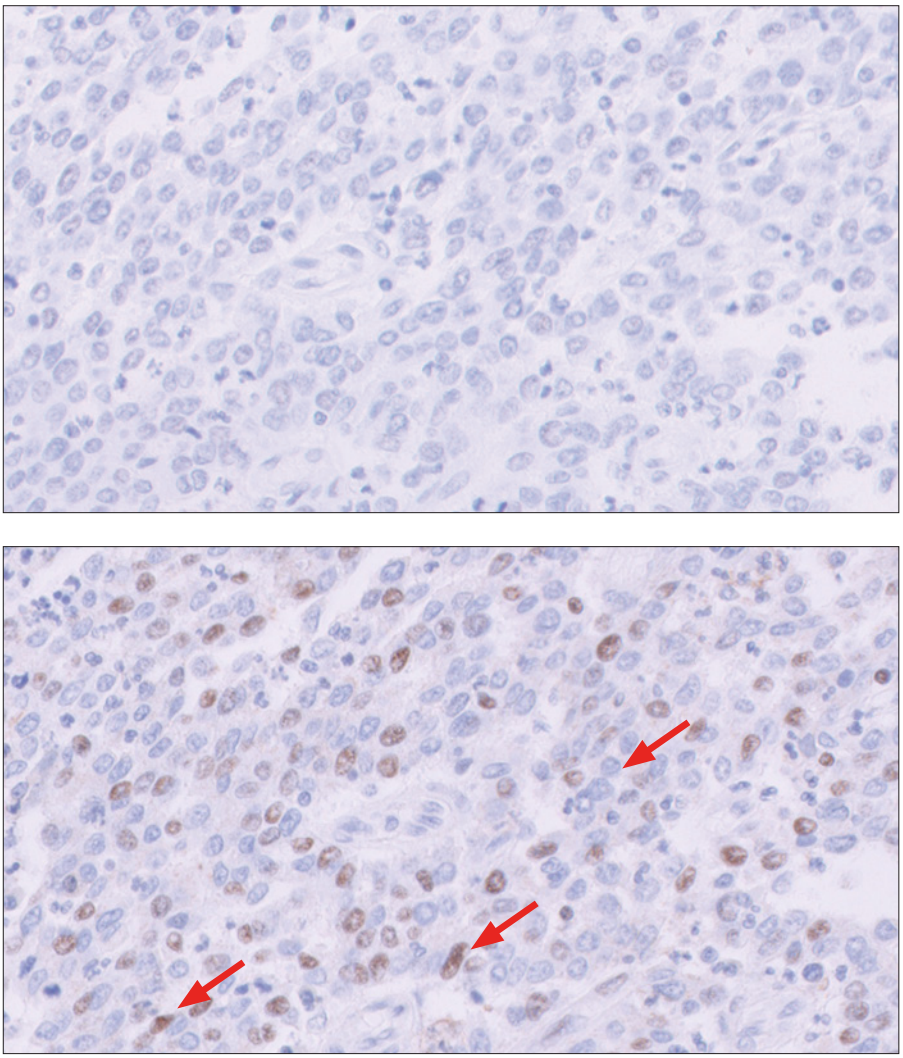


Figure 38b: Gastric or GEJ adenocarcinoma specimen with nuclear staining of tumor cells absent from the NCR-stained slide (top) and present in the PD-L1 stained slide (arrows)(bottom), indicating that this nuclear staining is specific staining. Since the nucleus is a cell compartment that is not scorable, and the nuclear reactivity does not interfere with evaluation of membrane staining on tumor cells, the PD-L1 slide should be considered evaluable (20x magnification).

Nonspecific Staining

Nonspecific staining is defined as chromogen-related staining and is not related to the anti-PD-L1 antibody/PD-L1 antigen interaction that can be visible either on both the NCR and PD-L1 stained slides, or only on the NCR slide. It is caused by several factors. These factors include, but are not limited to:

- Pre-analytic fixation and processing of the specimen, including use of fixatives other than neutral buffered formalin (not recommended)
- Incomplete removal of paraffin from the section
- Incomplete rinsing of reagents from slides during staining
- Drying of slides; ensure slides remain wet with buffer while loading onto Autostainer Link 48, prior to initiating run and during the staining procedure
- Cross-reactivity of the secondary antibody in the detection system
- Reagent trapping (tissue folding; tissue drying; hydrophobic or ionic interactions with 'sticky' tissues or substances such as cartilage, muscle fibers, dense fibrosis, mucin, necrotic debris)

The nonspecific staining of the NCR-stained test section is useful in determining the level of nonspecific staining in the PD-L1 stained test section.

All specimens must have ≤ 1+ nonspecific staining to be considered acceptable for PD-L1 expression evaluation.

When staining of cell nuclei is present in both the specimen slide stained with the PD-L1 primary antibody as well as in the NCR-stained slide, or only in the NCR-stained slide, it is considered nonspecific nuclear staining and, if present, should be ≤ 1+ intensity within the scorable tumor region(s) of both the PD-L1 and NCR-stained slides and excluded from scoring. If the intensity of the nonspecific nuclear staining within scorable tumor region(s) is > 1+ in the NCR-stained slide, or both the NCR- and PD-L1 stained slide, the PD-L1 slide should be marked as non-evaluable and a retest of the specimen should be performed.

Nonspecific staining with PD-L1 IHC 22C3 pharmDx is rare.

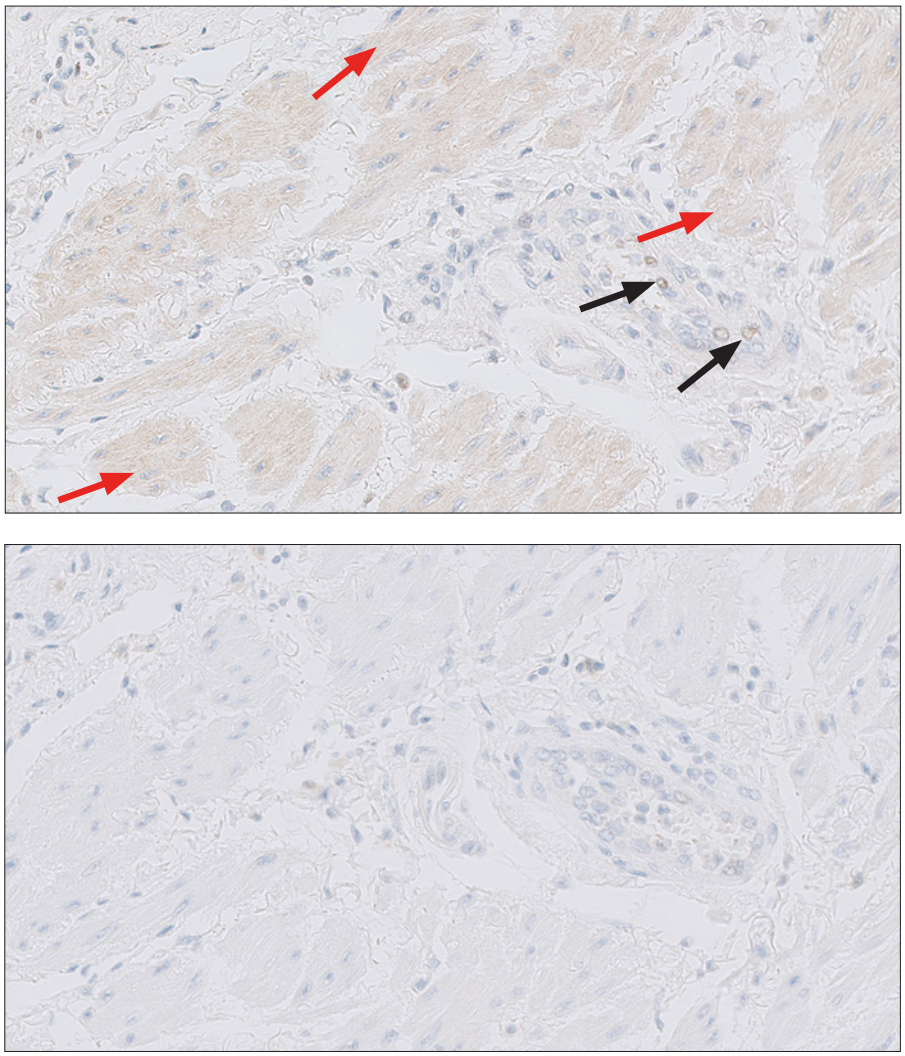


Figure 39a: Esophageal adenocarcinoma specimen stained with NCR (top) exhibiting acceptable (≤1+ intensity) nonspecific staining on smooth muscle cells (red arrows) and occasional endothelial cell nuclei (black arrows). Note that in this case, the nonspecific staining is not also present in the PD-L1 slide (bottom) (20x magnification).

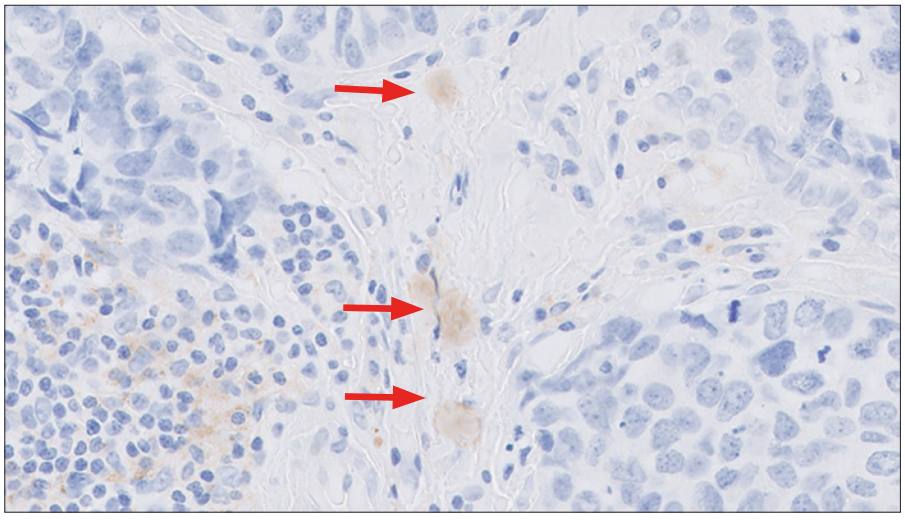


Figure 39b Negative Control Reagent (NCR) exhibiting nonspecific staining in TNBC (arrows) (20× magnification).

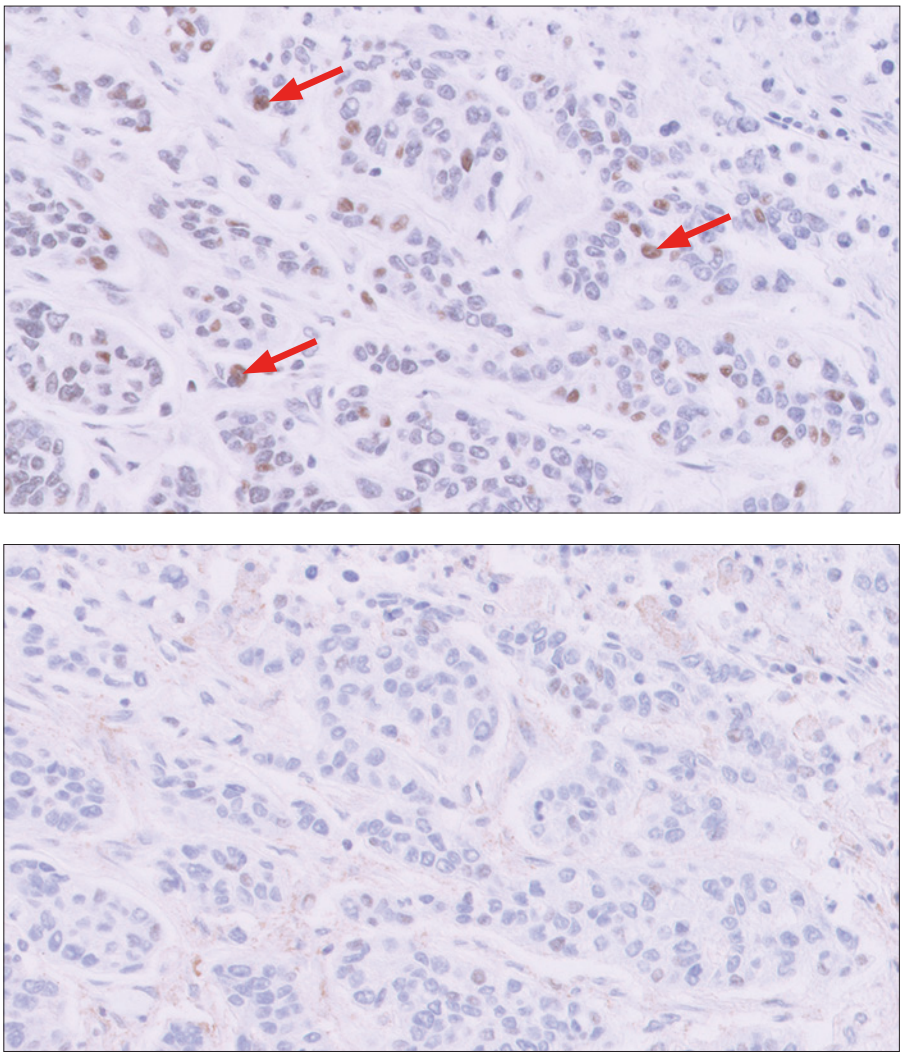


Figure 39c: Gastric or GEJ adenocarcinoma specimen exhibiting nonspecific nuclear staining (arrows) that is present in both the NCR-(top) and PD-L1 (bottom) stained slides, but is stronger (≥1+) intensity in the NCR slide. As the acceptance criteria for nonspecific staining, including nuclear staining, in the NCR and PD-L1 slides or NCR slide alone is ≤1+ intensity, the PD-L1 stained slide in this case should be considered non-evaluable (20x magnification).

Key Point

All specimens must have ≤ 1+ nonspecific staining, including nuclear staining

Edge Artifact

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

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

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

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

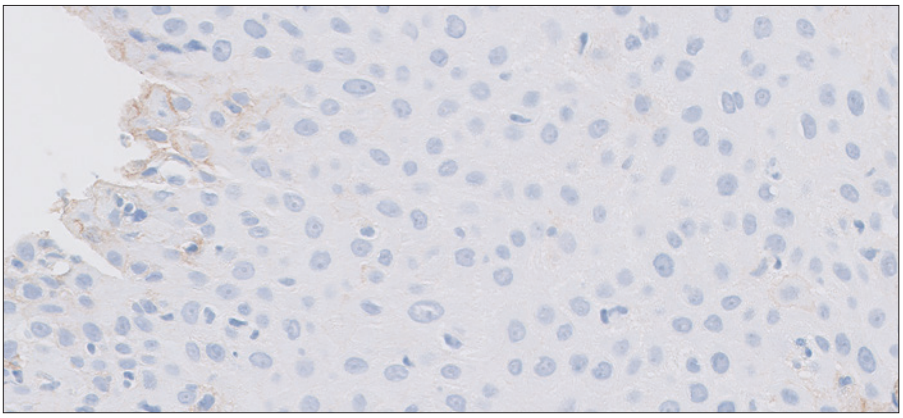


Figure 40: Edge staining should be excluded from the score (20× magnification).
Note: Squamous cell carcinoma from the cervix is depicted.

Key Point

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

Crush Artifact

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

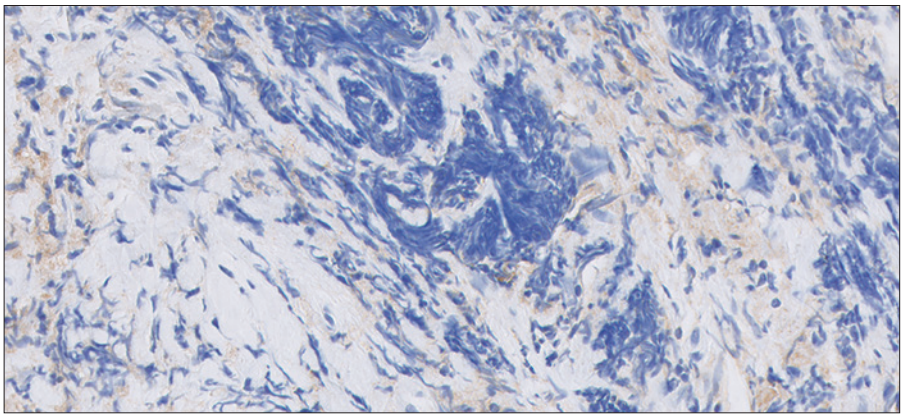


Figure 41: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting crush artifact; crush artifact should be excluded from the score (20× magnification).

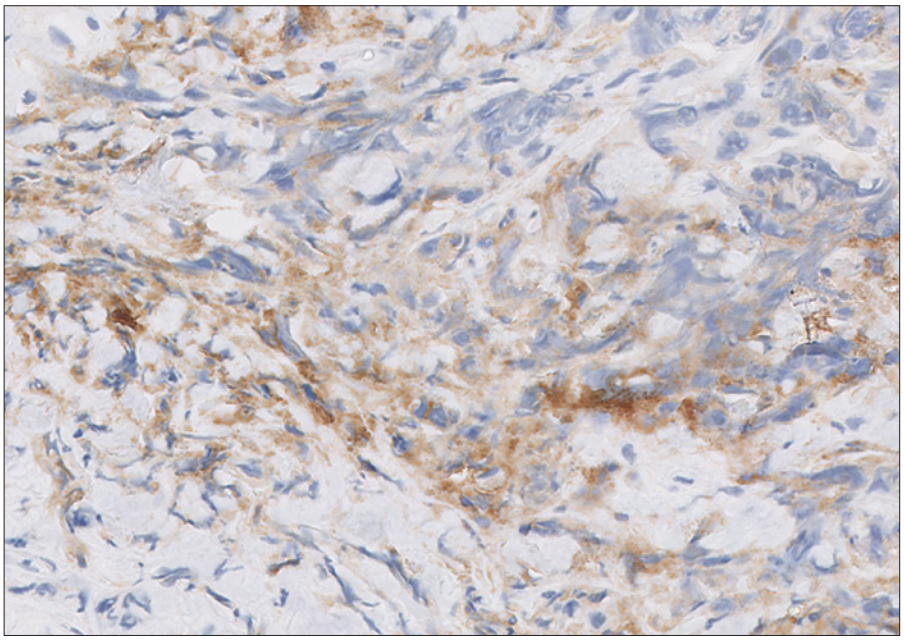


Figure 42: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting crush artifact; crush artifact should be excluded from the score (20× magnification).

Key Point

Scoring of crush artifact should be avoided

Poor Fixation

Standardization of fixation is very important when using PD-L1 IHC 22C3 pharmDx. Suboptimal fixation of tissues may give erroneous results.

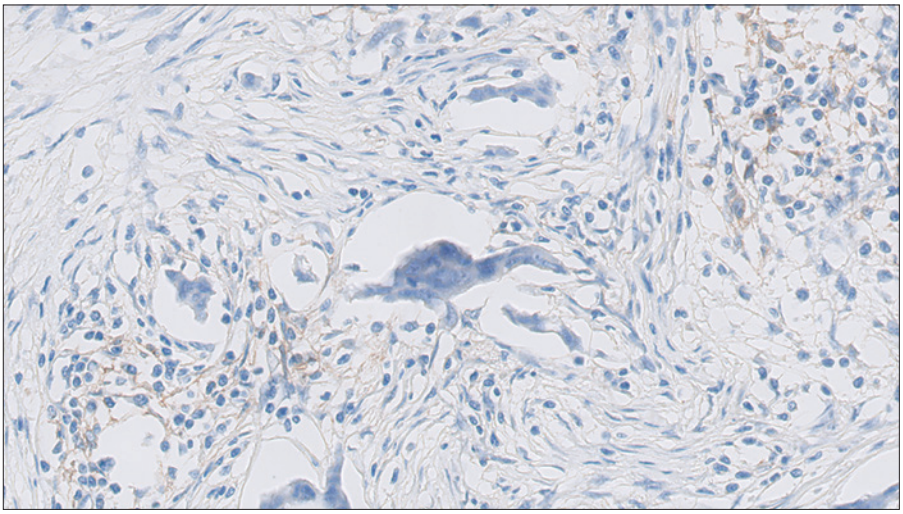


Figure 43: TNBC specimen exhibiting poor tissue fixation (20× magnification).

Key Point

Proper fixation is important for accurate PD-L1 assessment

Necrosis

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

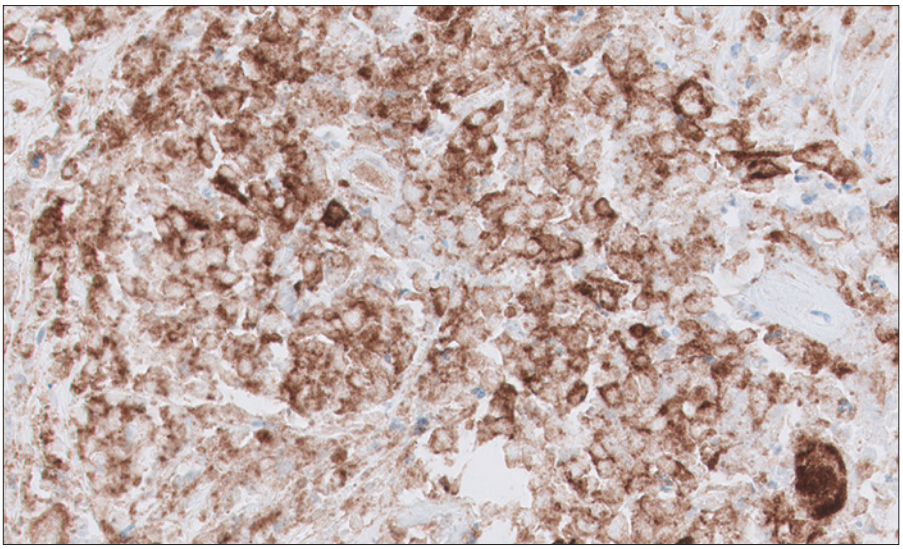


Figure 44: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting staining of necrosis; necrosis staining should be excluded from the score (20× magnification).

Key Point

Scoring of necrotic areas should be excluded from the CPS calculation

Hemosiderin

Hemosiderin pigment may be present in TNBC tissue and will often exhibit a golden-brown color which may be confused with DAB staining. This pigment should be ignored and excluded from the score. Utilization of the NCR slide can be useful to help distinguish hemosiderin pigment from DAB staining.

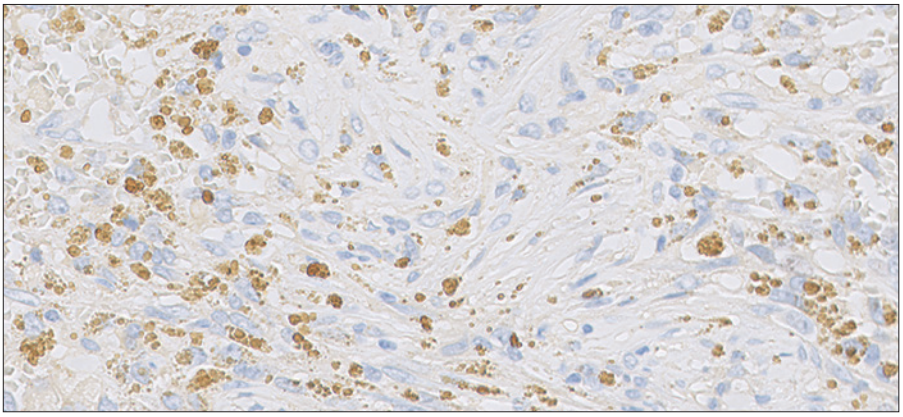


Figure 45: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting golden-brown hemosiderin pigment (20× magnification).

Key Point

Hemosiderin pigment should be excluded from the CPS calculation

PD-L1 IHC 22C3 pharmDx CPS Case Examples

CPS < 10 Case Examples

Case 1: CPS < 10

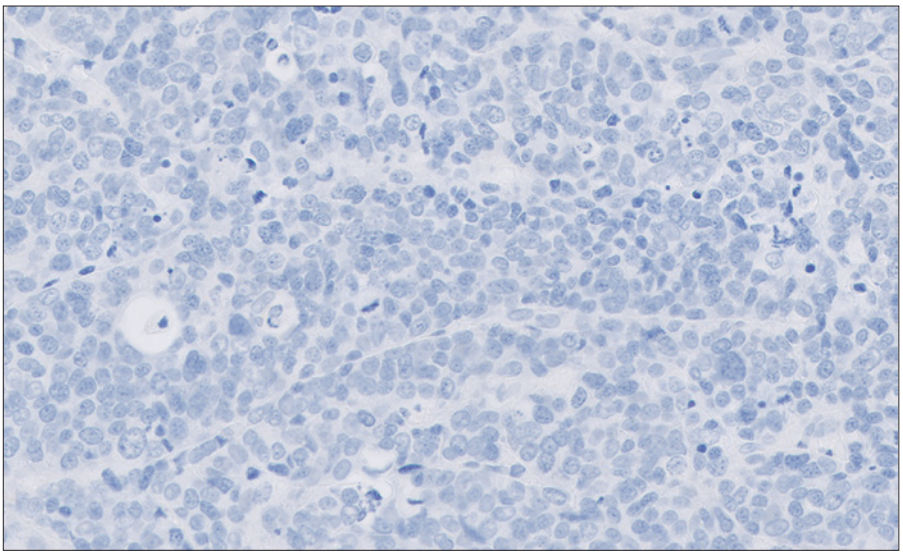


Figure 46: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 0 (20× magnification).

Case 2: CPS < 10

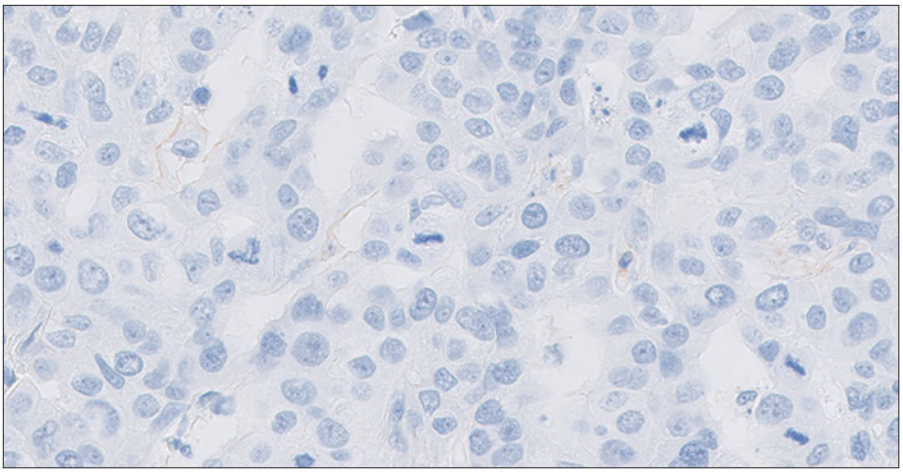


Figure 47: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 0 (20× magnification).

Case 3: CPS < 10

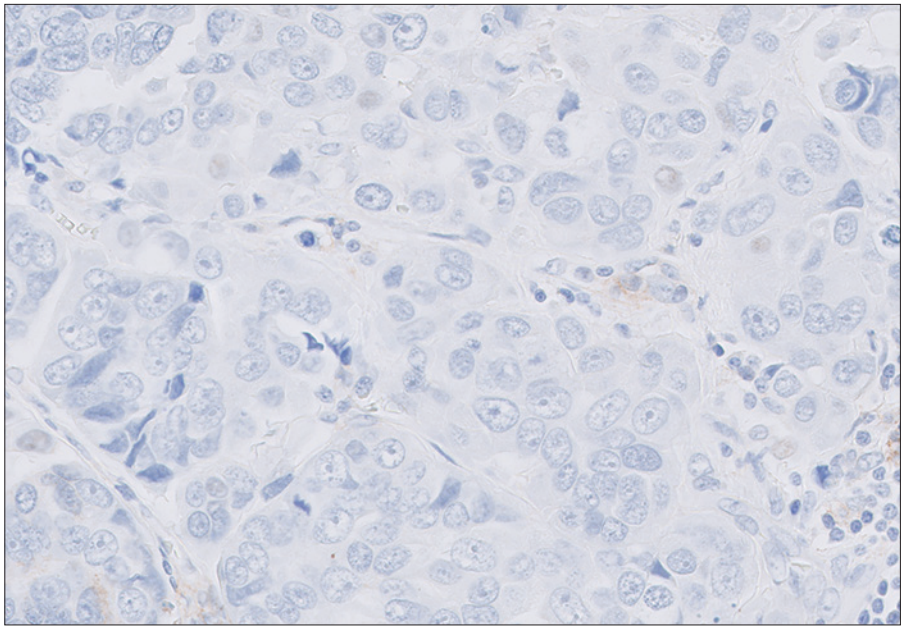


Figure 48: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 1, however any numerical CPS between 1–2 could be assigned to this image (20× magnification).

Case 4: CPS < 10

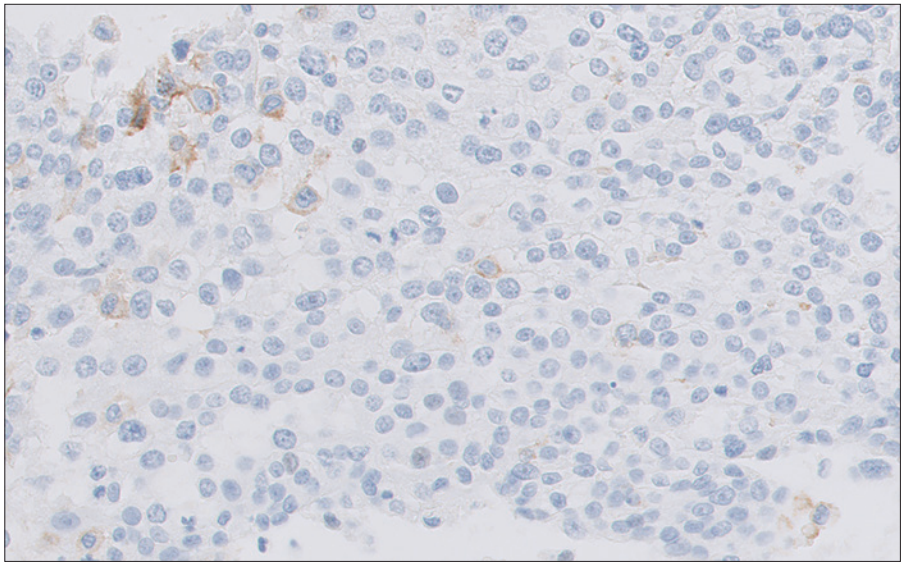


Figure 49: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 2, however any numerical CPS between 1–3 could be assigned to this image (20× magnification).

Case 5: CPS < 10

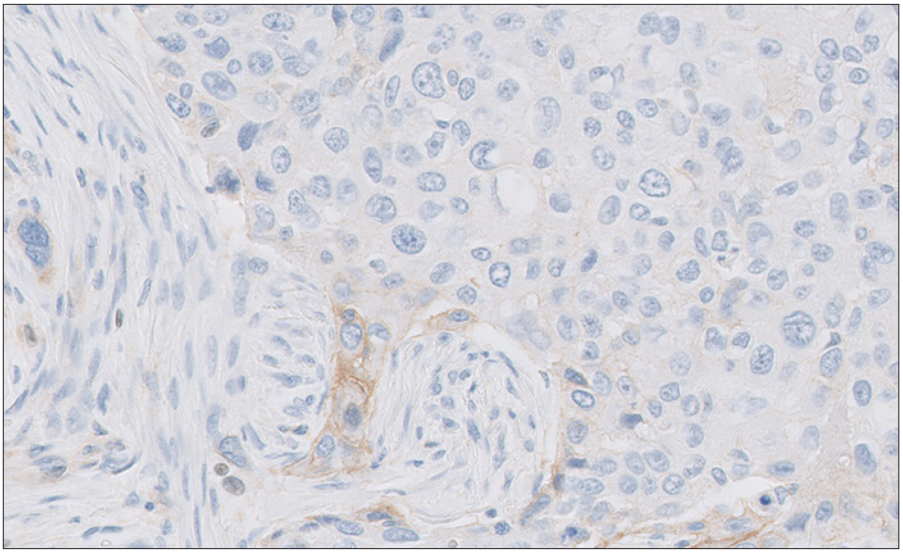


Figure 50: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 3, however any numerical CPS between 2–4 could be assigned to this image (20× magnification).

Near Cutoff Case Examples (CPS Range of 5–15)

Case 1: Near Cutoff (CPS Range of 5–15)

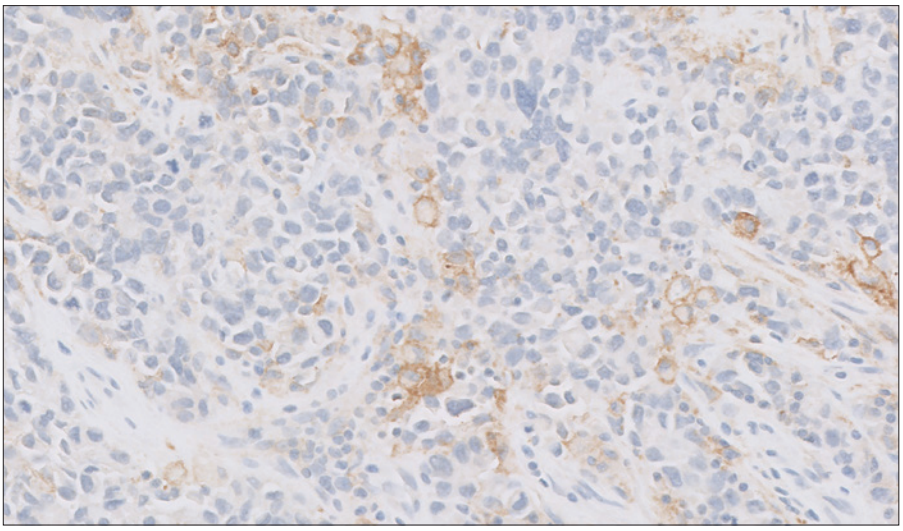


Figure 51: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 5, however any numerical CPS between 3–7 could be assigned to this image (20× magnification).

Case 2: Near Cutoff (CPS Range of 5–15)

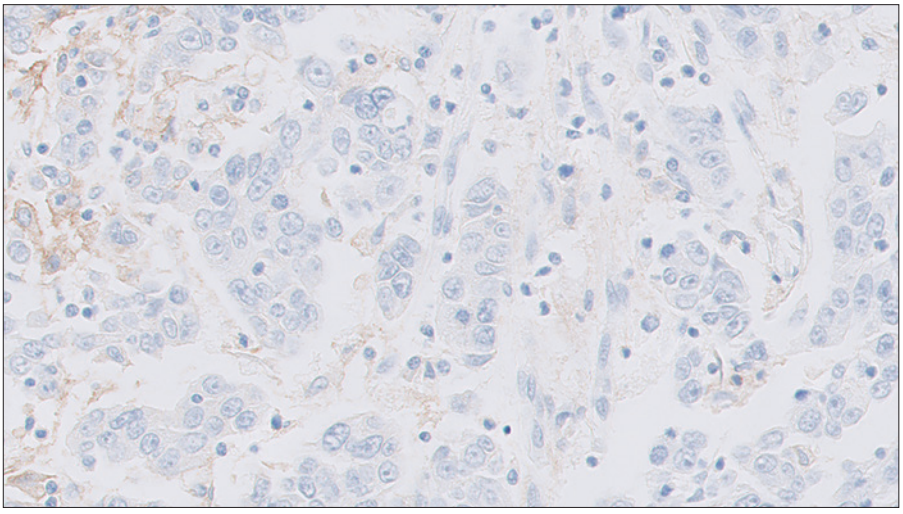


Figure 52: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 5, however any numerical CPS between 3–7 could be assigned to this image (20× magnification).

Case 3: Near Cutoff (CPS Range of 5–15)

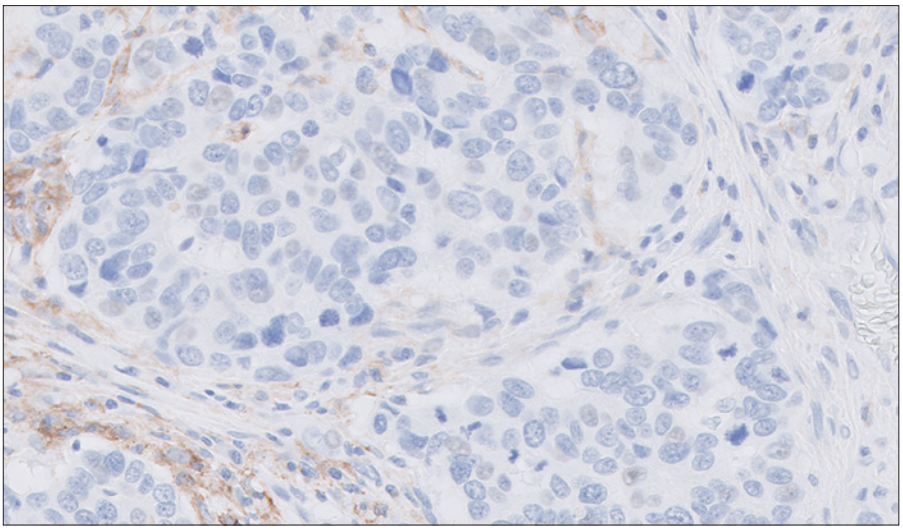


Figure 53: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 6, however any numerical CPS between 4–8 could be assigned to this image (20× magnification).

Case 4: Near Cutoff (CPS Range of 5–15)

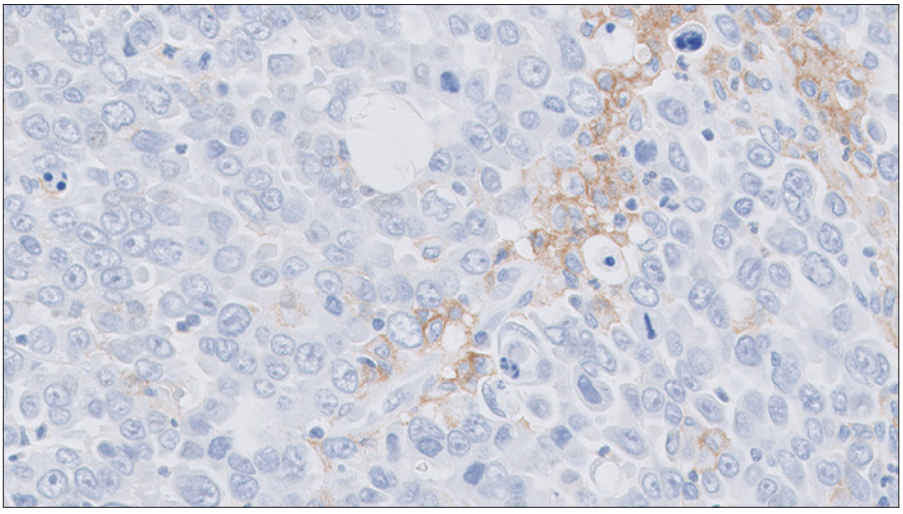


Figure 54: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 6, however any numerical CPS between 4–8 could be assigned to this image (20× magnification).

Case 5: Near Cutoff (CPS Range of 5–15)

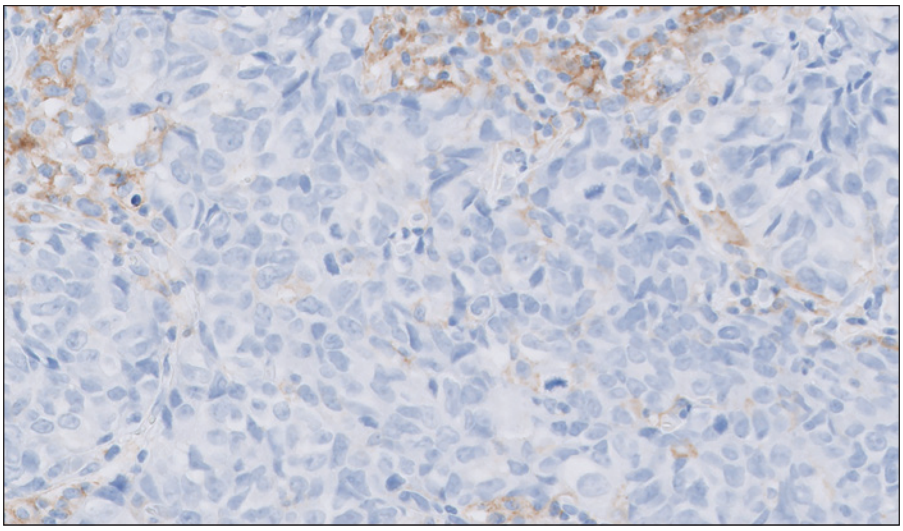


Figure 55: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).

Case 6: Near Cutoff (CPS Range of 5–15)

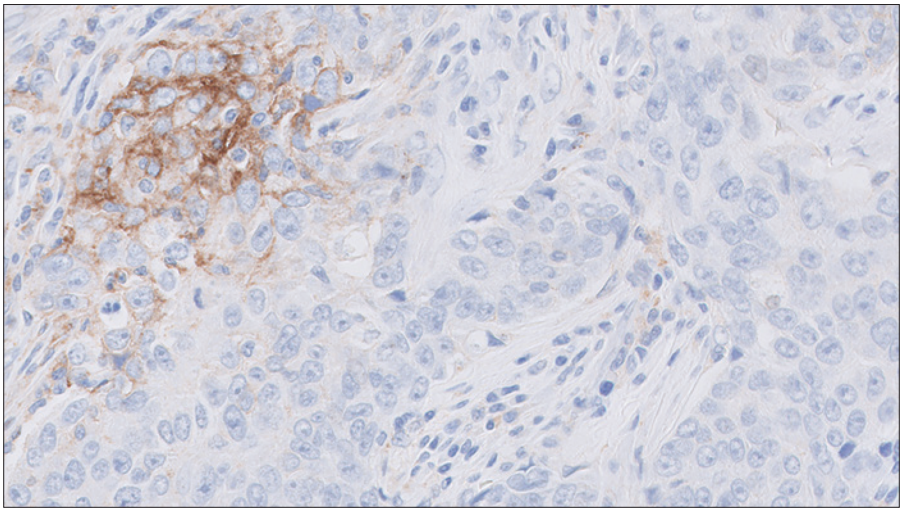


Figure 56: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 8, however any numerical CPS between 6–9 could be assigned to this image (20× magnification).

Case 7: Near Cutoff (CPS Range of 5–15)

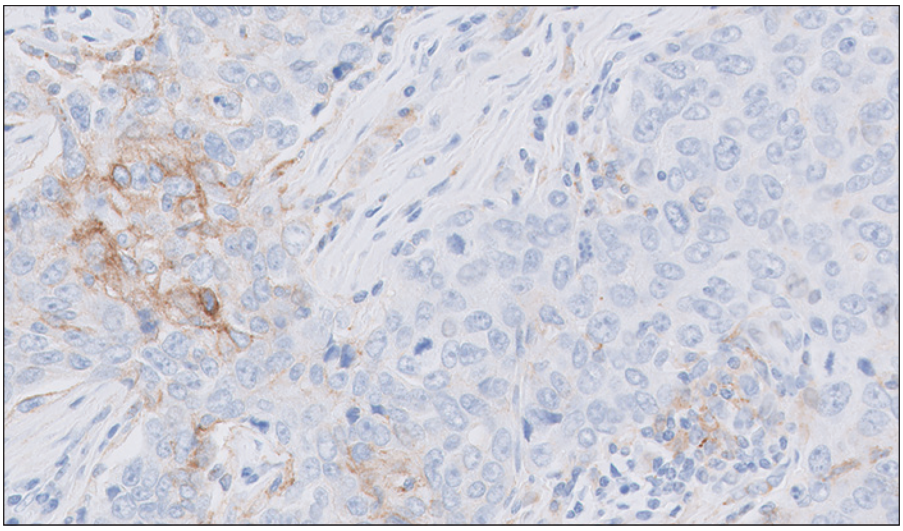


Figure 57: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 8, however any numerical CPS between 6–9 could be assigned to this image (20× magnification).

Case 8: Near Cutoff (CPS Range of 5–15)

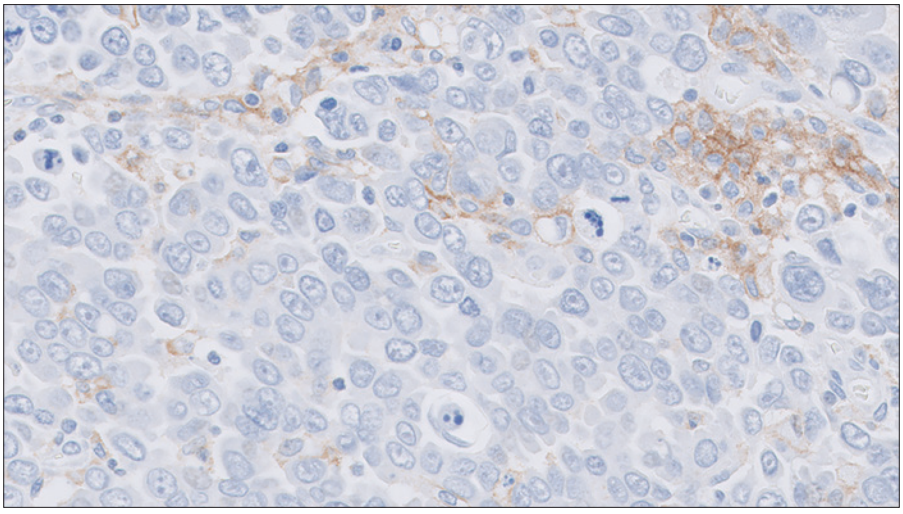


Figure 58: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 8, however any numerical CPS between 6–9 could be assigned to this image (20× magnification).

Case 9: Near Cutoff (CPS Range of 5–15)

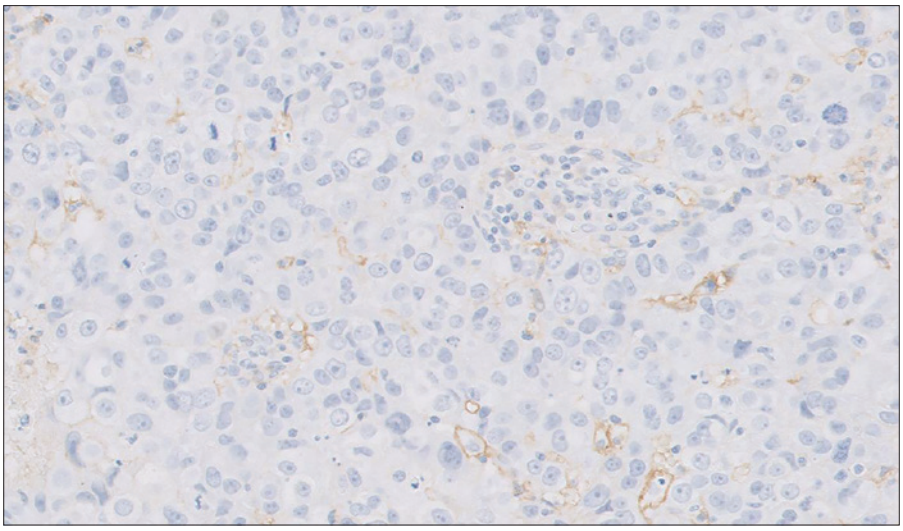


Figure 59: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 11, however any numerical CPS between 10–13 could be assigned to this image (20× magnification).

Case 10: Near Cutoff (CPS Range of 5–15)

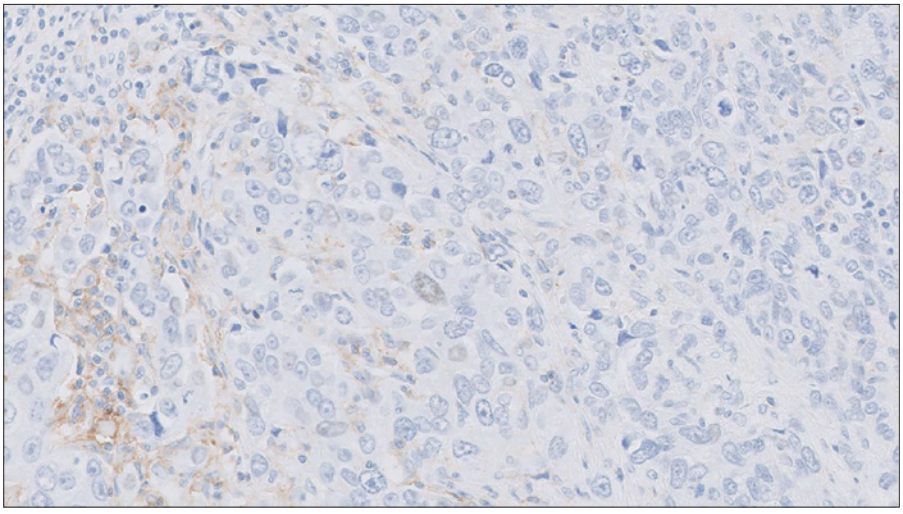


Figure 60: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 12, however any numerical CPS between 10–14 could be assigned to this image (20× magnification).

Case 11: Near Cutoff (CPS Range of 5–15)

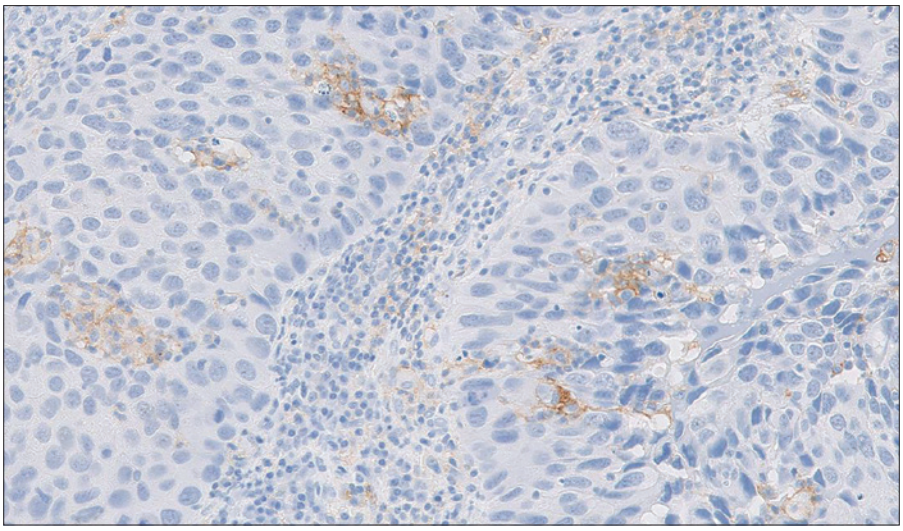


Figure 61: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 15, however any numerical CPS between 13–17 could be assigned to this image (20× magnification).

CPS ≥ 10 Case Examples

Case 1: CPS ≥ 10

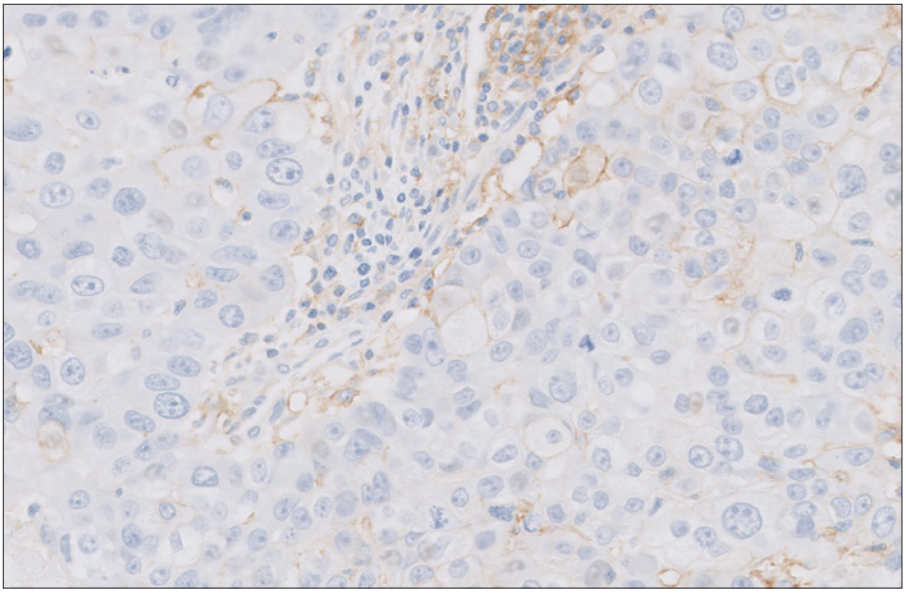


Figure 62: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 16, however any numerical CPS between 14–18 could be assigned to this image (20× magnification).

Case 2: CPS ≥ 10

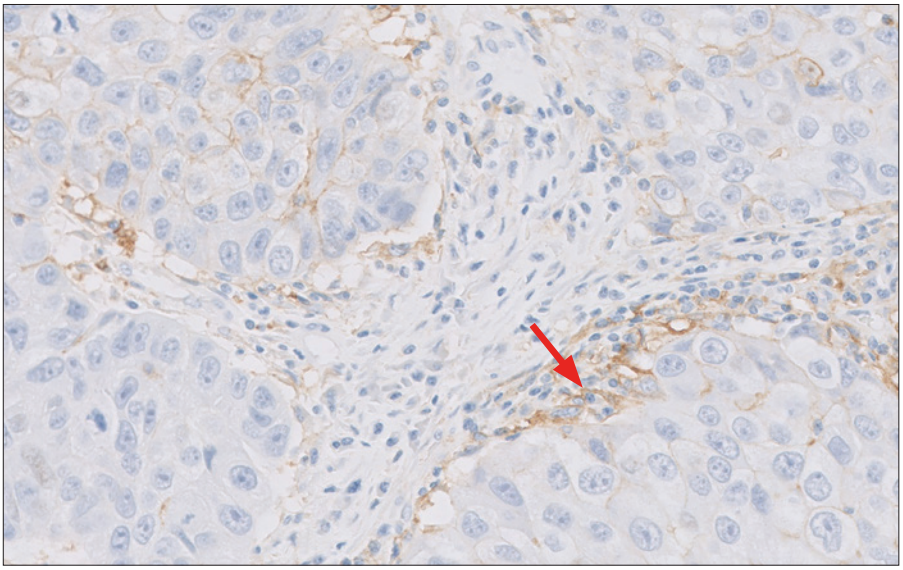


Figure 63: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 30, however any numerical CPS between 27–33 could be assigned to this image (20× magnification).

Note: Ensure that PD-L1 staining plasma cells adjacent to tumor (arrow) are excluded from the numerator of the score.

Case 3: CPS ≥ 10

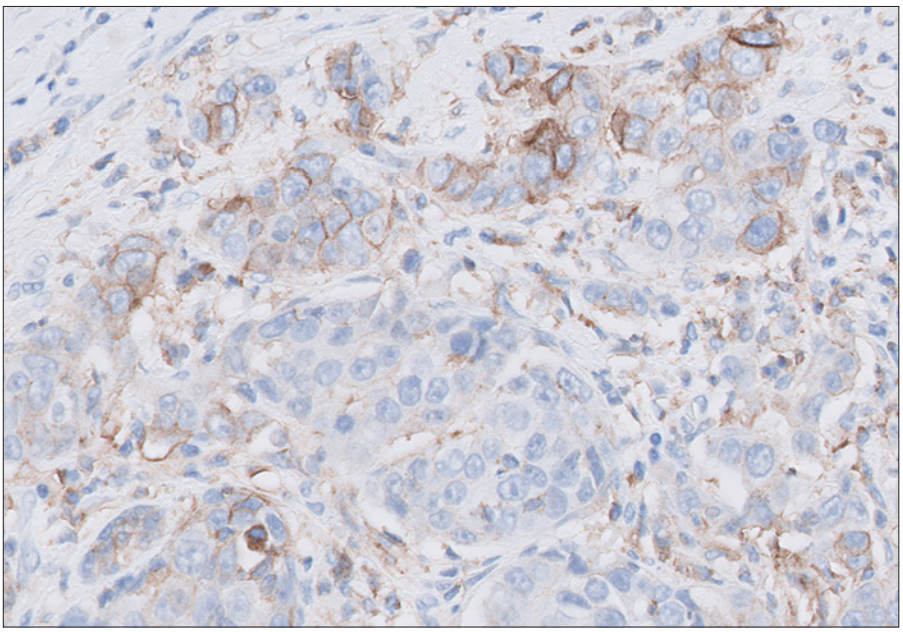


Figure 64: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 30, however any numerical CPS between 27–33 could be assigned to this image (20× magnification).

Case 4: CPS ≥ 10

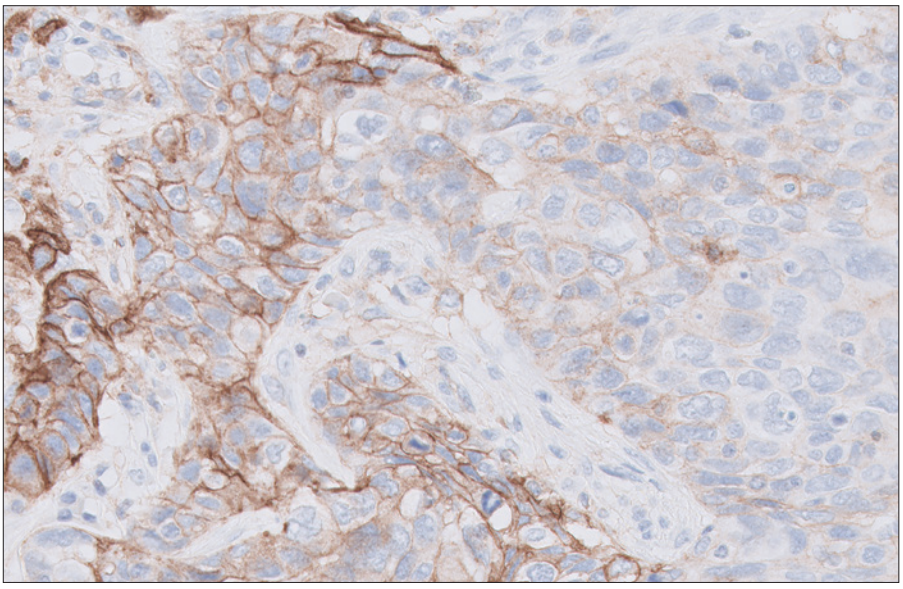


Figure 65: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 60, however any numerical CPS between 57–63 could be assigned to this image (20× magnification).

Case 5: CPS ≥ 10

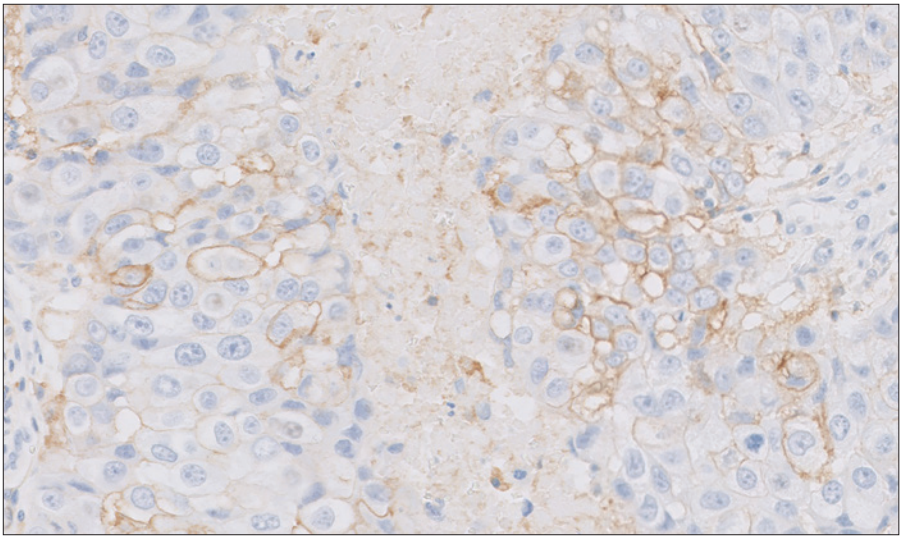


Figure 66: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 75, however any numerical CPS between 72–78 could be assigned to this image (20× magnification).

Case 6: CPS ≥ 10

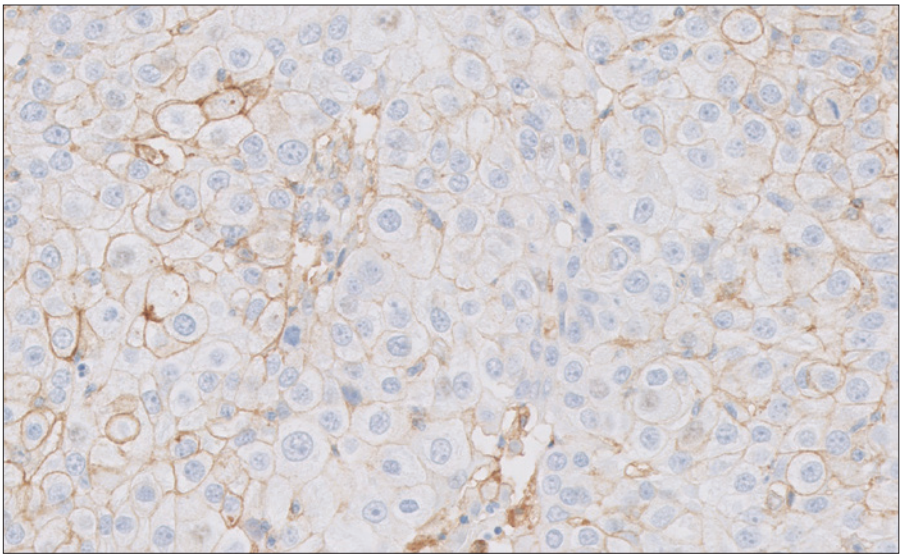


Figure 67: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 85, however any numerical CPS between 82–88 could be assigned to this image (20× magnification).

Case 7: CPS ≥ 10

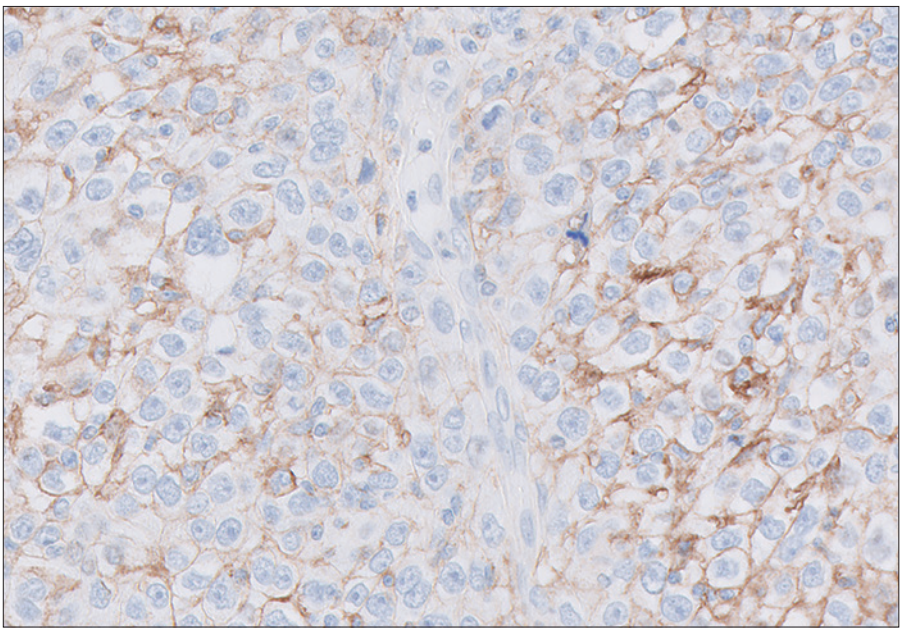


Figure 68: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 94, however any numerical CPS between 91–97 could be assigned to this image (20× magnification).

Case 8: CPS ≥ 10

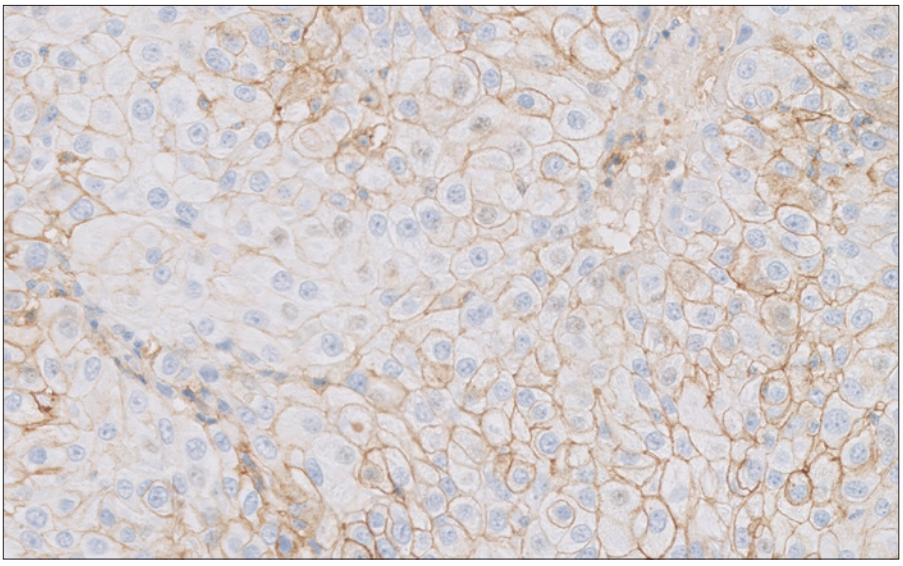


Figure 69: TNBC specimen stained with PD-L1 IHC 22C3 pharmDx primary antibody exhibiting a CPS of 100 (20× magnification).

Control Cell Line (CCL) Appendix

Passing CCL

Passing PD-L1 Negative CCL

- No cells with membrane staining*
- Nonspecific staining < 1+ intensity*

* Note that staining of a few cells in the MCF-7 cell pellet may occasionally be observed. The following acceptance criteria are applicable: the presence of ≤ 10 total cells with distinct cell membrane staining and/or nonspecific staining with ≥ 1+ intensity within the boundaries of the MCF-7 cell pellet are acceptable.

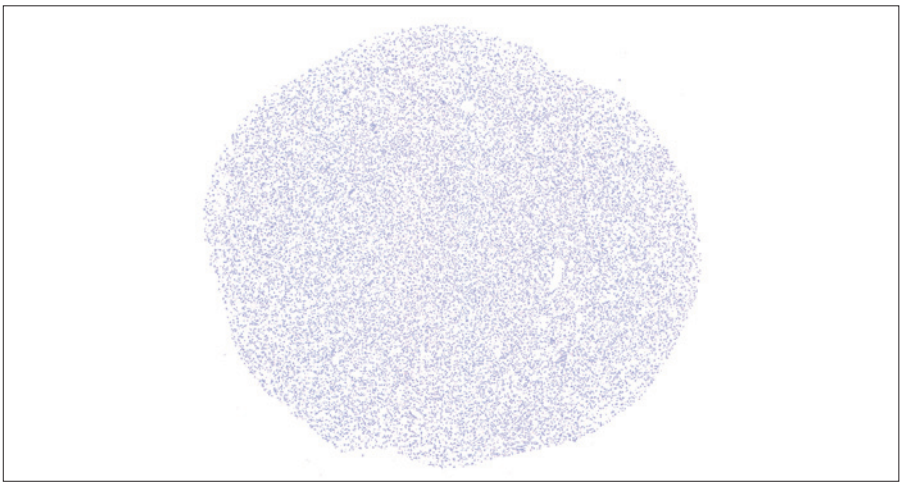


Figure 70: Ideal MCF-7 cell pellet (2× magnification).

Passing PD-L1 Positive CCL

- Cell membrane staining of ≥ 70% of cells
- ≥ 2+ average staining intensity of cells with membrane staining
- Nonspecific staining is < 1+ intensity

Figure 71: Ideal NCI-H226 cell pellet (2× magnification).

Borderline Passing CCL

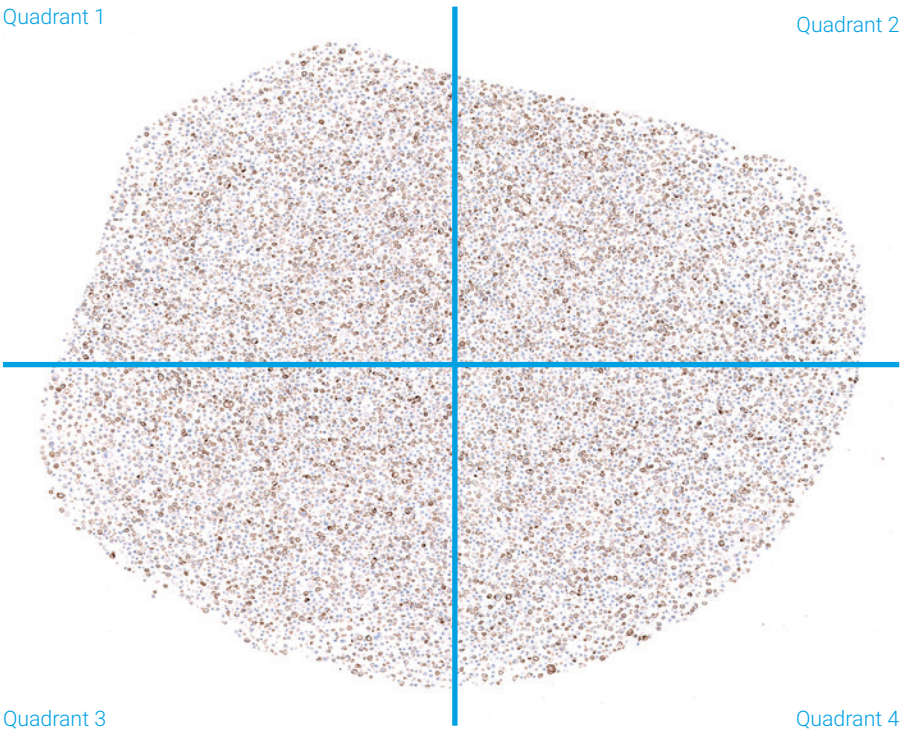
Borderline Passing vs. Passing PD-L1 Positive CCL

Borderline Passing PD-L1 positive CCL

Figure 72: NCI-H226 cell pellet (2× magnification).

Evaluation Strategy for Borderline Passing PD-L1 Positive CCL

For a borderline PD-L1 positive CCL, to determine the total percentage of cells staining in the cell pellet and the average staining intensity of all staining cells in the pellet, the cell pellet can be split into quadrants and inspected at 20× magnification.



Quadrant 1

In Quadrant 1 approximately 70% of cells exhibit membrane staining, and the average staining intensity of all staining cells in this quadrant is ≥ 2+.

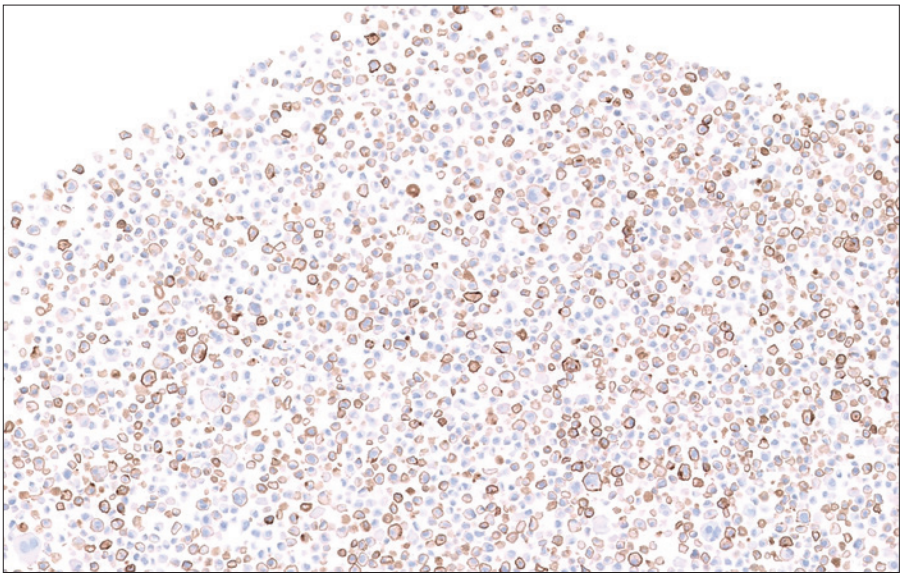


Figure 73: NCI-H226 cell pellet (5× magnification).

Quadrant 2

In Quadrant 2 approximately 75% of cells exhibit membrane staining, and the average staining intensity of all staining cells in this quadrant is ≥ 2+.

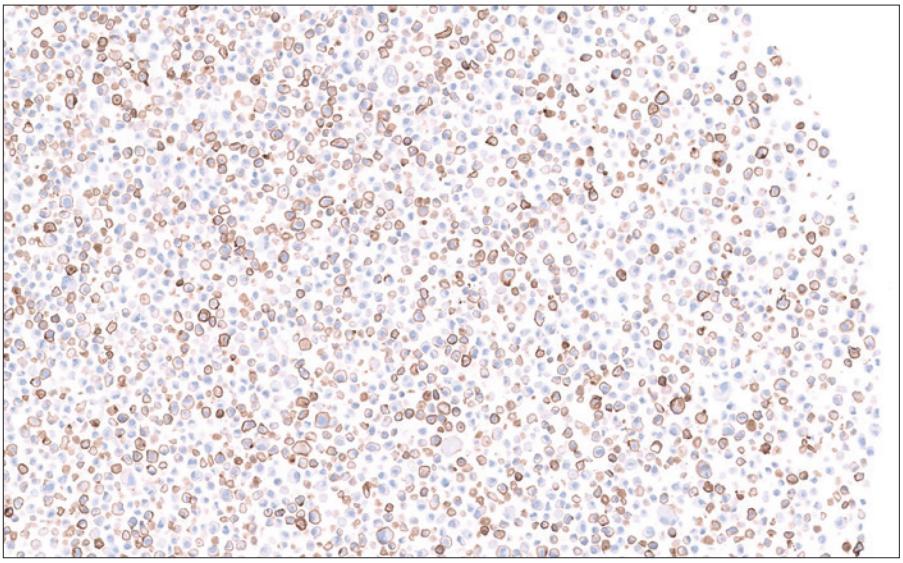


Figure 74: NCI-H226 cell pellet (5× magnification).

Quadrant 3

In Quadrant 3 approximately 70% of cells exhibit membrane staining, and the average staining intensity of all staining cells in this quadrant is ≥ 2+.

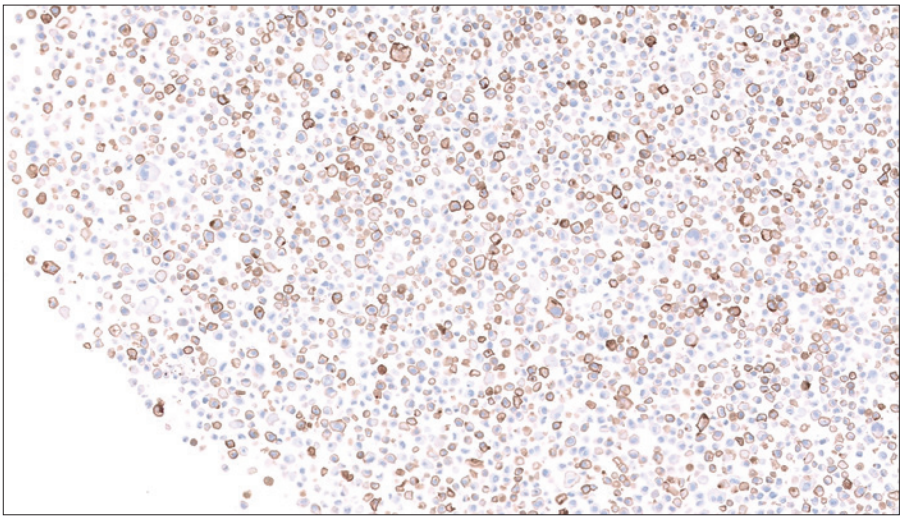


Figure 75: NCI-H226 cell pellet (5× magnification).

Quadrant 4

In Quadrant 4 approximately 65% of cells exhibit membrane staining, and the average staining intensity of all staining cells in this quadrant is ≥ 2+.

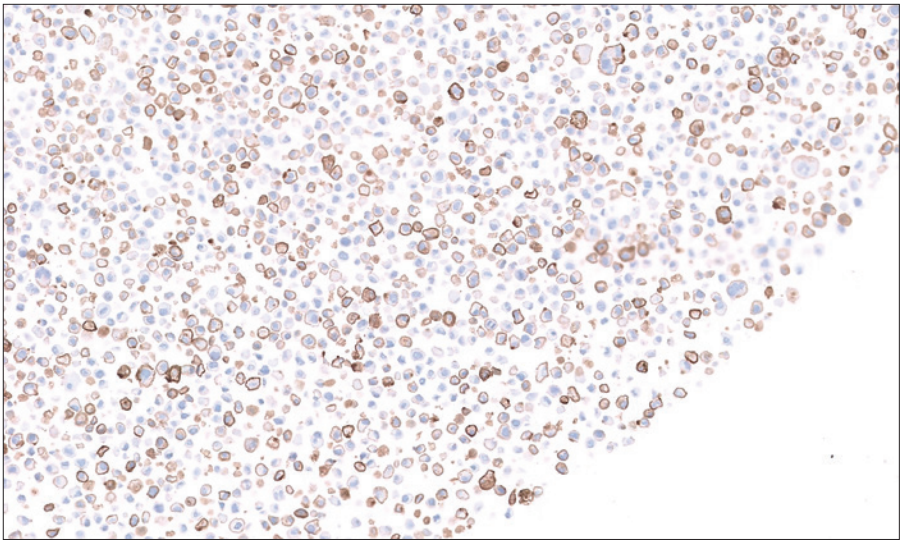
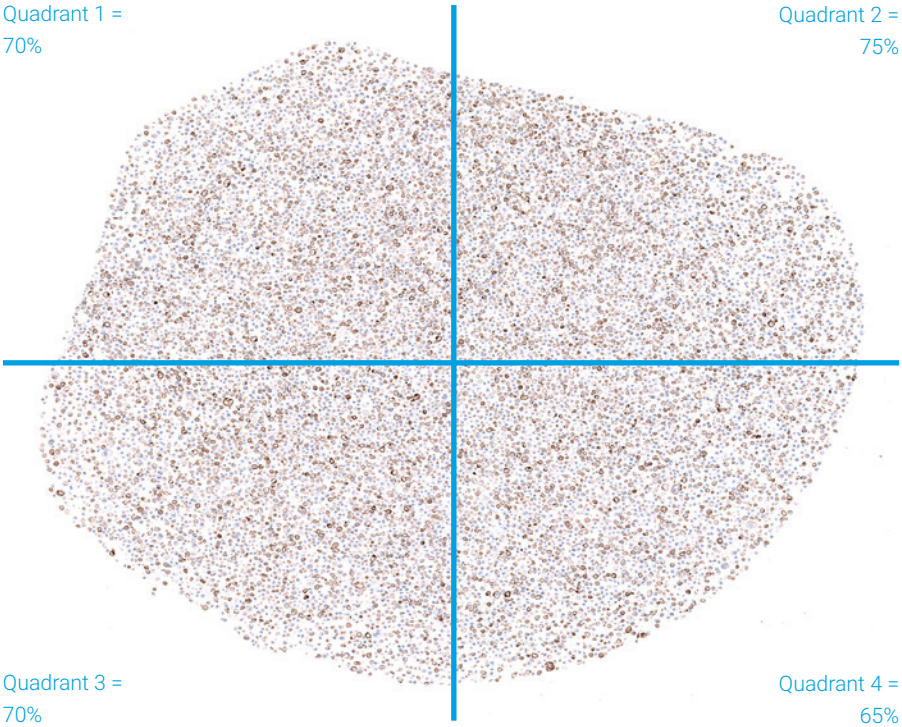


Figure 76: NCI-H226 cell pellet (5× magnification).

Calculation

1. Calculate the average percentage of cells exhibiting membrane staining across all 4 quadrants to estimate the total percentage of cells exhibiting membrane staining across the entire PD-L1 positive CCL pellet
2. Determine whether the average staining intensity across all cells with membrane staining in the pellet is ≥ 2+ intensity



$$\frac{70 + 75 + 70 + 65}{4} = 70$$

- The overall percentage of cells with membrane staining = 70%
- The average staining intensity of all cells with membrane staining in the cell pellet is ≥ 2+

NCI 226 positive control cell pellet meets acceptance criteria.

Failed CCL

Example 1: Passing PD-L1 Negative CCL with Failed PD-L1 Positive CCL

Passing PD-L1 negative CCL

- No cells with membrane staining.*
- Nonspecific staining < 1+ intensity.*

* Note that staining of a few cells in the MCF-7 cell pellet may occasionally be observed. The following acceptance criteria are applicable: the presence of ≤ 10 total cells with distinct cell membrane staining and/or nonspecific staining with ≥ 1+ intensity within the boundaries of the MCF-7 cell pellet are acceptable.

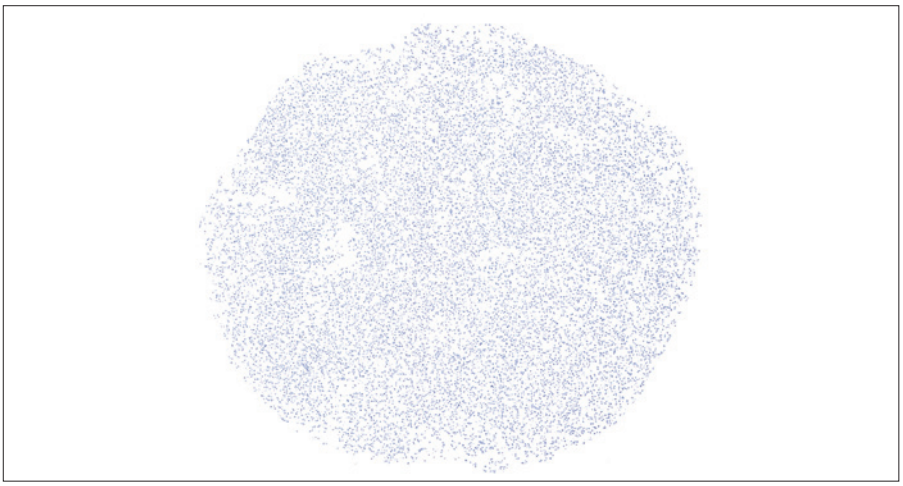


Figure 77: MCF-7 cell pellet (2× magnification).

Failed PD-L1 positive CCL

- Less than 70% of cells exhibit membrane staining, and the average staining intensity across all cells with membrane staining in the pellet is < 2+

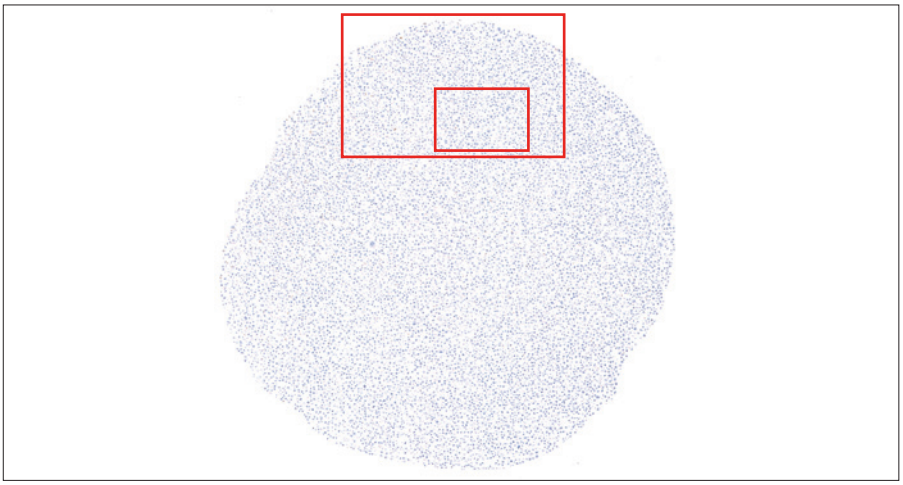


Figure 78: NCI-H226 cell pellet (2× magnification).

See following images for higher magnification images depicting details of failure.

Failed PD-L1 positive CCL (10×)

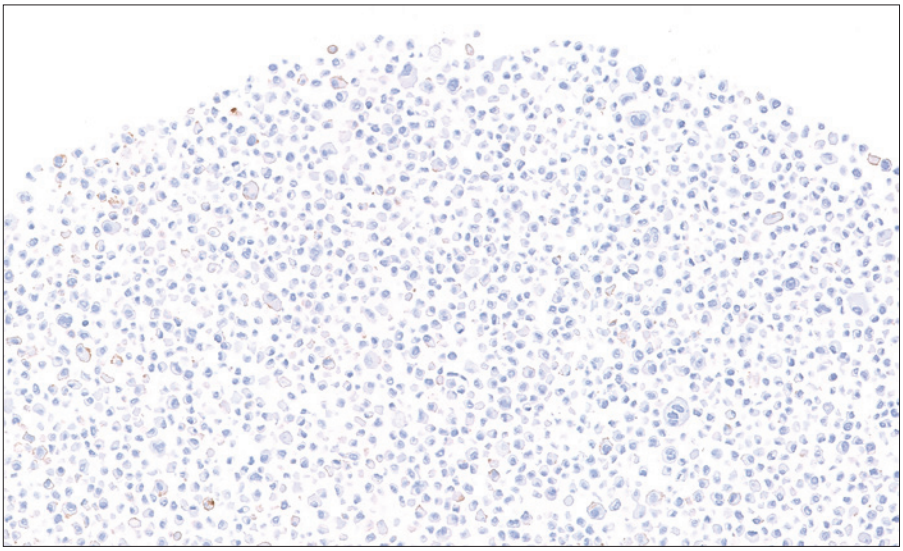


Figure 79: NCI-H226 cell pellet (10× magnification).

Failed PD-L1 positive CCL (20×)

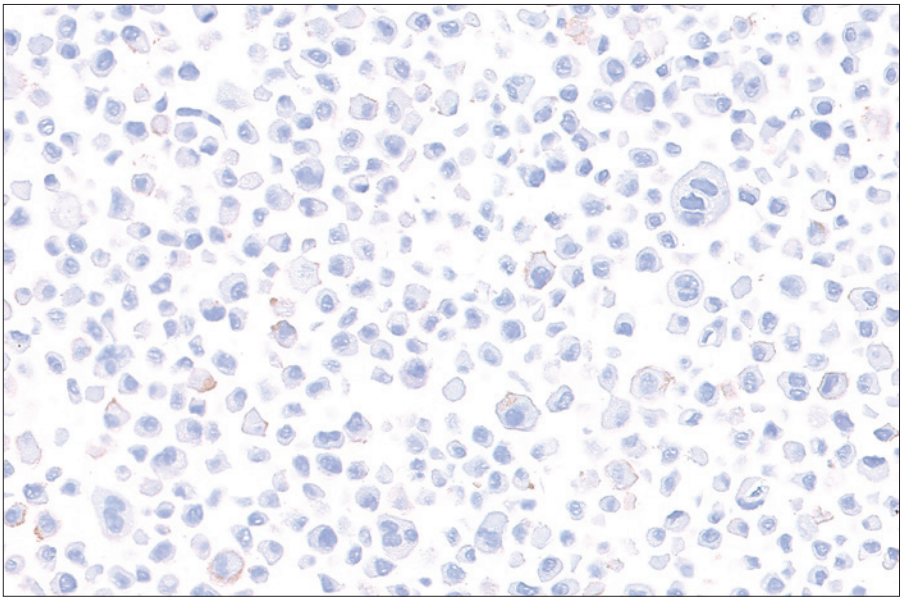


Figure 80: NCI-H226 cell pellet (20× magnification).

Example 2: Passing PD-L1 Negative CCL with Failed PD-L1 Positive CCL

Passing PD-L1 negative CCL

- No cells with membrane staining.*
- Nonspecific staining < 1+ intensity.*

* Note that staining of a few cells in the MCF-7 cell pellet may occasionally be observed. The following acceptance criteria are applicable: the presence of ≤ 10 total cells with distinct cell membrane staining and/or nonspecific staining with ≥ 1+ intensity within the boundaries of the MCF-7 cell pellet are acceptable.

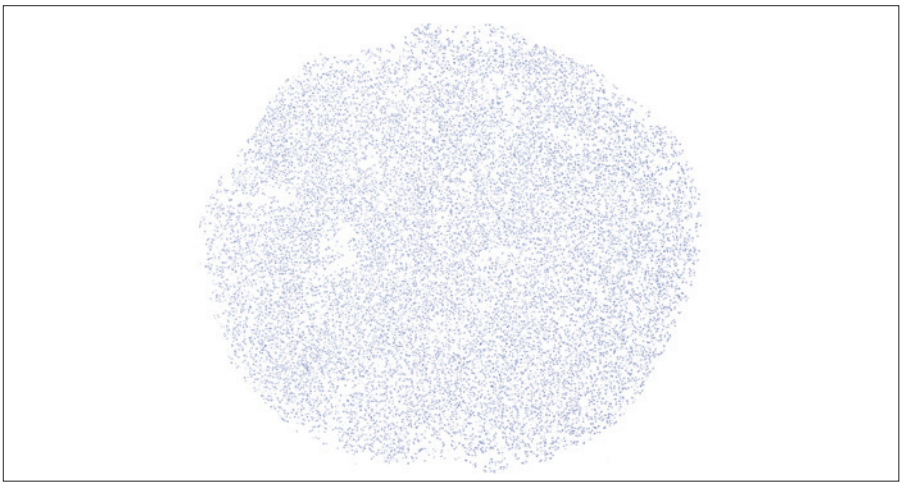


Figure 81: MCF-7 cell pellet (2× magnification).

Failed PD-L1 positive CCL

- Less than 70% of cells exhibit membrane staining, and the average staining intensity across all cells with membrane staining in the pellet is < 2+

Figure 82: NCI-H226 cell pellet (2× magnification).

See following images for higher magnification images depicting details of failure.

Failed PD-L1 positive CCL (10×)

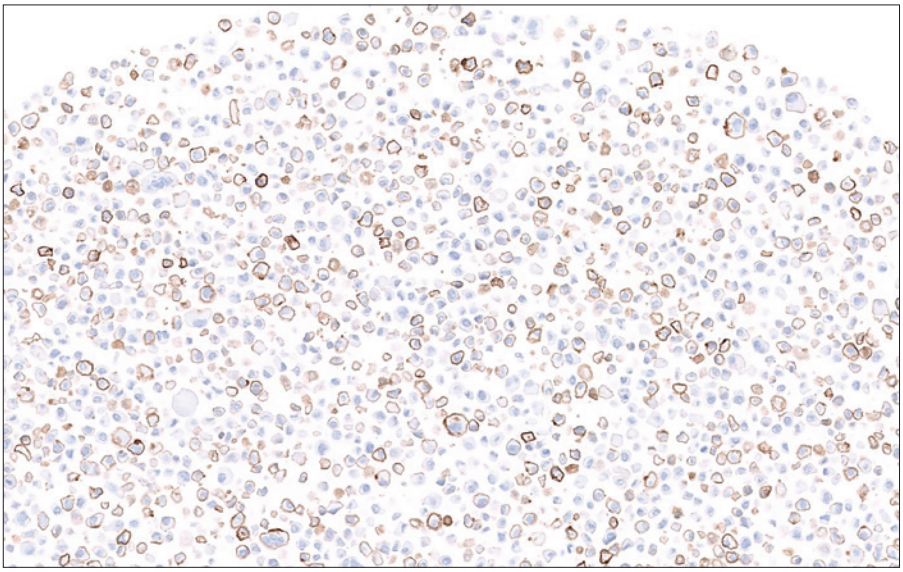


Figure 83: NCI-H226 cell pellet (10× magnification).

Failed PD-L1 positive CCL (20×)

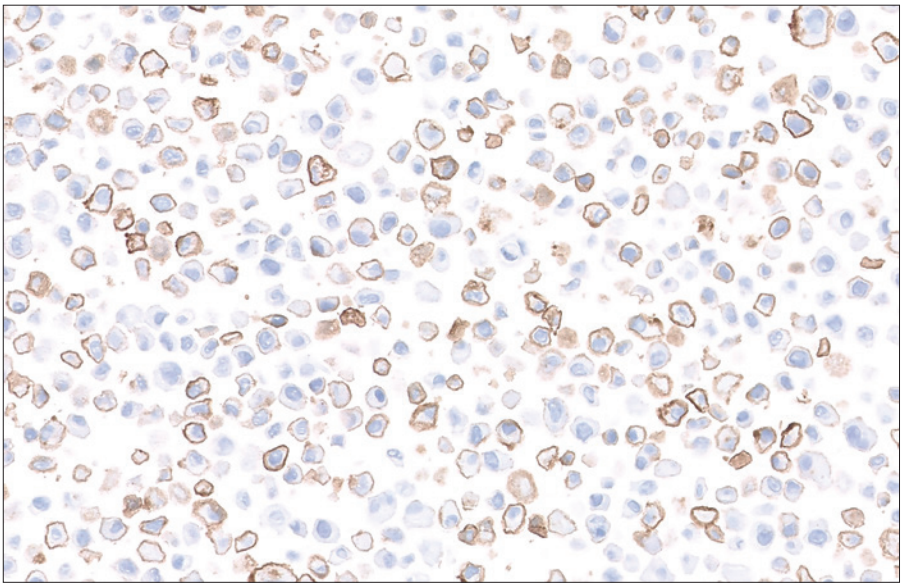


Figure 84: NCI-H226 cell pellet (20× magnification).

Example 3: Passing PD-L1 Positive CCL with Failed PD-L1 Negative CCL

Passing PD-L1 positive CCL

- Cell membrane staining of ≥ 70% of cells
- ≥ 2+ average staining intensity of cells with membrane staining
- Nonspecific staining is < 1+

Figure 85: NCI-H226 cell pellet (2× magnification).

Failed PD-L1 negative CCL

- Nonspecific (nuclear) staining is ≥ 1+ staining intensity
- There are > 10 total cells with distinct cell membrane or nonspecific nuclear staining that is ≥ 1+ intensity

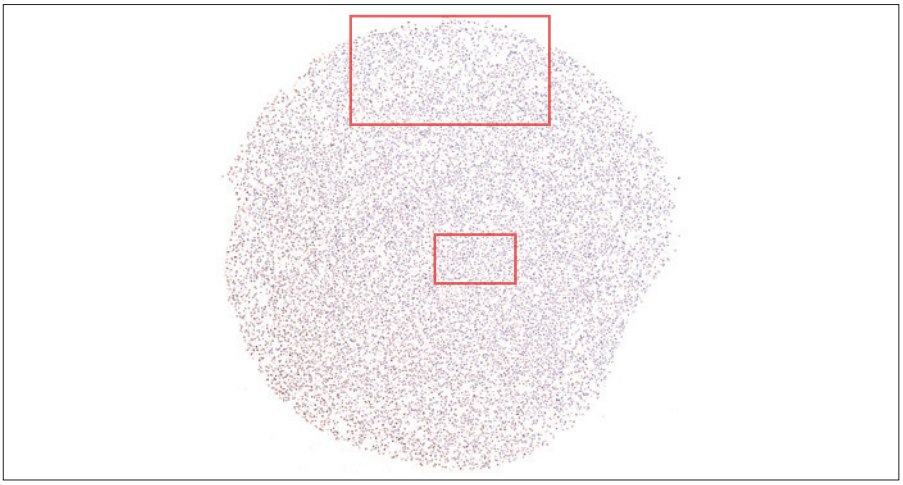


Figure 86: MCF-7 cell pellet (2× magnification).

See following images for higher magnification images depicting details of failure.

Failed PD-L1 negative CCL (10×)

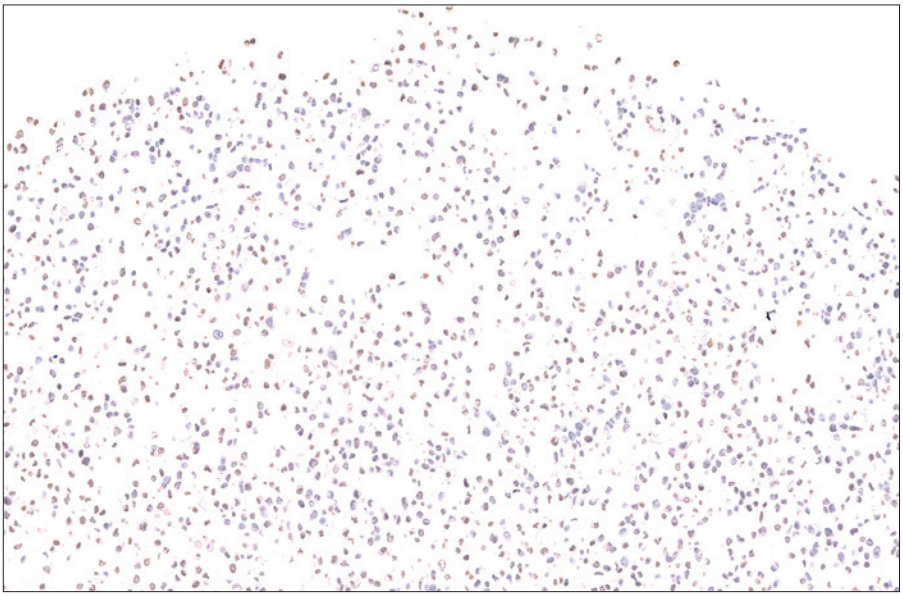


Figure 87: MCF-7 cell pellet (10× magnification).

Failed PD-L1 negative CCL (20×)

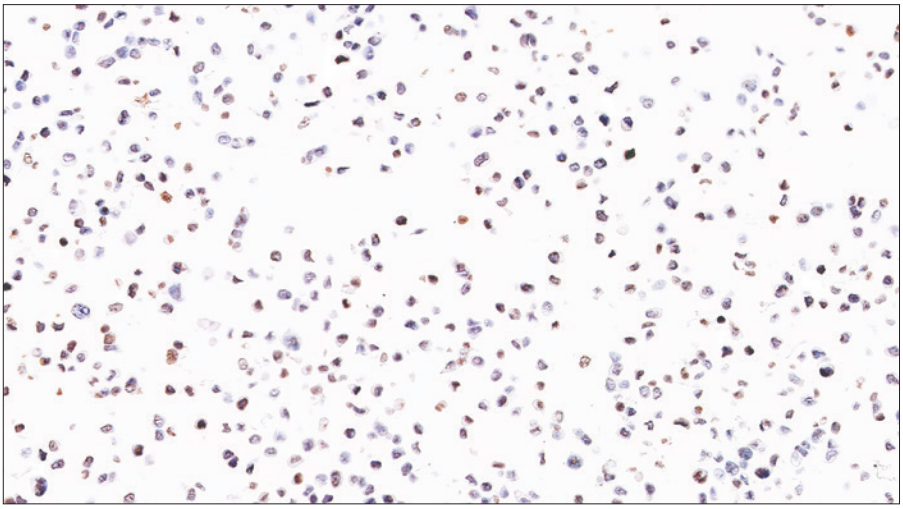


Figure 88: MCF-7 cell pellet (20× magnification).

Troubleshooting Guide

Troubleshooting Guidelines for PD-L1 IHC 22C3 pharmDx

For further troubleshooting help, contact your local Agilent representative.

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

Continued on next page

Problem	Probable Cause	Suggested Action
1× Target Retrieval Solution does not meet pH specifications	pH meter is not calibrated correctly	Ensure pH meter is calibrated per manufacturer's recommendations. After re-calibration, re-test the pH of 1× Target Retrieval Solution. Do not modify the pH of 1× Target Retrieval Solution. If the pH is outside the acceptable range (6.1 ± 0.2), discard 1× Target Retrieval Solution. Prepare new 1× Target Retrieval Solution. Check the pH of the new 1× Target Retrieval Solution
	Inferior quality water is used to dilute the Target Retrieval Solution concentrate	Ensure that distilled or deionized water is used to prepare 1× Target Retrieval Solution
	Incorrect Target Retrieval Solution is used	Ensure that the correct Target Retrieval Solution specified in "Materials Required but not Supplied" and/or "Reagent Preparation" section(s) is used
Nonspecific >1+ nuclear staining on PD-L1 and/or NCR slides	Specimen slides dried prior to initiating the Autostainer Link 48 staining procedure	Ensure slides remain wet with 1x Envision FLEX Wash Buffer after Deparaffinization, Rehydration, and Target Retrieval (3-in-1) procedure and after loading on the Autostainer Link 48. Ensure that the Autostainer Link 48 lid is properly closed to prevent reagent evaporation during the staining procedure.
	Improper manual slide rinsing with 1x EnVision FLEX Wash Buffer before loading on the Autostainer Link 48	Slide racks should be placed one rack at a time on the Autostainer Link 48 and then 1x EnVision FLEX Wash Buffer should be manually applied to the slides using a wash bottle. Ensure slides remain wet prior to initiating the Autostainer Link 48 procedure.

Note: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call Agilent Pathology Support for further assistance. Additional information on staining techniques and specimen preparation can be found in the Education Guide: Immunohistochemical Staining Methods (available from Agilent Technologies).

Clinical Performance Evaluation

The efficacy of KEYTRUDA in combination with paclitaxel, paclitaxel protein-bound, or gemcitabine and carboplatin was investigated in KEYNOTE-355 (NCT02819518), a multicenter, double-blind, randomized, placebo-controlled trial conducted in 847 patients with locally recurrent unresectable or metastatic TNBC, regardless of tumor PD-L1 expression, who had not been previously treated with chemotherapy in the metastatic setting. Patients with active autoimmune disease that required systemic therapy within 2 years of treatment or a medical condition that required immunosuppression were ineligible. Randomization was stratified by chemotherapy treatment (paclitaxel or paclitaxel protein-bound vs. gemcitabine and carboplatin), tumor PD-L1 expression (CPS ≥ 1 vs. CPS < 1) according to the PD-L1 IHC 22C3 pharmDx kit, and prior treatment with the same class of chemotherapy in the neoadjuvant setting (yes vs. no). According to pre-specified analysis plan, the study analysis population included patient populations with tumor PD-L1 expression CPS ≥ 1 and CPS ≥ 10.

Patients were randomized (2:1) to one of the following treatment arms; all study medications were administered via intravenous infusion:

- KEYTRUDA 200 mg on Day 1 every 3 weeks in combination with paclitaxel protein-bound 100 mg/m^2 on Days 1, 8 and 15 every 28 days, paclitaxel 90 mg/m^2 on Days 1, 8, and 15 every 28 days, or gemcitabine 1000 mg/m^2 and carboplatin AUC 2 mg/mL/min on Days 1 and 8 every 21 days.
- Placebo on Day 1 every 3 weeks in combination with paclitaxel protein-bound 100 mg/m^2 on Days 1, 8 and 15 every 28 days, paclitaxel 90 mg/m^2 on Days 1, 8, and 15 every 28 days, or gemcitabine 1000 mg/m^2 and carboplatin AUC 2 mg/mL/min on Days 1 and 8 every 21 days.

Assessment of tumor status was performed at Weeks 8, 16, and 24, then every 9 weeks for the first year, and every 12 weeks thereafter. The main efficacy outcome measure was PFS as assessed by BICR according to RECIST v1.1, modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ tested in the subgroup of patients with CPS ≥ 10. Additional efficacy outcome measures were OS as well as ORR and DoR as assessed by BICR.

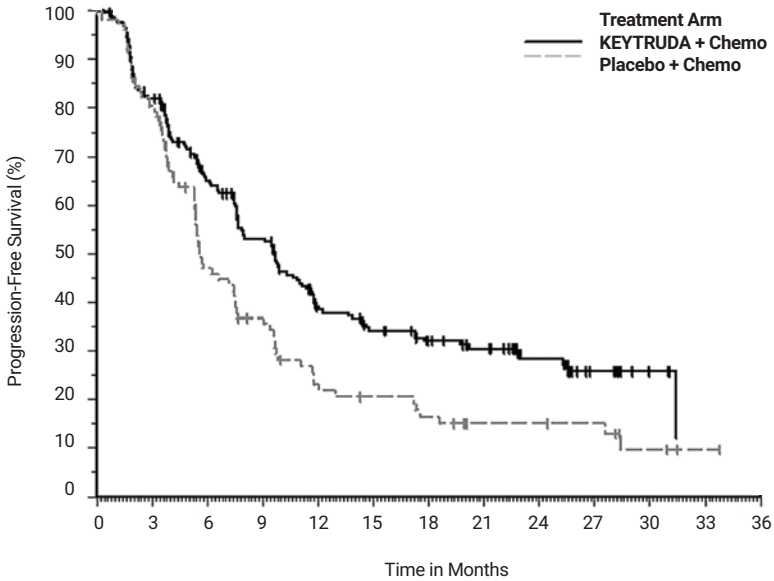
The study population characteristics for patients were: median age of 53 years (range: 22 to 85), 21% age 65 or older; 100% female; 68% White, 21% Asian, and 4% Black; 60% ECOG PS of 0 and 40% ECOG PS of 1; and 68% were post-menopausal status. Seventy-five percent of patients had tumor PD-L1 expression CPS ≥ 1 and 38% had tumor PD-L1 expression CPS ≥ 10.

Table 5 and Figure 89 summarize the efficacy results for KEYNOTE-355.

Table 5: Efficacy Results in KEYNOTE-355 (CPS ≥ 10)

Endpoint	KEYTRUDA 200 mg every 3 weeks with chemotherapy n=220	Placebo every 3 weeks with chemotherapy n=103
PFS		
Number of patients with event (%)	136 (62%)	79 (77%)
Median in months (95% CI)	9.7 (7.6, 11.3)	5.6 (5.3, 7.5)
Hazard ratio* (95% CI)	0.65 (0.49, 0.86)	
p-Value†	0.0012	
ORR		
Objective confirmed response rate (95% CI)	53% (46, 60)	40% (30, 50)
Complete response rate	17%	13%
Partial response rate	36%	27%
DoR		
Median in months (95% CI)	19.3 (9.9, 29.8)	7.3 (5.3, 15.8)

** Based on stratified Cox regression model*
† One-sided p-Value based on stratified log-rank test



Number at Risk

KEYTRUDA + Chemo:	220	173	122	96	63	52	44	37	25	12	5	0	0
Placebo + Chemo:	103	80	41	30	18	15	12	8	8	7	3	1	0

Figure 89: Kaplan-Meier curve for progression-free survival in KEYNOTE-355 (CPS ≥ 10).

References

- Keytruda [package insert]. Merck Sharp & Dohme LLC, a subsidiary of Merck & Co., Inc., Rahway, NJ, USA; 2020.
- PD-L1 IHC 22C3 pharmDx [Instructions for Use]. Carpinteria, CA: Dako, Agilent Pathology Solutions; 2020.
- Herbst, R.S.; Soria, J.C.; Kowanetz, M.; Fine, G.D.; Hamid, O.; Gordon, M.S.; Sosman, J.A.; McDermott, D.F.; Powderly, J.D.; Gettinger, S.N.; Kohrt, H.E.; Horn, L.; Lawrence, D.P.; Rost, S.; Leabman, M.; Xiao, Y.; Mokatrin, A.; Koeppen, H.; Hegde, P.S.; Mellman, I.; Chen, D.S.; Hodi, F.S. Predictive Correlates of Response to the Anti-PD-L1 Antibody MPDL3280A in Cancer Patients. *Nature.* 2014, *515* (7528), 563–567.
- Tumeh, P.C.; Harview, C.L.; Yearley, J.H.; Shintaku, I.P.; Taylor, E.J.; Robert, L.; Chmielowski, B.; Spasic, M.; Henry, G.; Ciobanu, V.; West, AN.; Carmona, M.; Kivork, C.; Seja, E.; Cherry, G.; Gutierrez, A.J.; Grogan, T.R.; Mateus, C.; Tomasic, G.; Glaspy, J.A.; Emerson, R.O.; Robins, H.; Pierce, R.H.; Elashoff, D.A.; Robert, C.; Ribas, A. PD-1 Blockade Induces Responses by Inhibiting Adaptive Immune Resistance. *Nature.* 2014, *515* (7528), 568–571.
- Hanahan, D.; Weinberg, R.A. Hallmarks of Cancer: The Next Generation. *Cell.* 2011, *144* (5), 646–674.
- Sharpe, A.H.; Freeman, G.J. The B7-CD28 Superfamily. *Nat. Rev. Immunol.* 2002, *2* (2), 116–126.
- Keir, M.E.; Butte, M.J.; Freeman, G.J.; Sharpe, A.H. PD-1 and its Ligands in Tolerance and Immunity. *Annu. Rev. Immunol.* 2008, *26,* 677–704.
- Freeman, G.J.; Long, A.J.; Iwai, Y.; Bourque, K.; Chernova, T.; Nishimura, H.; Fitz, L.J.; Malenkovich, N.; Okazaki, T.; Byrne, M.C.; Horton, H.F.; Fouser, L.; Carter, L.; Ling, V.; Bowman, M.R.; Carreno, B.M.; Collins, M.; Wood, C.R.; Honjo, T. Engagement of the PD-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation. *J. Exp. Med.* 2000, *192* (7), 1027–1034.
- Dong, H.; Strome, S.E.; Salomao, D.R.; Tamura, H.; Hirano, F.; Flies, D.B.; Roche, P.C.; Lu, J.; Zhu, G.; Tamada, K.; Lennon, V.A.; Celis, E.; Chen, L. Tumor-associated B7-H1 Promotes T-cell Apoptosis: A Potential Mechanism of Immune Evasion. *Nat. Med.* 2002, *8* (8), 793–800.
- Brahmer, J.R.; Tykodi, S.S.; Chow, L.Q.; Hwu, W.J.; Topalian, S.L.; Hwu, P.; Drake, C.G.; Camacho, L.H.; Kauh, J.; Odunsi, K.; Pitot, H.C.; Hamid, O.; Bhatia, S.; Martins, R.; Eaton, K.; Chen, S.; Salay, T.M.; Alaparthy, S.; Grosso, J.F.; Korman, A.J.; Parker, S.M.; Agrawal, S.; Goldberg, S.M.; Pardoll, D.M.; Gupta, A.; Wigginton, J.M. Safety and Activity of Anti-PD-L1 Antibody in Patients with Advanced Cancer. *N. Engl. J. Med.* 2012, *366* (26), 2455–2465.

- Topalian, S.L.; Hodi, F.S.; Brahmer, J.R.; Gettinger, S.N.; Smith, D.C.; McDermott, D.F.; Powderly, J.D.; Carvajal, R.D.; Sosman, J.A.; Atkins, M.B.; Leming, P.D.; Spigel, D.R.; Antonia, S.J.; Horn, L.; Drake, C.G.; Pardoll, D.M.; Chen, L.; Sharfman, W.H.; Anders, R.A.; Taube, J.M.; McMiller, T.L.; Xu, H.; Korman, A.J.; Jure-Kunkel, M.; Agrawal, S.; McDonald, D.; Kollia, G.D.; Gupta, A.; Wigginton, J.M.; Sznol, M. Safety, Activity, and Immune Correlates of Anti-PD-1 Antibody in Cancer. *N. Engl. J. Med.* 2012, *366* (26)*,* 2443–2454.
- Roach, C.; Zhang, N.; Corigliano, E.; Jansson, M.; Toland, G.; Ponto, G.; Dolled-Filhart, M.; Emancipator, K.; Stanforth, D.; Kulangara, K. Development of a Companion Diagnostic PD-L1 Immunohistochemistry Assay for Pembrolizumab Therapy in Non-small-cell Lung Cancer. *Appl. Immunohistochem. Mol. Morphol.* 2016, *24,* 392–397.
- Cortes, J.; Cescon, D.W.; Rugo, H.S.; et al. KEYNOTE-355: Randomized, double-blind, phase III study of pembrolizumab + chemotherapy versus placebo + chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer. *J. Clin. Oncol.* 2020, *15* (Supp 1000), abstr.
- Data on file. Agilent Technologies, Inc.

Notes

For PD-L1 testing,

Choose PD-L1 IHC 22C3 pharmDx—the ONE Leading Assay with KEYTRUDA® (pembrolizumab)

KEYNOTE

The **ONE** PD-L1 assay used in KEYTRUDA clinical trials[1,2]

The **ONE** PD-L1 assay first approved with KEYTRUDA in every indication that requires PD-L1 testing[1,2]

The **ONE** PD-L1 assay trusted worldwide to test hundreds of thousands of patients for KEYTRUDA[3]

1. PD-L1 IHC 22C3 pharmDx [Instructions for Use]. Carpinteria, CA: Dako, Agilent Pathology Solutions; 2020. **2.** Keytruda [package insert]. Merck Sharp & Dohme LLC, a subsidiary of Merck & Co., Inc., Rahway, NJ, USA; 2020. **3.** Data on file. Agilent Technologies, Inc.

www.agilent.com

U.S. and Canada
1-800-227-9770
agilent_inquiries@agilent.com

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

D62310_2.00

Published in the USA, November 21, 2023
29389 2023NOV21

Agilent
Trusted Answers