PD-L1 IHC 22C3 pharmDx Interpretation Manual – Esophageal Squamous Cell Carcinoma (ESCC)

FDA-approved for in vitro diagnostic use
Rx only
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 04
Introduction 06
PD-L1 Overview 08
PD-L1 IHC 22C3 pharmDx Overview 10
Kit Configuration (SK006) 11
Technical Considerations 12
Specimen Preparation 12
In-house Control Tissue 12
Optional Additional In-house Control: Tonsil Tissue 13
Tissue Processing 13
PD-L1 IHC 22C3 pharmDx Staining Procedure 14
Technical Checklist 17
Slide Evaluation 18
General Considerations 18
Specimen Adequacy 18
Evaluating Controls 19
Slide Evaluation Flowchart 23
Combined Positive Score 24
Definition of Combined Positive Score (CPS) 24
CPS Numerator Inclusion and Exclusion Criteria 24
Determining Combined Positive Score 25
Suggested Methods 27
Interpretation of CPS 30
Identifying Patients With ESCC for Treatment 31
PD-L1 IHC 22C3 pharmDx Testing Scheme 31
Reporting Results 32
Combined Positive Score Summary and Examples 33
Key Considerations in Scoring PD-L1 IHC 22C3 pharmDx Stained Specimens 33
Image Guide for Interpretation of PD-L1 IHC 22C3 pharmDx Staining in ESCC 34
Artifacts 48
PD-L1 IHC 22C3 pharmDx CPS Case Examples 53
CPS < 10 Case Examples 53
CPS ≥ 10 Case Examples 55
Near Cut-off Case Examples 57
(CPS Range of Greater Than or Equal to 1 but Less Than 10) 57
Near Cut-off Case Examples 60
(CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20) 60
Troubleshooting Guide 62
Clinical Performance Evaluation 63
References 66
Intended Use

For in vitro diagnostic use.

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

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

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

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

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

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

ESCC 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 provided with PD-L1 IHC 22C3 pharmDx, Code SK006 or visit www.agilent.com.
Assay Interpretation
The clinical interpretation of any staining, or the absence of staining, must be complemented by the evaluation of proper controls. Evaluation must be made by a qualified pathologist within the context of the patient’s clinical history and other diagnostic tests. This product is intended for in vitro diagnostic (IVD) use.

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

Photomicrographs
The included photomicrographs are of ESCC, except for Figure 36 which is squamous cell carcinoma from the cervix, and Figures 34, 35b, and 37 which are esophageal adenocarcinoma.

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

Tissue samples supplied by BioIVT.
The data and biospecimens used in this project were provided by US Biolab, Rockville, MD and by SageBio LLC, Sharon, MA, USA with appropriate ethics approval and through Trans-Hit Biomarkers Inc.
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.

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.

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 upregulation in ESCC 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-181) 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.
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 ESCC 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 ESCC 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.
Kit Configuration (SK006)

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, (50x)
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) and EnVision FLEX Hematoxylin (Code K8008) are required but not included in the kit.

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

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

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

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

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

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

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

Tonsil stained with PD-L1 should be pre-screened to exhibit strong staining in portions of the crypt epithelium and weak-to-moderate staining of the follicular macrophages in the germinal centers. PD-L1 expression of the endothelium, fibroblasts, 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) and stain within 4.5 months of sectioning, or at room temperature up to 25 °C and stain within 1 month of sectioning.
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. Please refer to the Product-specific Limitations Section on page 16 for Target Retrieval Solution limitations in ESCC specimens.

**EnVision FLEX Wash Buffer**

Dilute EnVision FLEX Wash Buffer (20×) 1:20 using distilled or deionized water (reagent-quality water). Store unused 1× buffer at 2–8 °C for no more than 1 month. Discard if cloudy in appearance.
DAB+ Substrate-Chromogen Solution
Add 1 drop of DAB+ Chromogen per mL of DAB+ Substrate Buffer and mix. Prepared DAB+ Substrate-Chromogen is stable for 5 days if stored in the dark at 2–8 °C. Mix the DAB+ Substrate-Chromogen Solution thoroughly prior to use. Any precipitate developing in the solution will not affect staining quality.

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

Controls to Assess Staining Quality
The following quality controls should be included in each staining run:

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

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

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

Staining and Counterstaining

- Place the Autostainer rack with slides on the Autostainer Link 48
- Ensure slides remain wet with buffer while loading and prior to initiating the run. Dried tissue sections may display increased non-specific staining
- Select the PD-L1 IHC 22C3 pharmDx protocol. The instrument performs the staining and counterstaining procedures by applying the appropriate reagent, monitoring the incubation time, and rinsing slides between reagents
- Counterstain slides using EnVision FLEX Hematoxylin, Code K8008

Mounting

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

Product-specific Limitations

- Laboratories should pay particular attention to the pH of the Target Retrieval Solution for pre-treatment of ESCC specimens as pH 5.9 may affect PD-L1 staining performance
- The studies carried out to assess TRS use up to three times in esophageal cancer did not meet acceptance criteria for qualitative evaluation of PD-L1 expression status, therefore TRS reuse is not recommended for ESCC specimens
## 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 _________________

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular preventive maintenance is performed on the Autostainer Link 48 and PT Link?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>PD-L1 IHC 22C3 pharmDx is used before the expiration date printed on the outside of the box?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>All PD-L1 IHC 22C3 pharmDx components, including Control Cell Line Slides, are stored in the dark at 2–8 °C?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>All PD-L1 IHC 22C3 pharmDx components, including Control Cell Line Slides, are equilibrated to room temperature (20–25 °C) prior to immunostaining?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Appropriate positive and negative control tissues from ESCC are identified?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Tissues are fixed in neutral buffered formalin?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Tissues are infiltrated with melted paraffin, at or below 60 °C?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Tissue sections of 4–5 µm are mounted on Dako FLEX IHC Microscope Slides or Superfrost Plus slides?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Specimens are oven-dried at 58 ± 2 °C for 1 hour?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Specimens are stained within 4.5 months of sectioning when stored in the dark at 2–8 °C (preferred) or within 1 month when stored in the dark at room temperature up to 25 °C?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>EnVision FLEX Target Retrieval Solution, Low pH is prepared properly? pH of 1× Target Retrieval Solution must be 6.1 ± 0.2.</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>EnVision FLEX Wash Buffer is prepared properly?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>DAB+ Substrate-Chromogen Solution is prepared properly?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Slides are counterstained with EnVision FLEX Hematoxylin?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>The Deparaffinization, Rehydration, and Target Retrieval 3-in-1 procedure is followed using PT Link?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Slides remain wet with buffer while loading and prior to initiating run on Autostainer Link 48?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>The PD-L1 IHC 22C3 pharmDx protocol is selected on Autostainer Link 48?</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>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.</td>
<td>☐</td>
<td>☐</td>
</tr>
</tbody>
</table>

Additional observations or comments:

________________________________________________________

________________________________________________________
PD-L1 IHC 22C3 pharmDx interpretation 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 be acceptable for use in IHC staining.

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

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

PD-L1 IHC 22C3 pharmDx Control Cell Line Slide

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

Evaluate the overall staining intensity using the following guide:

<table>
<thead>
<tr>
<th>0</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+</td>
<td>Weak intensity</td>
</tr>
<tr>
<td>2+</td>
<td>Moderate intensity</td>
</tr>
<tr>
<td>3+</td>
<td>Strong intensity</td>
</tr>
</tbody>
</table>

Positive Control Cell Pellet

The following staining is acceptable for the PD-L1 positive cell pellet (Figure 7):
- Cell membrane staining of ≥ 70% of cells
- ≥ 2+ average staining intensity
- Non-specific staining < 1+ intensity

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

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

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

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

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

Positive and Negative In-house Control Tissue (ESCC)

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

![Figure 9: Positive in-house control tissue (20× magnification).](image)
The ideal ESCC negative control tissue should demonstrate no staining of tumor cells and immune cells (Figure 10). However, because prevalence of PD-L1 expression on immune cells is high, a few staining immune cells are acceptable. Examine the negative in-house control tissue to determine the expected staining. The variety of different cell types present in most tissue sections offers internal negative control sites; this should be verified by the user. If inappropriate staining occurs in the in-house control tissues, results with the patient specimen should be considered invalid.

![Figure 10](image)

**Optional Control Tissue**

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

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

![Figure 11](image)

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

Do not use in-house control tissue as an aid in interpretation of patient results.
Negative Control Reagent (NCR)
Examine the slides stained with the NCR to identify non-specific background staining that may interfere with PD-L1 staining interpretation, making the specimen non-evaluable. Satisfactory performance is indicated by the absence of staining (Figure 12).

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

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

NCR-stained slides indicate non-specific background staining and allow for better interpretation of patient specimens stained with the primary antibody.
Figure 13: Recommended order of slide evaluation.
**Combined Positive Score**

**Definition of Combined Positive Score (CPS)**

PD-L1 expression in ESCC 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:

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

*Macrophages and histiocytes are considered the same cells

**CPS Numerator Inclusion and Exclusion Criteria**

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

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

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

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

<table>
<thead>
<tr>
<th>Tissue Elements</th>
<th>Included in the Numerator</th>
<th>Excluded from the Numerator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Cells</td>
<td>Convincing partial or complete linear membrane staining (at any intensity) of viable invasive tumor cells</td>
<td>- Non-staining tumor cells&lt;br&gt;- Tumor cells with only cytoplasmic staining&lt;br&gt;- Non-invasive neoplasia (including carcinoma in situ)</td>
</tr>
<tr>
<td>Immune Cells</td>
<td>Membrane and/or cytoplasmic* staining (at any intensity) of mononuclear inflammatory cells (MICs) within tumor nests and adjacent supporting stroma*:&lt;br&gt;- Lymphocytes (including lymphocyte aggregates)&lt;br&gt;- Macrophages‡&lt;br&gt;Only MICs directly associated with the response to the tumor are scored</td>
<td>- Non-staining MICs&lt;br&gt;- MICs associated with non-invasive neoplasia (including carcinoma in situ)&lt;br&gt;- MICs associated with benign structures&lt;br&gt;- MICs (including lymphoid aggregates) not directly associated with the response to the tumor&lt;br&gt;- Neutrophils, eosinophils, and plasma cells</td>
</tr>
<tr>
<td>Other Cells</td>
<td>Not included</td>
<td>- Benign epithelial cells&lt;br&gt;- Stromal cells (including fibroblasts)&lt;br&gt;- Necrotic cells and/or cellular debris</td>
</tr>
</tbody>
</table>

* 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

<table>
<thead>
<tr>
<th>Tissue Elements</th>
<th>Included in the Denominator</th>
<th>Excluded from the Denominator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Cells</td>
<td>All viable invasive tumor cells</td>
<td>- Non-viable tumor cells&lt;br&gt;- Non-invasive neoplasia (including carcinoma in situ)</td>
</tr>
<tr>
<td>Immune Cells</td>
<td>Not included</td>
<td>All immune cells</td>
</tr>
<tr>
<td>Other Cells</td>
<td>Not included</td>
<td>- Benign cells&lt;br&gt;- Stromal cells (including fibroblasts)&lt;br&gt;- Necrotic cells and/or cellular debris</td>
</tr>
</tbody>
</table>
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:*

\[
CPS = \frac{\# \text{ PD-L1 staining cells}}{\text{Total # of viable tumor cells}} \times 100 = \frac{\sim80 \text{ PD-L1 staining cells}}{100 \text{ tumor cells}} \times 100 = 80
\]

*CPS of entire tumor area: 10% \times 80 = CPS 8*

**Clinical Interpretation:** CPS < 10

---

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

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

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

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

Calculate the Combined Positive Score of the entire tumor area:

Assessment:
Combined Positive Score:

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

\[
(80 + 30 + 50 + 100) / 4 \approx \text{CPS 65}
\]

Clinical Interpretation: CPS ≥ 10

Figure 15: Example with heterogeneous PD-L1 staining area.
First: Evaluate the specimen for perceptible and convincing staining as described in “Determining Combined Positive Score” on page 25.

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

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

Calculate the Combined Positive Score of the entire tumor area:

**Assessment:**

**Combined Positive Score:**

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

**Clinical Interpretation:** CPS ≥ 10

* Including tumor cells, lymphocytes, macrophages

**Figure 16:** Example of near cut-off specimen.
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**

<table>
<thead>
<tr>
<th>CPS</th>
<th>PD-L1 Expression Level</th>
<th>Image (20× magnification)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10</td>
<td>CPS is less than 10</td>
<td></td>
</tr>
<tr>
<td>≥ 10</td>
<td>CPS is greater than or equal to 10</td>
<td></td>
</tr>
</tbody>
</table>
PD-L1 IHC 22C3 pharmDx is the only companion diagnostic indicated as an aid in identifying patients with ESCC for treatment with KEYTRUDA® (pembrolizumab).

Clinical Validation of PD-L1 IHC 22C3 pharmDx in Previously Treated Patients With ESCC

The clinical validity of PD-L1 IHC 22C3 pharmDx in evaluating PD-L1 expression in previously treated patients with ESCC is based on the KEYTRUDA KEYNOTE-181 study sponsored by Merck & Co. Specimens from patients with esophageal cancer who progressed on or after one prior line of standard therapy for advanced disease (advanced/metastatic adenocarcinoma or squamous cell carcinoma) were tested for PD-L1 expression using PD-L1 IHC 22C3 pharmDx. In the KEYNOTE-181 clinical trial, 42.8% of enrolled patients had ESCC 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 is presented in the Clinical Performance Evaluation section on pages 63–65.

Table 4: PD-L1 Prevalence in Patients with Recurrent or Metastatic ESCC Enrolled in KEYNOTE-181*

<table>
<thead>
<tr>
<th>PD-L1 Expression</th>
<th>CPS &lt; 10</th>
<th>CPS ≥ 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence % (n)</td>
<td>57.2% (210)</td>
<td>42.8% (157)</td>
</tr>
</tbody>
</table>

* Prevalence calculation based on patients with known PD-L1 expression (patients with unknown PD-L1 expression, n=6) and excludes patients with specimens outside the stability window (n=28)

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.

Recurrent locally advanced or metastatic ESCC patient specimen

Use PD-L1 IHC 22C3 pharmDx to determine PD-L1 expression

Pathologist reports numerical CPS and PD-L1 expression level

CPS < 10

CPS ≥ 10

Oncologist determines treatment

Recurrent locally advanced or metastatic ESCC patients with disease progression after one or more prior lines of systemic therapy with PD-L1 expression level CPS ≥ 10 are indicated for treatment with KEYTRUDA monotherapy

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) is indicated for the treatment of patients with recurrent locally advanced or metastatic ESCC with disease progression after one or more prior lines of systemic therapy whose tumors exhibit PD-L1 expression level CPS ≥ 10 as determined by an FDA-approved test. See KEYTRUDA prescribing information for details.
Combined Positive Score Summary and Examples

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

By definition, PD-L1 staining cells in ESCC 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 ESCC 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 ESCC, except for Figure 36, which is squamous cell carcinoma of the cervix, and Figures 34, 35b, and 37, which are esophageal adenocarcinoma.
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 ESCC 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 20x magnification.

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

Figure 18a: ESCC specimen stained with PD-L1 primary antibody exhibiting 1+ linear membrane staining of tumor cells (arrows) (20x magnification).
Figure 18b: ESCC specimen stained with PD-L1 primary antibody exhibiting 2+ linear membrane staining of tumor cells (arrows) (20× magnification).

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

**Key Point**

Perceptible and convincing linear membrane staining of tumor cells at any intensity should be included in the CPS numerator.
Partial Linear Membrane Staining

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

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

**Key Point**

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

Linear Membrane and Cytoplasmic Staining

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

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

**Key Point**

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

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

**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 ESCC 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 (arrows) (20× magnification).](image)

**Key Point**
Multinucleate tumor cells can be seen in ESCC and follow the same criteria for inclusion/exclusion as mononucleate tumor cells.
Imune 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: ESCC specimen stained with PD-L1 primary antibody exhibiting staining of tumor-associated lymphocytes (arrows) (20× magnification).

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

Multinucleate giant cells can be seen in ESCC 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.

![ESCC specimen stained with PD-L1 primary antibody exhibiting staining of multinucleate giant cells (arrows) (20× magnification).](image)

Figure 24: ESCC specimen stained with PD-L1 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 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 and tumor-associated mononuclear inflammatory cells (MICs) are indistinguishable from each other and exhibit PD-L1 primary antibody staining (20× magnification).

Figure 25b: Corresponding H&E to reference when tumor 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 5× magnification, several areas of PD-L1 staining mononuclear inflammatory cells are visible. To demonstrate which immune cells to include in the numerator, zoom in to 20× magnification on the boxed fields (5× magnification).

Figure 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

ESCC 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: ESCC specimen with small tumor cells (20× magnification).](image)

Medium Cell Size

![Figure 28: ESCC specimen with medium tumor cells (20× magnification).](image)
Large Cell Size

Figure 29: ESCC 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: ESCC specimen stained with PD-L1 primary antibody exhibiting only cytoplasmic staining of tumor cells (red arrows) (20× magnification). **Note:** Tumor-associated mononuclear inflammatory cells in the upper right corner exhibit cytoplasmic PD-L1 staining and should be included in the numerator (black arrows).](image)

**Key Point**

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

![Figure 31: ESCC specimen stained with PD-L1 primary antibody exhibiting staining of benign epithelial cells (red arrows) and associated mononuclear inflammatory cells (black arrows), both of which should be excluded from the score (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.

Carcinoma In Situ (CIS)

![Figure 32a: Hematoxylin and eosin (H&E) section demonstrating ESCC in situ (CIS) (arrow) (10× magnification).]
Figure 32b: Any tumor cells that are part of the CIS component should be excluded from the numerator and denominator (red arrow). Any mononuclear inflammatory cells (MICs) (black arrows) associated with the CIS component should be excluded from the numerator (10× magnification).

**Key Point**

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

Stromal Cells

Figure 33: PD-L1 staining on stromal cells (arrows) (20× magnification).

**Key Point**

Stromal cells exhibiting PD-L1 staining should be excluded from the score
### Other Immune Cells Excluded from CPS

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

**Key Point**

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

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

Non-specific Background Staining

Background staining is defined as diffuse, non-specific staining of a specimen. It is caused by several factors. These factors include, but are not limited to:

- Pre-analytic fixation and processing of the specimen
- Incomplete removal of paraffin from the section
- Incomplete rinsing of slides during staining
- Drying of slides; ensure slides remain wet with buffer while loading onto Autostainer Link 48 and prior to initiating run
- Improper deparaffinization procedure
- Incomplete rinsing of reagents from slides

The non-specific background staining of the NCR-stained test section is useful in determining the level of background staining in the PD-L1 stained test section. All specimens must have ≤ 1+ non-specific background staining.

The use of fixatives other than neutral buffered formalin may be a source of background staining and is not recommended. Background staining with PD-L1 IHC 22C3 pharmDx is rare.
Figure 35a: ESCC specimen stained with PD-L1 primary antibody exhibiting non-specific staining; non-specific background staining (red arrows) should be excluded from the score. Weak nuclear staining is also present and should be ignored (black arrows) (20× magnification).

Figure 35b: Negative Control Reagent (NCR) exhibiting non-specific background staining in esophageal adenocarcinoma (arrows) (20× magnification).

**Key Point**

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

Commonly, edge artifact is linked to the following pre-analytic factors:
- Thick tissue sections
- Drying of tissue prior to fixation or during staining procedure

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

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

![Figure 36: Edge staining should be excluded from the score (20× magnification). Note: Squamous cell carcinoma from the cervix is depicted.](image)

**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 37: Esophageal adenocarcinoma specimen stained with PD-L1 primary antibody exhibiting crush artifact; crush artifact should be excluded from the score (20× magnification).](image)

**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 38: ESCC 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 ESCC specimens and should be excluded from the score.

![Image of ESCC specimen stained with PD-L1 primary antibody exhibiting staining of necrosis; necrosis staining should be excluded from the score (20× magnification).](image)

**Figure 39:** ESCC specimen stained with PD-L1 primary antibody exhibiting staining of necrosis; necrosis staining should be excluded from the score (20× magnification).

**Key Point**

Scoring of necrotic areas should be excluded from the CPS calculation
PD-L1 IHC 22C3 pharmDx
CPS Case Examples

CPS < 10 Case Examples

Case 1: CPS < 10

![ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 0 (20× magnification).](image1)

Figure 40: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 0 (20× magnification).

Case 2: CPS < 10

![ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 0 (20× magnification).](image2)

Figure 41: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 0 (20× magnification).
Case 3: CPS < 10

Figure 42: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 3, however any numerical CPS between 2–4 could be assigned to this image (20× magnification).

Case 4: CPS < 10

Figure 43: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 4, however any numerical CPS between 2–4 could be assigned to this image (20× magnification).
Case 5: CPS ≥ 10

Figure 44: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 23, however any numerical CPS between 20–30 could be assigned to this image (20× magnification).

Case 6: CPS ≥ 10

Figure 45: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 40, however any numerical CPS between 35–45 could be assigned to this image (20× magnification).
Case 7: CPS ≥ 10

Figure 46: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 45, however any numerical CPS between 40–50 could be assigned to this image (20× magnification).

Case 8: CPS ≥ 10

Figure 47: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 72, however any numerical CPS between 70–80 could be assigned to this image (20× magnification).
Near Cut-off Case Examples (CPS Range of Greater Than or Equal to 1 but Less Than 10)

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

Figure 48: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).

Challenging Case 2: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

Figure 49: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).
Challenging Case 3: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

Figure 50: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 7, however any numerical CPS between 5–9 could be assigned to this image (20× magnification).

Challenging Case 4: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

Figure 51: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 4, however any numerical CPS between 2–6 could be assigned to this image (20× magnification).
Challenging Case 5: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

Figure 52: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 8, however any numerical CPS between 6–9 could be assigned to this image (20× magnification).

Challenging Case 6: Near Cut-off (CPS Range of Greater Than or Equal to 1 but Less Than 10)

Figure 53: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 5, however any numerical CPS between 3–7 could be assigned to this image (20× magnification).
Near Cut-off Case Examples (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

Challenging Case 7: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

Figure 54: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 14, however any numerical CPS between 12–16 could be assigned to this image (20× magnification).

Challenging Case 8: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

Figure 55: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 13, however any numerical CPS between 10–15 could be assigned to this image (20× magnification).
Challenging Case 9: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

Figure 56: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 16, however any numerical CPS between 13–19 could be assigned to this image (20× magnification).

Challenging Case 10: Near Cut-off (CPS Range of Greater Than or Equal to 10 but Less Than or Equal to 20)

Figure 57: ESCC specimen stained with PD-L1 antibody exhibiting a CPS of 16, however any numerical CPS between 13–19 could be assigned to this image (20× magnification).
# Troubleshooting Guide

## Troubleshooting Guidelines for PD-L1 IHC 22C3 pharmDx

For further troubleshooting help, contact your local Agilent representative.

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

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

The efficacy of KEYTRUDA was investigated in KEYNOTE-181 (NCT02564263), a multicenter, randomized, open-label, active-controlled trial that enrolled 628 patients with recurrent locally advanced or metastatic esophageal cancer who progressed on or after one prior line of systemic treatment for advanced disease. Patients with HER2/neu positive esophageal cancer were required to have received treatment with approved HER2/neu targeted therapy. All patients were required to have tumor specimens for PD-L1 testing at a central laboratory; PD-L1 status was determined using the PD-L1 IHC 22C3 pharmDx kit. Patients with a history of non-infectious pneumonitis that required steroids or current pneumonitis, active autoimmune disease, or a medical condition that required immunosuppression were ineligible.

Patients were randomized (1:1) to receive either KEYTRUDA 200 mg every 3 weeks or investigator’s choice of any of the following chemotherapy regimens, all given intravenously: paclitaxel 80–100 mg/m² on Days 1, 8, and 15 of every 4-week cycle, docetaxel 75 mg/m² every 3 weeks, or irinotecan 180 mg/m² every 2 weeks. Randomization was stratified by tumor histology (esophageal squamous cell carcinoma [ESCC] vs. esophageal adenocarcinoma [EAC]/Siewert type I EAC of the gastroesophageal junction [GEJ]), and geographic region (Asia vs. ex-Asia). Treatment with KEYTRUDA or chemotherapy continued until unacceptable toxicity or disease progression. Patients randomized to KEYTRUDA were permitted to continue beyond the first RECIST v1.1 (modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ)-defined disease progression if clinically stable until the first radiographic evidence of disease progression was confirmed at least 4 weeks later with repeat imaging. Patients treated with KEYTRUDA without disease progression could be treated for up to 24 months. Assessment of tumor status was performed every 9 weeks. The major efficacy outcome measure was OS evaluated in the following co-primary populations: patients with ESCC, patients with tumors expressing PD-L1 CPS ≥ 10, and all randomized patients. Additional efficacy outcome measures were PFS, ORR, and DoR, according to RECIST v1.1, modified to follow a maximum of 10 target lesions and a maximum of 5 target lesions per organ, as assessed by BICR.

A total of 628 patients were enrolled and randomized to KEYTRUDA (n=314) or investigator’s treatment of choice (n=314). Of these 628 patients, 167 (27%) had ESCC that expressed PD-L1 with a CPS ≥ 10. Of these 167 patients, 85 patients were randomized to KEYTRUDA and 82 patients to investigator’s treatment of choice [paclitaxel (n=50), docetaxel (n=19), or irinotecan (n=13)]. The baseline characteristics of these 167 patients were: median age of 65 years (range: 33 to 80), 51% age 65 or older; 84% male; 32% White and 68% Asian; 38% had an ECOG PS of 0 and 62% had an ECOG PS of 1. Ninety percent had M1 disease and 10% had M0 disease. Prior to enrollment, 99% of patients had received prior platinum-based treatment, and 84% had also received treatment with a fluoropyrimidine. Thirty-three percent of patients received prior treatment with a taxane.
The observed OS hazard ratio was 0.77 (95% CI: 0.63, 0.96) in patients with ESCC, 0.70 (95% CI: 0.52, 0.94) in patients with tumors expressing PD-L1 CPS ≥ 10, and 0.89 (95% CI: 0.75, 1.05) in all randomized patients. On further examination, in patients whose ESCC tumors expressed PD-L1 (CPS ≥ 10), an improvement in OS was observed among patients randomized to KEYTRUDA as compared with chemotherapy (hazard ratio of 0.64, 95% CI: 0.46, 0.90).

Ten (10) of the 167 ESCC participants with tumors expressing PD-L1 CPS ≥ 10 had specimens stained outside the stability window. These 10 participants have been excluded from the efficacy results summarized in Table 5 and the Kaplan-Meier curve for OS shown in Figure 58. The efficacy results for the population excluding the 10 participants with specimens outside the stability window are consistent with the efficacy conclusions detailed above.

**Table 5: Efficacy Results in Patients with Recurrent or Metastatic ESCC (CPS ≥ 10) in KEYNOTE-181 (Excluding 10 Participants with Specimens Outside the Stability Window)**

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>KEYTRUDA 200 mg every 3 weeks n=79</th>
<th>Chemotherapy n=78</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number (%) of patients with event</td>
<td>62 (78.5)</td>
<td>68 (87.2)</td>
</tr>
<tr>
<td>Median in months (95% CI)</td>
<td>10.3 (7.8, 13.6)</td>
<td>6.7 (4.8, 9.3)</td>
</tr>
<tr>
<td>Hazard ratio* (95% CI)</td>
<td>0.63 (0.45, 0.90)</td>
<td></td>
</tr>
<tr>
<td><strong>PFS (BCS per RECIST 1.1)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number (%) of patients with event</td>
<td>70 (88.6)</td>
<td>72 (92.3)</td>
</tr>
<tr>
<td>Median in months (95% CI)</td>
<td>3.2 (2.1, 4.3)</td>
<td>2.6 (2.1, 3.7)</td>
</tr>
<tr>
<td>Median follow-up (95% CI), months</td>
<td>3.2 (2.1, 4.4)</td>
<td>2.8 (2.1, 4.0)</td>
</tr>
<tr>
<td>Hazard ratio* (95% CI)</td>
<td>0.67 (0.48, 0.95)</td>
<td></td>
</tr>
<tr>
<td><strong>Objective Response Rate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORR (95% CI)</td>
<td>21.5 (13.1, 32.2)</td>
<td>7.7 (2.9, 16.0)</td>
</tr>
<tr>
<td>Number (%) of complete responses</td>
<td>3 (3.8)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>Number (%) of partial responses</td>
<td>14 (17.7)</td>
<td>5 (6.4)</td>
</tr>
<tr>
<td>Median duration of response in months (range)</td>
<td>10.3 (2.8, 18.8+)</td>
<td>7.7 (4.3, 16.8+)</td>
</tr>
</tbody>
</table>

* Cox proportional hazards model stratified by geographic region (Asia vs ex-Asia)

*+* indicates there is no progressive disease by the time of last disease assessment
Figure 58: Kaplan-Meier Curve for Overall Survival in KEYNOTE-181 (ESCC CPS ≥ 10). (Excluding 10 Participants with Specimens Outside the Stability Window)

Number of subjects at risk

Pembrolizumab 200 mg: 79 74 65 53 48 40 38 29 27 21 11 7 4 3 1 0
SOC: 78 70 51 40 33 22 17 14 10 8 4 4 3 2 2 1
References


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

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\(^3\)

---


---

Learn more:
https://www.agilent.com/chem/PDL122C3

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.

This information is subject to change without notice.

© Agilent Technologies, Inc. 2019
Published in the USA, July 30, 2019
29318 2019Jul30