PD-L1 IHC 28-8 pharmDx
Rx Only
Code SK005
50 tests for use with Autostainer Link 48

Agilent Dako

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Agilent

1. Intended Use

For in vitro diagnostic use.

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

PD-L1 protein expression is defined as the percentage of evaluable tumor cells exhibiting partial or complete membrane staining at any intensity.

Companion Diagnostic Indication

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

When used in accordance with approved therapeutic labeling:

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

PD-L1 expression (≥ 1% tumor cell expression), as detected by PD-L1 IHC 28-8 pharmDx in SCCHN may be associated with enhanced survival from OPDIVO®.

PD-L1 expression (≥ 1% tumor cell expression), as detected by PD-L1 IHC 28-8 pharmDx in UC may be associated with enhanced response rate and enhanced disease-free survival from OPDIVO®.

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

2. Summary and Explanation

Binding of the PD-1 ligands, PD-L1 and PD-L2, to the PD-1 receptor found on T cells, inhibits T-cell proliferation and cytokine production. Up-regulation of PD-1 ligands occurs in some tumors and signaling through this pathway can contribute to inhibition of active T-cell immune surveillance of tumors.[1] OPDIVO (nivolumab) is a human immunoglobulin G4 (IgG4) monoclonal antibody that binds to the PD-1 receptor and blocks its interaction with PD-L1 and PD-L2, releasing PD-1 pathway-mediated inhibition of the immune response, including the antitumor immune response.[2] In syngeneic mouse tumor models, blocking PD-1 activity resulted in decreased tumor growth.[3] Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is a negative regulator of T-cell activity. YERVOY (ipilimumab) is a monoclonal antibody that binds to CTLA-4 and blocks the interaction of CTLA-4 with its ligands, CD80/CD86. Blockade of CTLA-4 has been shown to augment T-cell activation and proliferation, which contributes to a general increase in antitumor immune response.[4] PD-1 and CTLA-4 inhibit antitumor immunity through complementary and non-redundant mechanisms. Clinical utility of PD-L1 IHC 28-8 pharmDx for the assessment of PD-L1 status in patients treated with OPDIVO alone or in combination with YERVOY has been investigated in NSCLC, SCCHN, and UC.

OPDIVO and YERVOY are trademarks owned by Bristol-Myers Squibb.

2.1 Non-Small Cell Lung Cancer (NSCLC) First-Line Treatment:

Clinical study CHECKMATE-227 investigated the clinical validity of PD-L1 IHC 28-8 pharmDx for the assessment of PD-L1 status in NSCLC patients treated with OPDIVO in combination with YERVOY.[5] Detection of PD-L1 expressing tumor cells in NSCLC patient specimens is associated with an overall survival benefit from OPDIVO + YERVOY treatment for the patient. Specimens from patients in OPDIVO and YERVOY clinical studies sponsored by Bristol-Myers Squibb were tested using PD-L1 IHC 28-8 pharmDx.

2.2 Non-Squamous Non-Small Cell Lung Cancer (nsNSCLC) Previously Treated:

Detection of PD-L1 expressing tumor cells in a nsNSCLC patient specimen may indicate an enhanced survival benefit to OPDIVO treatment for the patient. Specimens from patients in OPDIVO clinical studies sponsored by Bristol-Myers Squibb were tested using PD-L1 IHC 28-8 pharmDx. Clinical study CHECKMATE-057 investigated the clinical validity of PD-L1 IHC 28-8 pharmDx for the assessment of PD-L1 status in nsNSCLC patients treated with OPDIVO.[3,10] The anti-PD-L1 immunotherapeutic OPDIVO treatment effect has been correlated with PD-L1 expression in patients with advanced nsNSCLC.

2.3 Squamous Cell Carcinoma of the Head and Neck (SCCHN):

Detection of PD-L1 expressing tumor cells in SCCHN patient specimens may indicate an enhanced survival benefit to OPDIVO treatment for the patient. Specimens from patients in OPDIVO clinical studies sponsored by Bristol-Myers Squibb were tested using PD-L1 IHC 28-8 pharmDx. Clinical study CHECKMATE-141 investigated the clinical validity of PD-L1 IHC 28-8 pharmDx for the assessment of PD-L1 status in SCCHN patients treated with OPDIVO.[15]

2.4 Urothelial Carcinoma (UC):

Detection of PD-L1 expressing tumor cells in UC patient specimens may indicate an enhanced response rate benefit to OPDIVO treatment for the patient. Specimens from patients in OPDIVO clinical studies sponsored by Bristol-Myers Squibb were tested using PD-L1 IHC 28-8 pharmDx. Clinical study CHECKMATE-275 investigated the clinical validity of PD-L1 IHC 28-8 pharmDx for the assessment of PD-L1 status in UC patients treated with OPDIVO.[17]

Detection of PD-L1 expressing tumor cells in UC specimens may also indicate an enhanced disease-free survival benefit to OPDIVO as an adjuvant monotherapy for patients at high risk of recurrence.[18] Clinical study CHECKMATE-274 sponsored by Bristol-Myers Squibb investigated the clinical validity of PD-L1 IHC 28-8 pharmDx for the assessment of PD-L1 status in UC patients treated with OPDIVO as an adjuvant monotherapy.

3. Principle of Procedure

PD-L1 IHC 28-8 pharmDx contains optimized reagents and protocol required to complete an IHC staining procedure of FFPE specimens using PT Link Pre-treatment Module and Autostainer Link 48.[7] Following incubation with the primary monoclonal antibody to PD-L1 or the Negative Control Reagent (NCR), specimens are incubated with a linker antibody specific to the host species of the primary antibody, and then are incubated with a ready-to-use visualization reagent consisting of secondary antibody molecules and horseradish peroxidase molecules coupled to a dextran polymer backbone. The enzymatic conversion of the subsequently added chromogen results in precipitation of a visible reaction product at the site of the antigen. The color of the chromogenic reaction is modified by a chromogen enhancement reagent. The specimen may then be counterstained and coverslipped. Results are interpreted using a light microscope. Control Slides containing two FFPE human cell lines are provided to validate staining runs.

4. Materials Provided

PD-L1 IHC 28-8 pharmDx (Code SK005)

Each kit includes 19.5 mL of primary antibody and contains the reagents necessary to perform 50 tests in up to 15 individual runs. The materials listed below are sufficient for 50 tests (50 slides incubated with primary antibody to PD-L1 and 50 slides incubated with the corresponding NCR; 100 test slides in total). Additional primary antibody to PD-L1 is provided in the kit to stain 15 cell line control slides. The number of tests is based on the use of 2 x 150 µL per slide of each reagent except DAB+ and EnVision FLEX Target Retrieval Solution.

The kit provides materials sufficient for a maximum of 15 individual staining runs.

Quantity	*Description*
1 x 34.5 mL	**Peroxidase-Blocking Reagent** PEROXIDASE-BLOCKING REAGENT Buffered solution containing hydrogen peroxide, detergent and 0.015 mol/L sodium azide.
1 x 19.5 mL	**Primary Antibody: Monoclonal Rabbit Anti-PD-L1, Clone 28-8** MONOCLONAL RABBIT ANTI-PD-L1 CLONE 28-8 Monoclonal rabbit anti-PD-L1 in a buffered solution, containing stabilizing protein, and 0.015 mol/L sodium azide.
1 x 15 mL	**Negative Control Reagent*** NEGATIVE CONTROL REAGENT Monoclonal rabbit control IgG antibody in a buffered solution, containing stabilizing protein, and 0.015 mol/L sodium azide. *Monoclonal rabbit control IgG sold under license from Cell Signaling Technology.
1 x 34.5 mL	**LINKER, Anti-Rabbit** LINKER, ANTI-RABBIT Mouse secondary antibody against rabbit immunoglobulins in a buffered solution containing stabilizing protein and 0.015 mol/L sodium azide.
1 x 34.5 mL	**Visualization Reagent-HRP** VISUALIZATION REAGENT-HRP Dextran coupled with peroxidase molecules and goat secondary antibody molecules against rabbit and mouse immunoglobulins in a buffered solution containing stabilizing protein and an antimicrobial agent.
15 x 7.2 mL	**DAB+ Substrate Buffer** DAB+ SUBSTRATE BUFFER Buffered solution containing hydrogen peroxide and an antimicrobial agent.
1 x 5 mL	**DAB+ Chromogen** DAB+ CHROMOGEN 3,3'-diaminobenzidine tetrahydrochloride in an organic solvent.

1 x 34.5 mL **DAB Enhancer**

DAB ENHANCER

Cupric sulfate in water.

6 x 30 mL **EnVision FLEX Target Retrieval Solution, Low pH (50X)**

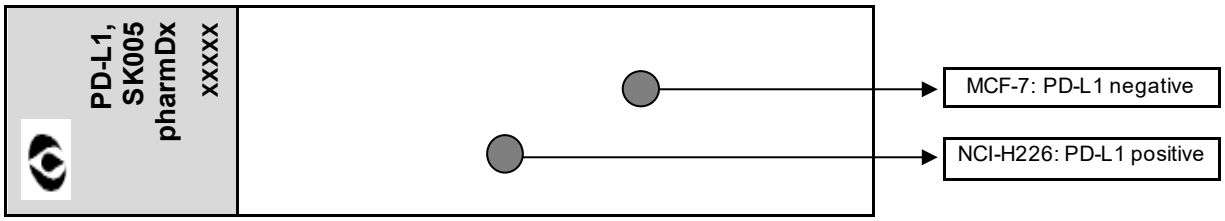
EnVision FLEX
TARGET RETRIEVAL SOLUTION
LOW pH (50X)

Buffered solution, pH 6.1, containing detergent and an antimicrobial agent.

15 slides **PD-L1 IHC 28-8 pharmDx Control Slides**

CONTROL SLIDES

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



**Dr. AF Gazdar and Dr. JD Minna at NIH are acknowledged for their contribution in developing NCI-H226 (ATCC Number: CRL-5826™).[8]
Note: All reagents included are formulated specifically for use with this kit. In order for the test to perform as specified, no substitutions, other than EnVision FLEX Target Retrieval Solution, Low pH (50x) (Code K8005) can be made. PD-L1 IHC 28-8 pharmDx has been tailored for use with Autostainer Link 48. Please refer to the User Guides for your Autostainer Link 48 and PT Link for further information.

5. Materials Required, but Not Supplied

PT Link Pre-treatment Module (Code PT100/PT101/PT200)
PT Link rinse station (Code PT109)
Autostainer Link 48 (Code AS480)
EnVision FLEX Wash Buffer (20x) (Code K8007)
EnVision FLEX Hematoxylin (Link) (Code K8008)
Distilled or deionized water (reagent-grade water)*
Timer
Positive and negative tissues to use as process controls (see 'Quality Control' Section 11 and Table 2)
Microscope slides: FLEX IHC Microscope Slides (Code K8020) or Superfrost Plus charged slides.
Coverslips
Permanent mounting medium and ancillary reagents required for mounting coverslips
Light microscope (4x–40x objective magnification)
pH meter (calibrated per manufacturer's recommendation)

*Note: Not all sources of distilled or de-ionized water may be of sufficient quality for IHC reagent preparation. Agilent recommends reagent-grade distilled or de-ionized water [corresponding to Clinical Laboratory Reagent Water (CLRW) standard as specified by Clinical & Laboratory Standards Institute (CLSI)[9]], or water similar in quality to be used for reagent preparation.

6. Precautions

1. For in vitro diagnostic use.
2. For professional users.
3. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, NaN_3 may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.[11]
4. Primary antibody, Negative Control Reagent, Linker, and Visualization Reagent contain material of animal origin.
5. Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection, and disposed of with proper precautions.[12]
6. Incubation times, temperatures, or methods other than those specified may give erroneous results.
7. Reagents have been optimally diluted. Further dilution may result in loss of antigen staining.
8. The Visualization Reagent, Liquid DAB+ chromogen and prepared DAB+ Substrate-Chromogen solution may be affected adversely if exposed to excessive light levels. Do not store system components or perform staining in strong light, such as direct sunlight.
9. Paraffin residue may lead to false negative results.
10. Use of reagent volumes other than recommended may result in loss of visible PD-L1 immunoreactivity.
11. Large tissue sections may require 3x150 µl of reagent.
12. As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper work procedures, the dangerous properties of the product and the necessary safety instructions. Please refer to Safety Data Sheet (SDS) for additional information.

13. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
14. Unused solution should be disposed of according to local, regional, national and international regulations.
15. For countries outside of the United States, see the local OPDIVO and YERVOY product labels for approved indications and expression cutoff values to guide therapy.
16. Hazard information is available in the Globally Harmonized System (GHS) insert contained within the product package. Safety Data Sheets are available on www.agilent.com or on request.
17. Contact Agilent Pathology Support via www.agilent.com to report any unusual staining.

7. Storage

Store all components of PD-L1 IHC 28-8 pharmDx, including Control Slides, in original container in the dark at 2-8 °C when not in use on Autostainer Link 48.

Do not use the kit after the expiration date printed on the outside of the kit box. If reagents are stored under any conditions other than those specified in this package insert, they must be validated by the user.

There are no obvious signs to indicate instability of this product; therefore, positive and negative controls should be run simultaneously with patient specimens.

8. Specimen Preparation

Specimens must be handled to preserve the tissue for IHC staining. Standard methods of tissue processing should be used for all specimens.

8.1 Paraffin-Embedded Sections

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

Tissue specimens should be cut into sections of 4-5 µm. After sectioning, tissues should be mounted on Superfrost Plus charged slides or Dako FLEX IHC Microscope Slides (Code K8020) and then placed in a 58 ± 2 °C oven for 1 hour.

8.2 Use of Decalcified Tissues

The use of PD-L1 IHC 28-8 pharmDx on decalcified tissues has not been validated and is not recommended.

8.3 Cut Section Storage

To preserve antigenicity, tissue sections, once mounted on slides, should be stored in the dark at 2-8 °C, or room temperature up to 25 °C, and stained within 4 months of sectioning **except** for squamous NSCLC stored at room temperature up to 25 °C. Squamous NSCLC tissue sections stored at room temperature up to 25 °C should be stained within 2 months of sectioning. Slide storage and handling conditions should not exceed 25 °C at any point post-mounting to ensure tissue integrity and antigenicity.

9. Reagent Preparation

The following reagents must be prepared prior to staining:

EnVision FLEX Target Retrieval Solution, Low pH (50x)

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

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

EnVision FLEX Wash Buffer (20x)

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

EnVision FLEX Wash Buffer (20x) is available as Code K8007.

DAB+ Substrate-Chromogen Solution

This solution should be mixed thoroughly prior to use. Any precipitate developing in the solution does not affect staining quality.

To prepare DAB+ Substrate-Chromogen Solution, add 1 drop of Liquid DAB+ Chromogen per 1 mL of DAB+ Substrate Buffer and mix. Prepared Substrate-Chromogen is stable for 5 days if stored in the dark at 2-8 °C.

Important Notes:

• **If using an entire bottle of DAB+ Substrate Buffer, add 9 drops of DAB+ chromogen**. Although the label states 7.2 mL, this is the useable volume and does not account for the "dead volume" in the bottle.

• The color of the Liquid DAB+ Chromogen in the bottle may vary from clear to lavender-brown. This will not affect the performance of this product. Dilute per the guidelines above. Addition of excess Liquid DAB+ Chromogen to the DAB+ Substrate Buffer will result in deterioration of the positive signal.

10. Staining Procedure on the Autostainer Link 48

Procedural Notes

The user should read these instructions carefully and become familiar with all components and instrumentation prior to use (see 'Precautions' Section 6).

All reagents should be equilibrated to room temperature (20-25 °C) prior to immunostaining. Likewise, all incubations should be performed at room temperature.

Do not allow tissue sections to dry during the staining procedure. Dried tissue sections may display increased nonspecific staining.

All of the required steps and incubation times for staining are preprogrammed in the DakoLink software. Please refer to the User Guides for Autostainer Link 48 and PT Link for further information on how to operate and maintain the instruments.

Note: The reagents and instructions supplied in this system have been designed for optimal performance when used with the recommended reagents and materials. Further dilution of the reagents or alteration of incubation times or temperatures may give erroneous or discordant results.

Staining Protocol

Please select the PD-L1 IHC 28-8 pharmDx staining protocol from the options in the DakoLink drop down menu.

All of the required steps and incubation times for staining are preprogrammed in the DakoLink software. If the appropriate PD-L1 IHC 28-8 pharmDx protocols are not on your server, please contact your local Technical Service Representative or Agilent Pathology Support to obtain the protocols.

Step 1: Deparaffinization, Rehydration and Target Retrieval (3-in-1) Procedure
Recommended procedure:

For details, please refer to the PT Link User Guide.

Set PT Link (Code PT100/PT101/PT200) Preheat and Cool to 65 °C. Set Heat to 97 °C for 20 minutes.

►Fill PT Link tanks with 1.5 L per tank of EnVision FLEX Target Retrieval Solution, Low pH, 1x working solution to cover the tissue sections.
►Preheat the EnVision FLEX Target Retrieval Solution to 65 °C.
►Immerse Autostainer racks containing mounted, FFPE tissue sections into the pre-heated EnVision FLEX Target Retrieval Solution, Low pH (1x working solution) in PT Link tank. Incubate for 20 minutes at 97 °C.
►As soon as target retrieval incubation time has been completed and the temperature has cooled to 65 °C, remove each Autostainer slide rack with the slides from the PT Link tank and **immediately** place the Autostainer rack with slides into a tank (e.g., PT Link Rinse Station, Code PT109) containing diluted, room temperature Wash Buffer (Code K8007).
►Immerse slides in diluted, room temperature Wash Buffer for 5 minutes.

Step 2: Staining procedure

After deparaffinization, rehydration and target retrieval (3-in-1) procedure, the Autostainer racks with slides are placed on Autostainer Link 48. Ensure slides remain wet with buffer while loading and prior to initiating run. The instrument will perform the staining process by applying the appropriate reagent, monitoring the incubation time and rinsing slides between reagents. The reagent times are preprogrammed in the DakoLink software as follows:

PD-L1 IHC 28-8 pharmDx Protocol
►Rinse in buffer
►Peroxidase Blocking Reagent (2x150 µL): 5 minutes
► Rinse in buffer
► Monoclonal Rabbit anti-PD-L1 or Negative Control Reagent (NCR) (2x150 µL): 30 minutes
► Rinse in buffer
► Linker Reagent (2x150 µL): 30 minutes
► Rinse in buffer
► Visualization Reagent-HRP (2x150 µL): 30 minutes
► Rinse in buffer
► Rinse in buffer: 5 minutes
►DAB+ solution (2x150 µL): 2x5 minutes
► Rinse in buffer
► DAB Enhancer (2x150 µL): 5 minutes
► Rinse in buffer
Counterstain*
►Hematoxylin (2x150 µL): 7 minutes
► Rinse in deionized water
► Rinse in buffer: 5 minutes
► Rinse in deionized water

Step 3: Counterstain*

Slides should be counterstained for 7 minutes with EnVision FLEX Hematoxylin (Link) (Code K8008). The Hematoxylin incubation time is preprogrammed in the protocol as shown above.

Step 4: Mounting
Non-aqueous, permanent mounting media is required.

Note: Some fading of stained slides over time may occur, depending on several factors including, but not limited to, counterstaining, mounting materials and methods, and slide storage conditions. To minimize fading, store slides in the dark at room temperature (20-25 °C).

11. Quality Control
Reagents in PD-L1 IHC 28-8 pharmDx have been quality controlled by immunohistochemistry using the target retrieval and staining procedures outlined above. Deviations in the recommended procedures for tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results. Quality controls should be included in each staining run. These quality controls are specified in Table 2 and include: a H&E stained patient tissue specimen; lab-supplied positive and negative control tissues; and an Agilent supplied Control Slide.[14] In the USA, consult the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry; see also CLSI Quality Assurance for Immunocytochemistry, Approved Guideline for additional information.[13]

12. Assay Verification
Prior to initial use of a staining system in a diagnostic procedure, the user should verify the assay's performance by testing it on a series of lab-supplied tissues with known IHC performance characteristics representing known positive and negative tissues. Refer to the quality control procedures outlined in the 'Quality Control' Section 11. These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Troubleshooting options for potential problems, their causes and suggested corrective actions are outlined in Table 20.

13. Staining Interpretation
For each staining run, slides should be examined in the order presented in Table 2 to determine the validity of the staining run and enable assessment of the staining of the sample tissue.

Staining interpretation for the PD-L1 IHC 28-8 pharmDx in various indications is summarized in Table 1.

Table 1: Staining Interpretation

PD-L1 IHC 28-8 pharmDx Staining Interpretation*

Slide evaluation should be performed by a pathologist using a light microscope. For evaluation of the PD-L1 immunohistochemical staining and scoring, 4x objective magnification can be used for initial assessment of the entire specimen followed by the 10-20x objectives for scoring (40x can be utilized for confirmation if needed). PD-L1 staining is indicated with a brown (3,3'-diaminobenzidine, DAB) reaction product.

PD-L1 protein expression is defined as the percentage of evaluable tumor cells exhibiting partial linear or complete circumferential plasma membrane staining at any intensity.

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

The entire specimen must be evaluated. All viable tumor cells on the entire PD-L1 stained patient slide must be evaluated and included in the PD-L1 scoring assessment. A minimum of 100 viable tumor cells must be present in the PD-L1 stained patient slide for the specimen to be considered adequate for evaluation. Refer to indication specific staining interpretation sections in this product insert for detailed guidelines.

Cytoplasmic staining, if present, is not considered for scoring purposes. Non-malignant cells and immune cells (e.g., infiltrating lymphocytes or macrophages) may also stain with PD-L1; however, these should not be included in the scoring for the determination of PD-L1 positivity.

Indication	Additional scoring considerations	PD-L1 Expression Cutoff
NSCLC	NA	≥ 1%
nsNSCLC	NA	≥ 1%, ≥ 5%, ≥ 10%
SCCHN	Cells within foci of dysplasia and carcinoma *in situ* are excluded from scoring. Accompanying H&E slides allow for the proper assessment of invasive carcinoma, carcinoma *in situ*, and adjacent normal epithelium.	≥ 1%
UC	Cells within foci of dysplasia and carcinoma *in situ* are excluded from scoring. Accompanying H&E slides allow for the proper assessment of invasive carcinoma, carcinoma *in situ*, and adjacent normal epithelium.	≥ 1%

*For additional guidance on PD-L1 scoring, please refer to the indication specific staining interpretation sections (Sections 13.1-13.3) and to the relevant PD-L1 IHC 28-8 pharmDx Interpretation Manual for each respective indication.

13.1 Staining Interpretation: Non-Squamous and Squamous Non-Small Cell Lung Cancer (NSCLC)
The entire specimen must be evaluated. All viable tumor cells on the entire PD-L1 stained patient slide must be evaluated and included in the PD-L1 scoring assessment. A minimum of 100 viable tumor cells should be present in the PD-L1 stained patient slide to determine the percentage of stained cells. For NSCLC specimens, record the percentage of viable tumor cells exhibiting partial linear or complete circumferential plasma membrane PD-L1 staining at any intensity.

13.2 Staining Interpretation: Squamous Cell Carcinoma of the Head and Neck (SCCHN)

This assay was validated for invasive SCCHN tissue samples and not for lesions with foci of dysplasia or carcinoma *in situ*. H&E stained slides should accompany each PD-L1 stained sample to allow proper assessment of invasive carcinoma, carcinoma *in situ*, and adjacent normal epithelium.

The entire specimen must be evaluated. All viable tumor cells on the entire PD-L1 stained patient slide must be included in the PD-L1 scoring assessment. A minimum of 100 viable tumor cells should be present in the PD-L1 stained patient slide to determine the percentage of stained cells. For SCCHN specimens, record the percentage of viable tumor cells exhibiting partial linear or complete circumferential plasma membrane PD-L1 staining at any intensity.

13.3 Staining Interpretation: Urothelial Carcinoma (UC)

This assay was validated for invasive UC tissue samples and not for lesions with foci of dysplasia or carcinoma *in situ*. H&E stained slides should accompany each PD-L1 stained sample to allow proper assessment of invasive carcinoma, carcinoma *in situ*, and adjacent normal epithelium.

The entire specimen must be evaluated. All viable tumor cells on the entire PD-L1 stained patient slide must be included in the PD-L1 scoring assessment. A minimum of 100 viable tumor cells should be present in the PD-L1 stained patient slide to determine the percentage of stained cells. For UC specimens, record the percentage of viable tumor cells exhibiting partial linear or complete circumferential plasma membrane PD-L1 staining at any intensity.

14. Slide Evaluation

Table 2: Recommended Order of Slide Evaluation

Specimens	Rationale	Requirements
1. H&E (Lab-supplied)	A hematoxylin and eosin (H&E) stain of the tissue specimen is evaluated first to assess tissue histology and preservation quality. Note: The H&E may be reviewed again in the context of the patient tissue slides stained with the Negative Control Reagent and primary antibody (steps 5 and 6).	PD-L1 IHC 28-8 pharmDx and H&E stains 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.
2. Control Slide (Agilent-supplied)	The Control Cell Line Slide stained with the PD-L1 primary antibody from PD-L1 IHC 28-8 pharmDx should be examined to ascertain that all reagents are functioning properly. The Control Slide contains the PD-L1-positive cell line pellet and PD-L1-negative cell line pellet.	One Control Slide should be stained with the primary antibody to PD-L1 in each staining run. *NCI-H226 (PD-L1-positive control cell line originating from human lung squamous cell carcinoma with positive PD-L1 protein expression) acceptance criteria:* • Plasma membrane staining of ≥ 80% of cells. • ≥2+ average staining intensity of cells with membrane staining. • Non-specific staining < 1+ intensity. *MCF-7 (PD-L1-negative control cell line originating from human breast adenocarcinoma with negative PD-L1 protein expression) acceptance criteria:* • No specific staining. • Non-specific 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 plasma membrane staining, and/or cytoplasmic staining with ≥ 1+ intensity within the boundaries of the MCF-7 cell pellet are acceptable. If either of the Control Cell Lines does not meet these criteria, all results with the patient specimens should be considered invalid.

Specimens	Rationale	Requirements
3. Positive Control Tissue Slides (Lab-supplied)	The Positive Control Tissue Slides stained with both PD-L1 primary antibody and Negative Control Reagent should be examined next. These slides verify that the fixation method and target retrieval process are effective. 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.	Controls should be biopsy/surgical specimens, fixed, processed and embedded as soon as possible in the same manner as the patient sample(s). Control tissue must represent one of the approved tumor indications for PD-L1 IHC 28-8 pharmDx as listed in the Intended Use, Section 1. Use intact specimens for interpretation of staining results as necrotic or degenerated cells often stain nonspecifically. The tissues selected for use as the Positive Tissue Controls should give weak to moderate positive staining when stained with PD-L1 to aid in detection of subtle changes in assay sensitivity. Two positive tissue control slides should be included in each staining run. **Slide stained with PD-L1**: Presence of brown plasma membrane staining on tumor cells should be observed. Non-specific staining should be ≤ 1+. **Slide stained with Negative Control Reagent**: No membrane staining. Non-specific staining should be ≤ 1+. If the positive tissue controls fail to demonstrate appropriate positive staining, results with the test specimens should be considered invalid.
4. Negative Control Tissue Slides (Lab-supplied)	The Negative Control Tissue Slides (known to be PD-L1 negative) stained with both PD-L1 primary antibody and Negative Control Reagent should be examined next to verify the specificity of the labeling of the target antigen by the primary antibody. Alternatively, negative portions of the Positive Control Tissue may serve as the Negative Control Tissue, but this should be verified by the user.	Controls should be biopsy/surgical specimens, fixed, processed and embedded as soon as possible in the same manner as the patient sample(s). Control tissue must represent one of the approved tumor indications for PD-L1 IHC 28-8 pharmDx as listed in the Intended Use, Section 1. Two negative tissue control slides should be included in each staining run. **Slide stained with PD-L1**: No membrane staining in tumor cells. Non-specific staining should be ≤ 1+. **Slide stained with Negative Control Reagent**: No membrane staining. Non-specific staining should be ≤ 1+. If specific plasma membrane staining occurs in the Negative Control Tissue Slides, results with the patient specimen should be considered invalid.
5. Patient tissue slide stained using the Negative Control Reagent	Examine patient specimens stained with the Negative Control Reagent from PD-L1 IHC 28-8 pharmDx. Negative Control Reagent is used in place of the primary antibody and aids in interpretation of specific staining at the antigen site.	Absence of plasma membrane staining verifies the specific labeling of the target antigen by the PD-L1 primary antibody. Non-specific staining should be ≤ 1+.
6. Patient tissue slide stained using the PD-L1 primary antibody	Examine the entire slide of the patient specimens stained with the PD-L1 primary antibody from PD-L1 IHC 28-8 pharmDx last. Refer to Summary and Explanation, Limitations, and Non-Clinical Performance Evaluation for specific information regarding PD-L1 IHC 28-8 pharmDx immunoreactivity.	Positive staining intensity should be assessed within the context of any non-specific staining observed on the Negative Control Reagent slide in the same run. As with any immunohistochemical test, a negative result means that the antigen was not detected, not necessarily that the antigen was absent in the cells/tissue assayed. For staining interpretation guidelines, refer to 'Staining Interpretation' Section 13.

15. Limitations

15.1 General Limitations

1. For prescription use only.
2. Immunohistochemistry is a multi-step diagnostic process that requires specialized training in the selection of the appropriate reagents; tissue selection, fixation, and processing; preparation of the immunohistochemistry slide, and interpretation of the staining results.
3. Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false-negative results. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.
4. Excessive or incomplete counterstaining may compromise proper interpretation of results.
5. Staining artifacts require re-test of the stained slides if the artifacts impair the interpretation of PD-L1 staining. Background staining may be evaluated by comparing tissue stained with primary antibody to tissue stained with Negative Control Reagent.

6. The clinical interpretation of positive staining or its absence must be evaluated within the context of clinical presentation, morphology and other histopathological criteria. The clinical interpretation of any staining, or its absence, must be complemented by morphological studies and proper controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist, who is familiar with the antibodies, reagents and methods used, to interpret the stained slide. Staining must be performed in a certified, licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
7. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit non-specific staining with horseradish peroxidase.[16]
8. Reagents may demonstrate unexpected reactions in previously untested tissue types. The possibility of unexpected reactions in tested tissue types cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues. Contact Agilent Pathology Support with documented unexpected reactions.
9. False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudo peroxidase activity (erythrocytes) and endogenous peroxidase activity (cytochrome c).[13]
10. The reagents and instructions supplied in this system have been designed for optimal performance. Further dilution of the reagents or alteration of incubation times or temperatures may give erroneous or discordant results.

15.2 Product-Specific Limitations

1. False-negative results could be caused by degradation of the antigen in the tissues over time. Specimens should be stained within the cut section storage criteria. Refer to 'Specimen Preparation' Section 8.
2. For optimal and reproducible results, the PD-L1 protein requires target retrieval pre-treatment when tissues are fixed in neutral buffered formalin and paraffin embedded.
3. Do not substitute reagents from different lot numbers of this product, or from kits of other manufacturers. The only exception is the EnVision FLEX Target Retrieval Solution, Low pH (50x), which, if required, is available as Code K8005.
4. Stained control cell lines should be used only for validation of the staining run and should not be used as a guide to score the staining reaction in patient tissue sections.
5. Use of PD-L1 IHC 28-8 pharmDx on tissues with fixatives other than 10% neutral buffered formalin has not been validated.
6. Excisional, incisional, punch or core needle biopsies were considered acceptable sample types for the clinical trials using PD-L1 IHC 28-8 pharmDx. Fine needle aspirates or other cytology specimens were insufficient for biomarker analysis and were excluded from the clinical trials using PD-L1 IHC 28-8 pharmDx.

16. Non-Clinical Performance Evaluation

16.1 Analytical Specificity for PD-L1 IHC 28-8 pharmDx

The primary antibody for PD-L1 IHC 28-8 pharmDx is a rabbit monoclonal anti-human PD-L1 antibody, clone 28-8. The immunogen used for the antibody generation is a purified recombinant human PD-L1 containing the extracellular domain (Phe19-Thr239) of human PD-L1. IHC staining with the PD-L1 primary antibody showed no cross-reactivity for PD-L2 exogenously expressed in Chinese hamster ovary (CHO) cells.

PD-L1 IHC 28-8 pharmDx specifically detects PD-L1 membrane protein expressed in tumor cells in FFPE tissues, which can be completely abolished by the addition of PD-L1 antigen. PD-L1 IHC 28-8 pharmDx does not detect PD-L1 membrane protein in PD-L1 knock-out tumor cells in which the PD-L1 gene is genetically deleted.

16.2 Normal and Neoplastic Tissues

Table 3 summarizes Monoclonal Rabbit Anti-Human PD-L1 immunoreactivity on the recommended panel of normal tissues. Table 4 summarizes Monoclonal Rabbit Anti-Human PD-L1 immunoreactivity on neoplastic tissues in multi-tumor tissue microarrays. All tissues were FFPE and stained with PD-L1 IHC 28-8 pharmDx according to the instructions in this package insert. PD-L1 IHC 28-8 pharmDx detected PD-L1 protein localized in the plasma membrane of cell types known to express the PD-L1 antigen such as immune cells and cells of epithelial origin mainly tumor cells.

Table 3: Summary of PD-L1 IHC 28-8 pharmDx Normal Tissue Reactivity

Tissue Type (# tested)	Positive Plasma Membrane Staining: Tissue Elements	Positive Cytoplasmic Staining: Tissue Elements
Adrenal (3)	3/3 Medullary cells	3/3 Medullary cells
Bladder (3)	1/3 Urothelium	1/3 Smooth muscle, urothelium
Bone marrow (3)	3/3 Megakaryocytes	3/3 Megakaryocytes
Breast (3)	0/3	0/3
Cerebellum (3)	0/3	0/3
Cerebrum (3)	0/3	0/3
Cervix (3)	1/3 Epithelium	1/3 Epithelium
Colon (3)	2/3 Macrophages	0/3
Esophagus (3)	0/3	0/3
Kidney (3)	3/3 Tubular epithelium	3/3 Tubular epithelium
Liver (3)	2/3 Immune cells	2/3 Immune cells
Lung (3)	3/3 Alveolar macrophages	0/3
Mesothelial cells (3)	0/3	0/3
Muscle, cardiac (3)	0/2*	0/2*
Muscle, skeletal (3)	0/2*	0/2*
Nerve, peripheral (3)	0/3	0/3
Ovary (3)	0/3	0/3
Pancreas (3)	3/3 Epithelium (mainly islet cells)	3/3 Epithelium (mainly islet cells)
Parathyroid (3)	3/3 Epithelium	0/3

Tissue Type (# tested)	Positive Plasma Membrane Staining: Tissue Elements	Positive Cytoplasmic Staining: Tissue Elements
Pituitary (3)	1/3 Anterior adenohypophysis	1/3 Anterior adenohypophysis 3/3 Posterior neurohypophysis
Prostate (3)	0/2*	0/2*
Salivary gland (3)	0/3	0/3
Skin (3)	0/3	1/3 Epithelium
Small intestine (3)	0/2*	0/2*
Spleen (3)	1/3 Macrophages 3/3 Littoral cell	0/3
Stomach (3)	0/3	0/3
Testis (3)	0/3	1/3 Leydig cells
Thymus (3)	3/3 Medullary epithelium	0/3
Thyroid (3)	0/3	0/3
Tonsil (3)	3/3 Crypt epithelium 3/3 Germinal center (immune cells)	0/3
Uterus (3)	0/3	0/3

* One of the three samples tested for this tissue type was observed to demonstrate drying artifact or strong background staining which precluded scoring, therefore the results from this sample are not reported.

Table 4: Summary of PD-L1 IHC 28-8 pharmDx Neoplastic Tissue Reactivity

Tumor Type	Location / Organ	PD-L1 positive/total (N=162)
Adenocarcinoma	Appendix	1/1
	Breast, DCIS	0/2
	Breast, invasive ductal	3/7
	Breast, invasive ductal metastatic to lymph node	1/1
	Bronchoalveolar carcinoma, lung	0/1
	Cervix, endocervical type	0/1
	Colon	2/5
	Colon, metastatic to liver	1/1
	Colon, mucinous	0/1
	Esophagus	1/1
	Gallbladder	2/4
	GI, metastatic to lung	0/1
	Head & neck, hard palate	0/1
	Lung	2/5
	Ovary	0/1
	Ovary, endometrioid	0/1
	Ovary, mucinous	0/1
	Ovary, serous	0/1
	Pancreas	1/2
	Pancreas, ductal	0/3
	Prostate	2/4
	Rectum	2/4
	Salivary/parotid gland	0/2
	Small Intestine	0/2
	Stomach	1/6
	Stomach, mucinous	0/1
	Thyroid, follicular	0/1
	Thyroid, follicular-papillary	0/1
	Thyroid, papillary	0/3
	Uterus, clear cell	1/1
	Uterus, endometrium	1/3
Adrenocortical carcinoma	Adrenal	0/1
Astrocytoma	Cerebrum	0/3
Basal cell carcinoma	Skin	0/1
Carcinoma	Nasopharyngeal, NPC	0/1
Chordoma	Pelvic cavity	0/1
Embryonal carcinoma	Testis	0/1
Ependymoma	Brain	0/1
Glioblastoma	Brain	0/1
Hepatoblastoma	Liver	0/1
Hepatocellular carcinoma	Liver	1/5
Islet cell tumor	Pancreas	0/1
Interstitialoma	Colon	0/1
	Rectum	0/1
	Small intestine	0/1
Large cell carcinoma	Lung	1/1
Liposarcoma	Abdominal cavity, mucinous	0/1

Tumor Type	Location / Organ	PD-L1 positive/total (N=162)
Lymphoma		
Anaplastic large cell	Lymph node	1/1
Diffuse B-cell	Lymph node	2/4
Hodgkin	Lymph node	2/2
Non-Hodgkin	Lymph node	1/1
Medullablastoma	Brain	0/1
Medullary carcinoma	Thyroid	0/1
Melanoma	Rectum	0/1
	Nasal cavity	0/1
Meningioma	Brain	0/2
Mesothelioma	Peritoneum	0/1
Neuroblastoma	Retroperitoneum	0/1
Neurofibroma	Soft tissue, lower back	0/1
Primitive neuroectodermal	Retroperitoneum	0/1
Renal cell carcinoma		
Papillary	Kidney	0/1
Clear cell	Kidney	0/6
Sarcoma		
Chondrosarcoma	Bone	0/1
Clear cell	Abdominal wall	0/1
Osteosarcoma	Bone	0/2
Leiomyosarcoma	Soft tissue, chest wall	0/1
	Bladder	0/1
Liposarcoma	Abdominal cavity, mucinous	0/1
Rhabdomyosarcoma	Soft tissue, embryonal	0/1
	Prostate	0/1
	Retroperitoneum	0/1
Synovial sarcoma	Pelvic cavity	0/1
Seminoma	Testis	0/2
Signet ring cell carcinoma	Metastatic colon signet ring cell carcinoma to ovary	0/1
	Colon	0/1
Small cell carcinoma	Lung	1/2
Spermatocytoma	Testis	0/2
Squamous cell carcinoma	Metastatic esophageal squamous cell carcinoma to lymph node	1/1
	Cervix	2/4
	Esophagus	4/7
	Head & neck	0/2
	Lung	1/3
	Skin	1/2
	Uterus	1/1
Thymoma	Mediastinum	1/1
Transitional cell carcinoma	Bladder	3/6
	Kidney	0/1

17. Performance Evaluation

17.1 Performance Evaluation: NSCLC ≥ 1% PD-L1 Expression Cutoff

17.1.1 Analytical Sensitivity: NSCLC

Analytical sensitivity of PD-L1 IHC 28-8 pharmDx was tested on 186 unique cases of human NSCLC FFPE specimens staged I to IV using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a range of 0-100% positive tumor cells and 0-3 staining intensity.

17.1.2 Precision/External Reproducibility: NSCLC

The Precision and External Reproducibility of PD-L1 IHC 28-8 pharmDx was evaluated at Agilent and three external testing sites respectively. Negative percent agreement (NPA), positive percent agreement (PPA), overall agreement (OA), and corresponding two-sided 95% confidence intervals were calculated and reported in Table 5 and Table 6. Comparisons were made using majority diagnostic outcome across all observations of a given specimen as reference.

Table 5: Precision of PD-L1 IHC 28-8 pharmDx: NSCLC ≥ 1% PD-L1 Expression Cutoff

Precision Studies	Method	% Agreement (95% CI)
		≥ 1% PD-L1 Expression Cutoff
Inter-instrument	Each of 90 NSCLC specimens with a range of PD-L1 IHC expression was tested twice on each of three Autostainer Link 48 instruments. A total of 539 comparisons made to majority call were performed.	NPA 97.8 (94.9-100.0) PPA 98.5 (96.2-100.0) OA 98.1 (96.3-99.6)
Inter-operator/analyst	Each of 90 NSCLC specimens with a range of PD-L1 IHC expression was tested twice by three operators on one Autostainer Link 48. A total of 539 comparisons made to majority call were performed.	NPA 99.0 (97.6-100.0) PPA 100.0 (98.5-100.0) OA 99.4 (98.7-100.0)
Inter-day	Each of 90 NSCLC specimens with a range of PD-L1 IHC expression was tested with one replicate over five non-consecutive days on the Autostainer Link 48 instrument. A total of 445 comparisons made to majority call were performed.	NPA 98.4 (96.0-100.0) PPA 99.0 (97.5-100.0) OA 98.7 (97.1-99.8)
Intra-run	Each of 90 NSCLC specimens with a range of PD-L1 IHC expression was tested with 5 replicates within a run on the Autostainer Link 48 instrument. A total of 447 comparisons made to majority call were performed.	NPA 98.6 (96.4-100.0) PPA 98.7 (97.0-100.0) OA 98.7 (97.3-99.8)
Inter-Lot	Each of 36 NSCLC specimens with a range of PD-L1 IHC expression was tested with two replicates with each of three assay build lots on the Autostainer Link 48 instrument. A total of 360 comparisons made to majority call were performed.	NPA 100 (97.3-100.0) PPA 99.1 (97.3-100.0) OA 99.4 (98.3-100.0)

Table 6: Reproducibility of the PD-L1 IHC 28-8 pharmDx: NSCLC ≥ 1% PD-L1 Expression Cutoff

Reproducibility	Method	% Agreement (95% CI)
		≥ 1% PD-L1 Expression Cutoff
Inter-site assay (three sites)	Each of 80 NSCLC specimens with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 1198 comparisons made to majority call.	NPA 98.5 (97.1-99.6) PPA 99.2 (97.6-100.0) OA 98.9 (97.8-99.7)
Intra-site assay	Each of 80 NSCLC specimens with a range of PD-L1 IHC expression was tested on five non-consecutive days. Intra-site analysis was performed for three sites on a total of 1198 comparisons made to majority call.	NPA 98.1 (96.6-99.4) PPA 99.6 (98.9-100.0) OA 99.0 (98.1-99.7)
Inter-observer (one observer at each of three sites)	Scoring of 130 NSCLC specimens with a range of PD-L1 IHC expression was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 1170 comparisons made to majority call.	NPA 95.8 (92.9-98.2) PPA 99.2 (97.8-100.0) OA 97.9 (96.5-99.0)
Intra-observer	Scoring of 130 NSCLC specimens with a range of PD-L1 IHC expression was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 1170 comparisons made to majority call.	NPA 97.7 (96.2-99.1) PPA 99.6 (99.0-100.0) OA 98.9 (98.2-99.5)

17.1.3 Clinical Performance Evaluation: NSCLC (First-line Treatment)

Clinical performance of PD-L1 IHC 28-8 pharmDx was evaluated in CHECKMATE-227, a Phase 3, randomized, open-label, multicenter, multi-part, trial in subjects with chemotherapy-naïve Stage IV or recurrent NSCLC, who were previously untreated for advanced disease. Clinical validation of the device in this study focuses on the cohort of subjects in Part 1a of the study, which was limited to the population of patients with PD-L1 expression ≥ 1%, treated with the combination of nivolumab with ipilimumab vs chemotherapy, and stratified by histology (squamous vs non-squamous). Among the patients randomized to Part 1a, 793 patients were randomized 1:1 to receive either nivolumab (OPDIVO) 3 mg/kg in combination with ipilimumab (YERVOY) 1 mg/kg (n=396) or histology-based platinum-doublet chemotherapy of gemcitabine with either cisplatin or carboplatin for squamous, and pemetrexed with cisplatin or carboplatin for non-squamous NSCLC (n=397). Pre-study (baseline) tumor tissue specimens were collected prior to randomization to conduct pre-planned analyses of efficacy according to predefined PD-L1 expression levels. The primary efficacy outcome measure was overall survival (OS). Additional efficacy outcome measures included BICR-assessed progression-free survival (PFS), overall response rate (ORR), and duration of response (DoR).

The baseline demographic and disease characteristics were generally balanced between randomized subjects in the nivolumab + ipilimumab and chemotherapy groups. The median age was 64 years (range: 26 to 87) with 49% of patients ≥ 65 years and 10% of patients ≥ 75 years, 76% White, 65% male. Baseline ECOG performance status was 0 (34%) or 1 (65%), 50% with PD-L1 ≥ 50%, 29% with squamous and 71% with non-squamous histology, 10% had brain metastases, and 85% were former/current smokers.

The study demonstrated a statistically significant improvement in OS for PD-L1 ≥ 1% patients and a clinically meaningful benefit in PFS, ORR, and DoR compared to platinum-doublet chemotherapy alone (median OS (95% CI) was 17.1 (15, 20.1) months with nivolumab + ipilimumab and 14.9 (12.7, 16.7) with chemotherapy, HR = 0.79 (97.72% CI: 0.65, 0.96); stratified log-rank test p-value = 0.0066). Efficacy results are presented in Table 7 and the Kaplan-Meier plot for OS is shown in Figure 1.

Table 7: Efficacy Results (PD-L1 ≥ 1%) - CHECKMATE-227

	Nivolumab and Ipilimumab (n=396)	Chemotherapy (n=397)
Overall Survival		
Events (%)	258 (65.2)	298 (75.1)
Median (months)[a] (95% CI)	17.1 (15, 20.1)	14.9 (12.7, 16.7)
Hazard ratio (97.72% CI)[b]	0.79 (0.65, 0.96)	
Stratified log-rank p-value	0.0066	

[a] Kaplan-Meier estimate.
[b] Based on a stratified Cox proportional hazard model.

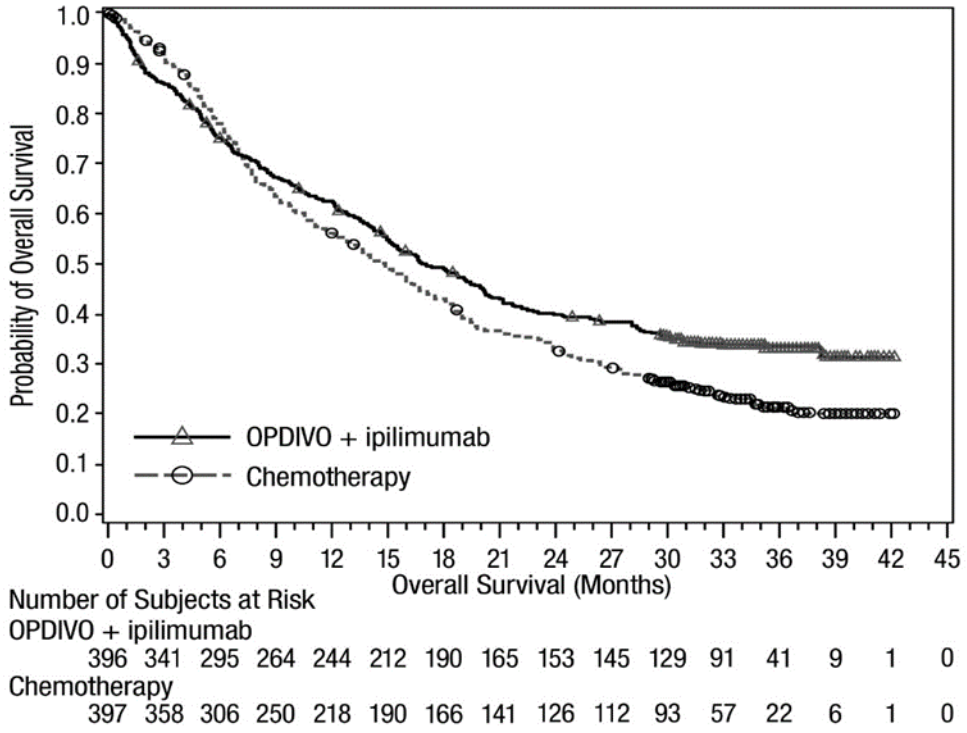


Figure 1: Overall Survival (PD-L1 ≥ 1%) - CHECKMATE-227

BICR-assessed PFS showed a HR of 0.82 (95% CI: 0.69, 0.97), with a median PFS of 5.1 months (95% CI: 4.1, 6.3) in the nivolumab and ipilimumab arm and 5.6 months (95% CI: 4.6, 5.8) in the platinum-doublet chemotherapy arm. The BICR-assessed confirmed ORR was 36% (95% CI: 31, 41) in the nivolumab and ipilimumab arm and 30% (95% CI: 26, 35) in the platinum-doublet chemotherapy arm. Median duration of response observed in the nivolumab and ipilimumab arm was 23.2 months and 6.2 months in the platinum-doublet chemotherapy arm.

17.2 Performance Evaluation: nsNSCLC ≥ 1%, ≥ 5%, ≥ 10% PD-L1 Expression Cutoffs

17.2.1 Analytical Sensitivity: nsNSCLC

Analytical sensitivity of PD-L1 IHC 28-8 pharmDx was tested on 112 unique cases of n nsNSCLC FFPE specimens staged I to IV using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a range of 0-100% positive tumor cells and 0-3 staining intensity.

17.2.2 Precision/External Reproducibility: nsNSCLC

The Precision and External Reproducibility of PD-L1 IHC 28-8 pharmDx was evaluated at Agilent and three external testing sites respectively. The performance data are provided in Table 8 and Table 9. Negative percent agreement (NPA), positive percent agreement (PPA) and overall agreement (OA) of independent pair-wise comparison of the tests were determined for each PD-L1

expression cutoff evaluated. The most frequently occurring observation was applied as a reference to calculate the NPA, PPA, OA and the corresponding 95% confidence intervals.

Table 8: Precision of PD-L1 IHC 28-8 pharmDx – nsNSCLC ≥ 1%, ≥ 5%, ≥ 10% PD-L1 Expression Cutoffs

Precision Studies	Method	% Agreement (95% CI)		
		≥ 1% PD-L1 Expression Cutoff	≥ 5% PD-L1 Expression Cutoff	≥ 10% PD-L1 Expression Cutoff
Inter-instrument	Each of 34 nsNSCLC specimens with a range of PD-L1 IHC expression was tested on each of three Autostainer Link 48 instruments, repeated twice. The slides were blinded and randomized prior to scoring. A total of 204 independent pair-wise comparisons were performed.	NPA 100.0 (96.9, 100.0) PPA 100.0 (95.6, 100.0) OA 100.0 (98.2, 100.0)	NPA 100.0 (96.9, 100.0) PPA 98.8 (93.6, 99.8) OA 99.5 (97.3, 99.9)	NPA 98.1 (93.5, 99.5) PPA 100.0 (96.2, 100.0) OA 99.0 (96.5, 99.7)
Inter-analyst	Each of 34 nsNSCLC specimens with a range of PD-L1 IHC expression was tested by three analysts, repeated twice on one Autostainer Link 48 instrument. The slides were blinded and randomized prior to scoring. A total of 204 independent pair-wise comparisons were performed.	NPA 100.0 (96.9, 100.0) PPA 100.0 (95.6 100.0) OA 100.0 (98.2, 100.0)	NPA 100.0 (96.9, 100.0) PPA 100.0 (95.6, 100.0) OA 100.0 (98.2, 100.0)	NPA 100.0 (96.6, 100.0) PPA 97.9 (92.7, 99.4) OA 99.0 (96.5, 99.7)
Inter-day	Each of 34 nsNSCLC specimens with a range of PD-L1 IHC expression was tested over five non-consecutive days on the Autostainer Link 48 instrument. The slides were blinded and randomized prior to scoring. A total of 170 independent pair-wise comparisons were performed.	NPA 100.0 (96.3, 100.0) PPA 100.0 (94.8, 100.0) OA 100.0 (97.8, 100.0)	NPA 100.0 (96.3, 100.0) PPA 100.0 (94.8, 100.0) OA 100.0 (97.8, 100.0)	NPA 100.0 (95.9, 100.0) PPA 98.8 (93.3, 99.8) OA 99.4 (96.7, 99.9)
Inter-lot	Each of 20 nsNSCLC specimens with a range of PD-L1 IHC expression was tested with two replicates with each of five reagent lots on the Autostainer Link 48 instrument. A total of 160 independent pair-wise comparisons were performed.	NPA 100.0 (94.3, 100.0) PPA 100.0 (96.2, 100.0) OA 100.0 (97.7, 100.0)	NPA 100.0 (95.4, 100.0) PPA 100.0 (95.4, 100.0) OA 100.0 (97.7, 100.0)	NPA 100.0 (96.4, 100.0) PPA 100.0 (93.6, 100.0) OA 100.0 (97.7, 100.0)
Intra-run	Each of 34 nsNSCLC specimens with a range of PD-L1 IHC expression was tested with five replicates within a run on the Autostainer Link 48 instrument. The slides were blinded and randomized prior to scoring. A total of 167 (≥ 10%) and 168 (≥ 1% and ≥ 5%) independent pair-wise comparisons were performed.	NPA 97.8 (92.3, 99.4) PPA 98.7 (93.1, 99.8) OA 98.2 (94.9, 99.4)	NPA 98.0 (93.0, 99.4) PPA 97.1 (89.9, 99.2) OA 97.6 (94.0, 99.1)	NPA 96.5 (90.1, 98.8) PPA 96.3 (89.8, 98.7) OA 96.4 (92.4, 98.3)

Table 9: Reproducibility of the PD-L1 IHC 28-8 pharmDx - nsNSCLC, tested at three external sites ≥ 1%, ≥ 5%, ≥ 10% PD-L1 Expression Cutoffs

Reproducibility	Method	% Agreement (95% CI)		
		≥ 1% PD-L1 Expression Cutoff	≥ 5% PD-L1 Expression Cutoff	≥ 10% PD-L1 Expression Cutoff
Inter-site assay (three sites)	Each of 24 nsNSCLC specimens with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 360 independent pair-wise comparisons.	NPA 89.6 (83.3, 93.7) PPA 87.1 (82.1, 90.9) OA 88.1 (84.3, 91.0)	NPA 88.0 (83.1, 91.6) PPA 92.6 (86.9, 95.9) OA 89.7 (86.2, 92.5)	NPA 90.8 (86.5, 93.9) PPA 94.2 (88.5, 97.2) OA 91.9 (88.7, 94.3)
Intra-site assay	Each of 24 nsNSCLC specimens with a range of PD-L1 IHC expression was tested on five non-consecutive days at each of three study sites. Intra-site analysis was performed for three sites on a total of 360 independent pair-wise comparisons.	NPA 95.3 (90.7, 97.7) PPA 96.7 (93.3, 98.4) OA 96.1 (93.6, 97.7)	NPA 96.6 (93.1, 98.3) PPA 93.6 (88.5, 96.5) OA 95.3 (92.6, 97.0)	NPA 97.7 (94.8, 99.0) PPA 92.9 (87.4, 96.1) OA 95.8 (93.2, 97.5)
Inter-observer (one observer at each of three sites)	Scoring of 30 nsNSCLC specimens with a range of PD-L1 IHC expression, stained with PD-L1 IHC 28-8 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 270 independent pair-wise comparisons.	NPA 84.0 (74.5, 90.4) PPA 92.6 (88.0, 95.5) OA 90.0 (85.8, 93.0)	NPA 94.4 (88.4, 97.4) PPA 94.4 (89.8, 97.1) OA 94.4 (91.0, 96.6)	NPA 93.3 (87.8, 96.5) PPA 88.9 (82.5, 93.2) OA 91.1 (87.1, 94.0)
Intra-observer (one observer at each of three sites)	Scoring of 30 nsNSCLC specimens with a range of PD-L1 IHC expression, stained with PD-L1 IHC 28-8 pharmDx, was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 270 independent pair-wise comparisons.	NPA 91.4 (83.2, 95.8) PPA 95.8 (91.9, 97.8) OA 94.4 (91.0, 96.6)	NPA 94.7 (89.0, 97.6) PPA 98.1 (94.5, 99.3) OA 96.7 (93.8, 98.2)	NPA 97.8 (93.7, 99.2) PPA 93.3 (87.8, 96.5) OA 95.6 (92.4, 97.4)

17.2.3 Clinical Performance Evaluation: nsNSCLC (Previously Treated)

Clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in CHECKMATE-057, a Phase 3, randomized, open-label study of nivolumab vs docetaxel in adult (≥ 18 years) subjects with advanced or metastatic nsNSCLC after failure of prior platinum doublet -based chemotherapy. A total of 582 subjects were randomized at 112 sites in 22 countries (Argentina, Australia, Austria, Brazil, Canada, Chile, Czech Republic, France, Germany, Hong Kong, Hungary, Italy, Mexico, Norway, Peru, Poland, Romania, Russian Federation, Singapore, Spain, Switzerland, and United States). Subjects were randomized 1:1 and stratified according to 1) prior use of maintenance therapy vs. no use of maintenance therapy and 2) second-line vs. third-line therapy. Pre-study (baseline) tumor tissue specimens were collected prior to randomization and prior to first treatment to conduct pre-planned analyses of efficacy according to predefined baseline PD-L1 expression levels (secondary objective). The primary endpoint was overall survival (OS). Other secondary endpoints were objective response rate (ORR), progression-free survival (PFS), and disease-related symptom improvement by 12 weeks, as measured by the Lung Cancer Symptom Scale (LCSS).

The baseline demographic and disease characteristics were generally balanced between randomized subjects in the nivolumab and docetaxel groups. The mean age was 62 years (range: 21 to 85) with 34% ≥65 years of age and 7% ≥75 years of age. The majority of patients were white (92%) and male (55%); baseline ECOG performance status was 0 (31%) or 1 (69%). Seventy-nine percent of patients were former/current smokers. Tumor specimens were collected from nsNSCLC tumors, consistent with the inclusion requirements for the study. Frequencies of PD-L1 expression at each of the predefined baseline expression levels in all randomized subjects in CHECKMATE-057 are presented in Table 10.

Table 10: Frequency of Pre-Study PD-L1 Expression in All Randomized Subjects with nsNSCLC – CHECKMATE-057

Population PD-L1 Expression Category	Nivolumab 3 mg/kg (N = 292)	Docetaxel (N = 290)	Total (N = 582)
Overall	292	290	582
PD-L1 Quantifiable at Baseline (N(%))	231 (79.1)	224 (77.2)	455 (78.2)
Baseline PD-L1 Expression ≥ 1%	123/231 (53.2)	123/224 (54.9)	246/455 (54.1)
Baseline PD-L1 Expression < 1%	108/231 (46.8)	101/224 (45.1)	209/455 (45.9)
Baseline PD-L1 Expression ≥ 5%	95/231 (41.1)	86/224 (38.4)	181/455 (39.8)
Baseline PD-L1 Expression < 5%	136/231 (58.9)	138/224 (61.6)	274/455 (60.2)
Baseline PD-L1 Expression ≥ 10%	86/231 (37.2)	79/224 (35.3)	165/455 (36.3)
Baseline PD-L1 Expression < 10%	145/231 (62.8)	145/224 (64.7)	290/455 (63.7)
Without PD-L1 Expression at Baseline (N(%))	61 (20.9)	66 (22.8)	127 (21.8)

Patients with PD-L1 expression by all predefined expression levels in the OPDIVO group were associated with enhanced survival compared to docetaxel, whereas survival was similar to docetaxel in patients with no PD-L1 expression. Meaningful differences in median OS were observed in nivolumab over docetaxel subgroups when analyzed by PD-L1 expression level. Median OS was 17.1, 18.2, and 19.4 months for nivolumab subjects compared to 9.0, 8.1, and 8.0 months for docetaxel subjects with ≥ 1%, ≥ 5%, and ≥ 10% PD-L1 expression levels, respectively. There were no differences in OS between the treatment groups in subjects with < 1%, < 5%, and < 10% expression levels, with ranges of median OS of 9.7 to 10.4 months for nivolumab and 10.1 to 10.3 months for docetaxel. The unstratified hazard ratios (HR) and median overall survival (OS) are presented in Figure 2. The Kaplan-Meier plot for subgroups by PD-L1 expression level is shown in Figure 3 and Figure 4.

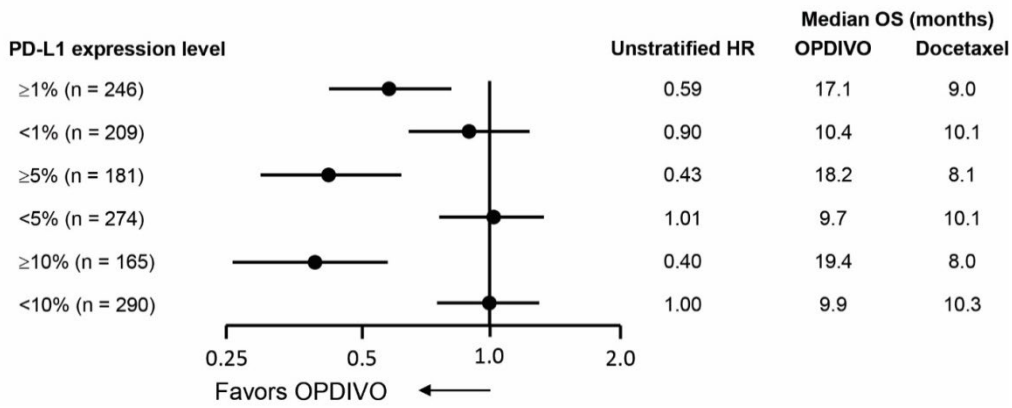


Figure 2: Forest Plot - OS Based on PD-L1 Expression in nsNSCLC Patients – CHECKMATE-057

Note: The unstratified hazard ratio and the corresponding 95% CI were estimated in a Cox proportional hazards model using the randomized arm as a single covariate

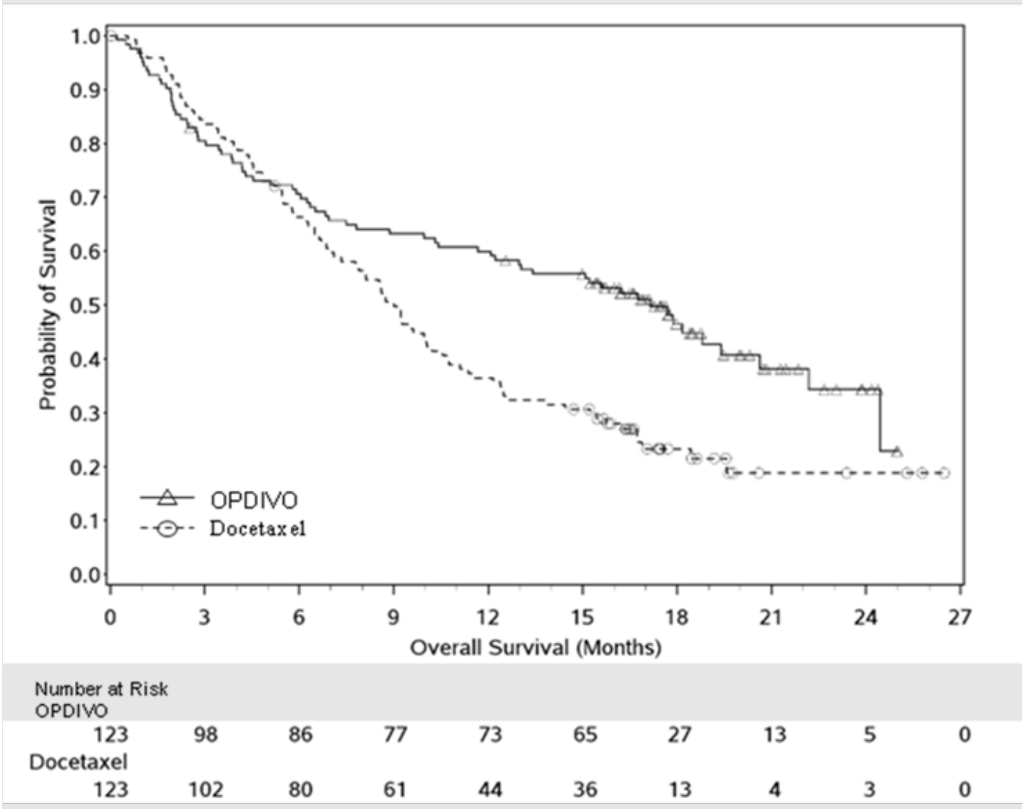


Figure 3: Overall Survival - nsNSCLC Patients with ≥ 1% PD-L1 Expression – CHECKMATE-057

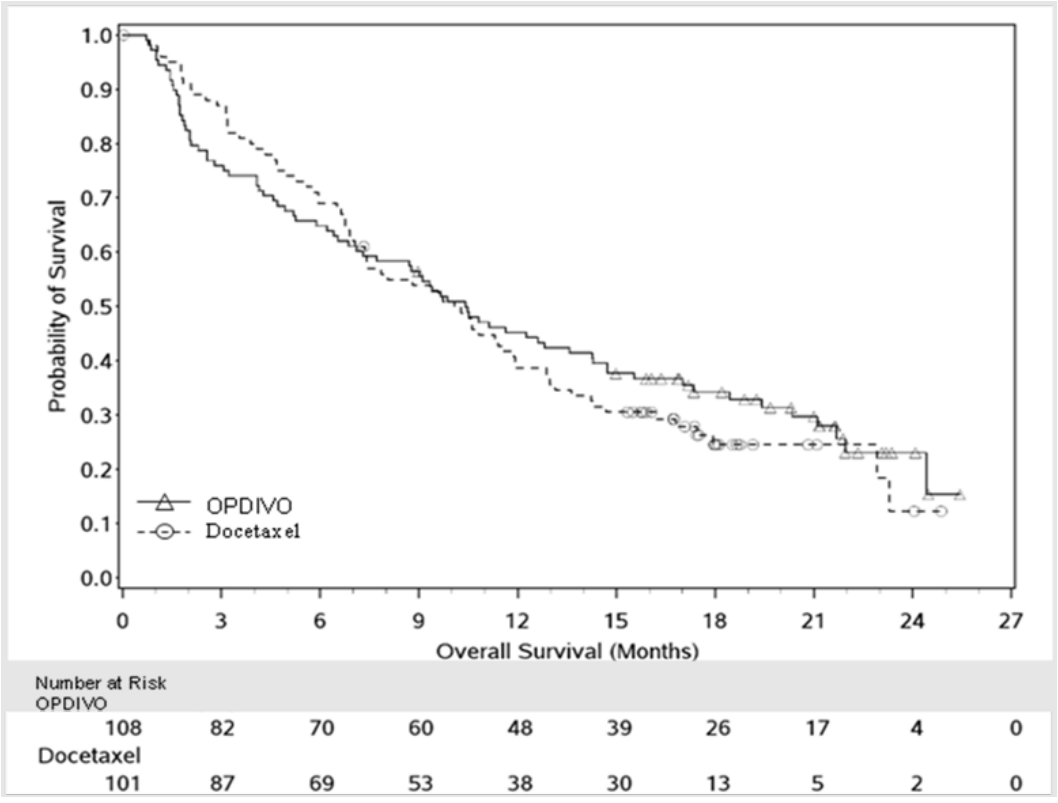


Figure 4: Overall Survival - nsNSCLC Patients with < 1% PD-L1 Expression – CHECKMATE-057

17.3 Performance Evaluation: SCCHN

17.3.1 Analytical Sensitivity: SCCHN

Analytical sensitivity of PD-L1 IHC 28-8 pharmDx was tested on 236 unique cases of SCCHN FFPE specimens staged I to IV using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a range of 0-95% positive tumor cells and 0-3 staining intensity.

17.3.2 Precision/External Reproducibility: SCCHN

The Precision and External Reproducibility of PD-L1 IHC 28-8 pharmDx was evaluated at Agilent and three external testing sites, respectively. Average negative agreement (ANA), average positive agreement (APA), overall agreement (OA), and corresponding 95% confidence intervals were performed and are reported in Table 11 and Table 12.

Table 11: Precision of PD-L1 IHC 28-8 pharmDx – SCCHN

Precision Study	Method	% Agreement (95% CI)
		≥ 1% PD-L1 Expression Cutoff
Inter-Lot	Each of 39 SCCHN specimens with a range of PD-L1 IHC expression was tested with each of three assay build lots on the Autostainer Link 48 instrument. A total of 115 pair-wise comparisons were performed.	ANA 100.0 (96.9, 100.0) APA 100.0 (96.6, 100.0) OA 100.0 (98.4, 100.0)

Table 12: Reproducibility of the PD-L1 IHC 28-8 pharmDx - SCCHN, tested at three external sites

Reproducibility	Method	% Agreement (95% CI)
		≥ 1% PD-L1 Expression Cutoff
Inter-site assay (three sites)	A set of 32 SCCHN specimens, with a range of PD-L1 IHC expression, was tested on five non-consecutive days at each of three sites. Inter-site analysis was performed between three sites on a total of 2400 pair-wise comparisons.	ANA 96.0 (91.5, 99.2) APA 96.3 (92.9, 99.2) OA 96.2 (92.2, 99.2)
Intra-site assay	A set of 32 SCCHN specimens, with a range of PD-L1 IHC expression, was tested on five non-consecutive days at each of three sites. Intra-site analysis was performed for three sites on a total of 960 pair-wise comparisons.	ANA 97.2 (94.7, 99.1) APA 97.4 (95.3, 99.2) OA 97.3 (95.0, 99.2)
Inter-observer (three observers)	Three separate scoring evaluations of a set of 38 SCCHN specimens, demonstrating a range of PD-L1 IHC expression, stained with PD-L1 IHC 28-8 pharmDx, were performed by three pathologists, with a minimum of a 14-day washout period between reads. Inter-observer analysis was performed between three pathologists on a total of 1026 pair-wise comparisons.	ANA 97.1 (94.6, 99.4) APA 97.1 (94.7, 99.4) OA 97.1 (94.7, 99.4)
Intra-observer	Three separate scoring evaluations of a set of 38 SCCHN specimens, demonstrating a range of PD-L1 IHC expression, stained with PD-L1 IHC 28-8 pharmDx, were performed by three pathologists, with a minimum of a 14-day washout period between reads. Intra-observer analysis was performed for three pathologists on a total of 342 pair-wise comparisons.	ANA 97.1 (94.2, 99.4) APA 97.1 (94.3, 99.4) OA 97.1 (94.2, 99.4)

17.3.3 Clinical Performance Evaluation: SCCHN

Clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in CHECKMATE-141, an open label, randomized Phase 3 clinical trial of nivolumab vs therapy of investigator's choice in recurrent or metastatic platinum-refractory squamous cell carcinoma of the head and neck (SCCHN). Patients were randomized at 55 sites in 15 countries (Argentina, Brazil, Canada, France, Germany, Hong Kong, Italy, Japan, Korea, Netherlands, Spain, Switzerland, Taiwan, United Kingdom, and United States of America). Subjects were randomized 2:1 (nivolumab: investigator's choice) and stratified according to prior cetuximab treatment (yes/no). Pre-study (baseline) tumor tissue specimens were collected prior to randomization and prior to first treatment to conduct pre-planned analyses of efficacy according to predefined baseline PD-L1 expression levels (exploratory objective). The major efficacy outcome measure was OS. Additional efficacy outcome measures were PFS and ORR.

In this trial, a total of 361 patients were randomized; 240 patients to OPDIVO and 121 patients to investigator's choice (45% received docetaxel, 43% received methotrexate, and 12% received cetuximab). The median age was 60 years (range: 28 to 83) with 31% ≥ 65 years of age, 83% were White, 12% Asian, and 4% were Black, and 83% male. Baseline ECOG performance status was 0 (20%) or 1 (78%), 76% were former/current smokers, 90% had Stage IV disease, 45% of patients received only one prior line of systemic therapy, the remaining 55% received two or more prior lines of systemic therapy, and 25% had HPV p16-positive tumors, 24% had HPV p16-negative tumors, and 51% had unknown status. Tumor specimens were collected from SCCHN tumors from either a primary or metastatic site, consistent with the inclusion requirements for the study. 327 subjects (out of 361 total subjects) had tumor tissue collected at baseline with the following site proportion: 29.7% primary, 52.0% metastasis, and 18.3% not reported. Frequencies of PD-L1 expression at each of the predefined baseline expression levels in all randomized subjects in CHECKMATE-141 are presented in Table 13.

Table 13: Frequency of Pre-Study PD-L1 Expression in All Randomized Subjects with SCCHN – CHECKMATE-141

Population PD-L1 Expression Category	Nivolumab 3 mg/kg (N = 240)	Investigator's Choice (N = 121)	Total (N = 361)
Overall	240	121	361
PD-L1 Quantifiable at Baseline (N (%))	161 (67.1)	99 (81.8)	260 (72.0)
Baseline PD-L1 Expression ≥ 1%	88/161 (54.7)	61/99 (61.6)	149/260 (57.3)
Baseline PD-L1 Expression < 1%	73/161 (45.3)	38/99 (38.4)	111/260 (42.7)
Without PD-L1 Expression at Baseline (N (%))	79 (32.9)	22 (18.2)	101 (28.0)

The Phase 3 CHECKMATE-141 trial demonstrated a statistically significant improvement in OS for subjects randomized to nivolumab as compared with investigator's choice at a pre-specified interim analysis (78% of the planned number of events for final analysis). The median OS was 7.5 months for nivolumab subjects compared to 5.1 months for investigator's choice subjects with a hazard ratio of 0.70 (95% CI: 0.53, 0.92).

In pre-specified exploratory subgroup analyses, using the PD-L1 IHC 28-8 pharmDx assay, the hazard ratio for survival was 0.89 (95% CI: 0.54, 1.45) with median survivals of 5.7 months and 5.8 months for the nivolumab and chemotherapy arms, respectively, in patients with < 1% PD-L1 expression. The HR for survival was 0.55 (95% CI: 0.36, 0.83) with median survivals of 8.7 months and 4.6 months for the nivolumab and chemotherapy arms, respectively, in subjects with ≥ 1% PD-L1 expression.[15] The unstratified hazard ratios (HR) and median overall survival (OS) are presented in Table 14. Hazard ratios and median overall survival by PD-L1 expression level subgroup are shown in Table15.

Table 14: Overall Survival of SCCHN Patients in CHECKMATE-141 (minimum 11.4 months follow up)

	OPDIVO (n=240)	Investigator's Choice (n=121)
Overall Survival		
Deaths (%)	133 (55%)	85 (70%)
Median (months) (95% CI)	7.5 (5.5, 9.1)	5.1 (4.0, 6.0)
Hazard ratio (95% CI)[a]	0.70 (0.53, 0.92)	
p-value[b,c]	0.0101	

[a] Based on stratified proportional hazards model.
[b] Based on stratified log-rank test.
[c] p-value is compared with 0.0227 of the allocated alpha for this interim analysis.

Table 15: Median Overall Survival of SCCHN Patients by PD-L1 Expression Level Subgroup – CHECKMATE-141

	Median Overall Survival in months	
PD-L1 Expression Level	Nivolumab	Investigators choice therapy
< 1%	5.7	5.8
≥ 1%	8.7	4.6
	Hazard Ratios (95% CI)	
	Nivolumab vs. Investigators Choice	
< 1%	0.89 (0.54, 1.45)	
≥ 1%	0.55 (0.36, 0.83)	

17.4 Performance Evaluation: UC

17.4.1 Analytical Sensitivity: UC

Analytical sensitivity of PD-L1 IHC 28-8 pharmDx was tested on 138 unique cases of human UC FFPE specimens staged III to IV using a manufactured production lot. Assessment of PD-L1 expression demonstrated staining across a range of 0-90% positive tumor cells and 0-3 staining intensity.

17.4.2 Precision/External Reproducibility: UC

The Precision and External Reproducibility of PD-L1 IHC 28-8 pharmDx was evaluated at Agilent and three external testing sites respectively. Average negative agreement (ANA), average positive agreement (APA), overall agreement (OA), and corresponding 95% confidence intervals were performed and are reported in Table 16 and Table 17.

Table 16: Precision of PD-L1 IHC 28-8 pharmDx – UC

Precision Study	Method	% Agreement (95% CI)
		≥ 1% PD-L1 Expression Cutoff
Combined Precision	Each of 29* urothelial carcinoma specimens with a range of PD-L1 IHC expression was tested in the following conditions: 5 operators/instruments/days using one assay lot. A total of 286 independent pair-wise comparisons were performed.	ANA 96.6 (91.7, 100.0) APA 96.4 (90.3, 100.0) OA 96.5 (91.0, 100.0)
Inter-observer	Each of 72 urothelial carcinoma specimens with a range of PD-L1 IHC expression were read 3 times by 3 different pathologists with a 2-week washout period in between reads. A total of 1944 independent pair-wise comparisons were performed.	ANA 90.3 (85.2, 94.8) APA 93.1 (89.2, 96.4) OA 91.9 (87.6, 95.7)
Intra-observer	Each of 72 urothelial carcinoma specimens with a range of PD-L1 IHC expression were read 3 times by the same pathologist with a 2-week washout period in between reads. A total of 3 pathologists were used and 648 independent pair-wise comparisons were performed.	ANA 95.2 (92.6, 97.4) APA 96.6 (94.6, 98.2) OA 96.0 (93.8, 97.8)
Inter-lot	Each of 29* urothelial carcinoma specimens with a range of PD-L1 IHC expression was tested with 3 different assay build lots on the Autostainer Link 48 instrument. A total of 87 independent pair-wise comparisons were performed.	ANA 97.8 (93.8, 100.0) APA 97.6 (92.3, 100.0) OA 97.7 (93.1, 100.0)
Intra-run	Each of 29* urothelial carcinoma specimens with a range of PD-L1 IHC expression was tested with five replicates within the same run by the same operator on the same Autostainer Link 48 instrument. A total of 290 independent pair-wise comparisons were performed.	ANA 96.3 (90.3, 100.0) APA 96.8 (92.7, 100.0) OA 96.6 (91.7, 100.0)

*A total of 30 urothelial carcinoma specimens were tested in combined precision, inter-lot, and intra-run studies, one specimen was found to be not evaluable.

Table 17: Reproducibility of the PD-L1 IHC 28-8 pharmDx – UC, tested at three external sites

Reproducibility	Method	% Agreement (95% CI)
		≥ 1% PD-L1 Expression Cutoff
Inter-site assay (three sites)	Each of 46 urothelial carcinoma specimens with a range of PD-L1 IHC expression was tested on five non-consecutive days. Inter-site analysis was performed between three sites on a total of 3440 pair-wise comparisons.	ANA 88.1 (82.2, 93.7) APA 85.8 (77.7, 92.7) OA 87.0 (80.5, 93.2)
Intra-site assay	Each of 46 urothelial carcinoma specimens with a range of PD-L1 IHC expression was tested on five non-consecutive days. Intra-site analysis was performed for three sites on a total of 1376 pair-wise comparisons.	ANA 93.2 (89.5, 96.5) APA 91.9 (87.2, 96.0) OA 92.6 (88.6, 96.2)
Inter-observer (one observer at each of three sites)	Scoring of 78 urothelial carcinoma specimens with a range of PD-L1 IHC expression was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Inter-observer analysis was performed between three sites on a total of 2106 pair-wise comparisons.	ANA 91.9 (87.8, 95.7) APA 92.5 (88.5, 96.1) OA 92.2 (88.1, 95.9)
Intra-observer	Scoring of 78 urothelial carcinoma specimens with a range of PD-L1 IHC expression was performed by three pathologists, one at each of three study sites, on three non-consecutive days. Intra-observer analysis was performed for three sites on a total of 702 pair-wise comparisons.	ANA 96.1 (94.0, 98.0) APA 96.4 (94.4, 98.2) OA 96.3 (94.3, 98.0)

17.4.3 Clinical Performance Evaluation: UC

Clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in CHECKMATE-275, A phase II single arm clinical trial of nivolumab in subjects with metastatic or unresectable urothelial cancer who have progressed or recurred following treatment with a platinum agent. A total of 270 subjects were randomized to nivolumab at 63 sites in 11 countries (Australia, Belgium, Czech Republic, Finland, Germany, Italy, Japan, Poland, Spain, Sweden, and United States of America). Pre-study (baseline) tumor tissue specimens were systematically collected and PD-L1 status determined prospectively to define PD-L1 subgroups for pre-specified efficacy analysis. Major efficacy outcome measures included confirmed objective response rate (ORR) as assessed by independent radiographic review committee (IRRC) using Response Evaluation Criteria in Solid Tumors (RECIST v1.1) and duration of response (DOR).

The median age was 66 years (range 38 to 90), 78% were male, 86% of patients were white. Twenty-seven percent had non-bladder urothelial carcinoma and 84% had visceral metastases. Thirty-four percent of patients had disease progression following prior platinum-containing neoadjuvant or adjuvant therapy. Twenty-nine percent of patients had received ≥ 2 prior systemic regimens in the metastatic setting. Thirty-six percent of patients received prior cisplatin only, 23% received prior carboplatin only, and 7% were treated with both cisplatin and carboplatin in the metastatic setting. Forty-six percent of patients had an ECOG performance status of 1. Eighteen percent of patients had a hemoglobin < 10 g/dL, and twenty-eight percent of patients had liver metastases at baseline. Patients were included regardless of their PD-L1 status.

In study CHECKMATE-275, of the 270 patients, 46% were defined as having PD-L1 expression of ≥ 1% (defined as ≥ 1% of tumor cells expressing PD-L1). The remaining 54% of patients, were classified as having PD-L1 expression of < 1% (defined as < 1% of tumor cells expressing PD-L1). Confirmed ORR in all patients and the two PD-L1 subgroups are summarized in Table 18. Median time to response was 1.9 months (range; 1.6-7.2).

Table 18: Efficacy Results for CHECKMATE-275 (UC)

	All Treated Subjects N=270	PD-L1 < 1% N=146	PD-L1 ≥ 1% N=124
Confirmed Objective Response Rate n (%)	53 (19.6%)	22 (15.1%)	31 (25.0%)
95% CI	15.1 – 24.9	9.7 – 21.9	17.7 – 33.6
Complete Response Rate	7 (2.6%)	1 (0.7%)	6 (4.8%)
Partial Response Rate	46 (17.0%)	21 (14.4%)	25 (20.2%)
Median Duration of Response* Months (range)	10.3 (1.9+, 12.0+)	7.6 (3.7+,12.0+)	NE (1.9+, 12.0+)

*Estimated from the Kaplan-Meier Curve

17.4.4Clinical Performance Evaluation: UC

Clinical utility of PD-L1 IHC 28-8 pharmDx was evaluated in CHECKMATE-274, A Phase 3 Randomized, Double-blind, Multi-center Study of Adjuvant Nivolumab versus Placebo in Subjects with High Risk Invasive Urothelial Carcinoma that are at high risk of recurrence after undergoing radical resection. Patients were randomized at 170 sites in 30 countries (Argentina, Australia, Austria, Belgium, Brazil, Canada, Chile, China, Colombia, Denmark, France, Germany, Greece, Ireland, Israel, Italy, Japan, South Korea, Mexico, Netherlands, Peru, Poland, Romania, Russia, Spain, Sweden, Switzerland; Taiwan, United Kingdom [UK], and United States of America [USA]). Subjects were randomized 1:1 to nivolumab (n=353) or placebo (n=356) and stratified by pathologic nodal status (N+ vs. N0/x with < 10 nodes removed vs. N0 with ≥10 nodes removed), tumor PD-L1 expression (≥ 1%, < 1%/indeterminate), and use of cisplatin neo-adjuvant chemotherapy. Pre-study (baseline) tumor tissue specimens were collected prior to randomization to conduct pre-planned analyses of efficacy according to predefined PD-L1 expression levels. The primary efficacy outcome measure was disease free survival (DFS). Additional efficacy outcome measures included non-urothelial tract recurrence free survival (NUTRFS), distant metastasis-free survival (DMFS), and time to recurrence (TTR).

The baseline demographic and disease characteristics were generally balanced between randomized subjects in the nivolumab and placebo groups. The median age was 67.0 years (range: 30 - 92), 75.6% White, 76.2% male. Baseline ECOG performance status was 0 (62.8%), 1 (34.8%), or 2 (2.3%). The predominant tumor type was urinary bladder (79.0% of subjects) and 17.9%, 57.8%, and 16.8% of all randomized subjects had Stage pT2, Stage pT3, and Stage pT4A disease (tumor stage) at the time of resection, respectively.

Frequencies of PD-L1 expression in all randomized subjects in CHECKMATE-274 are presented in Table 19.

Table 19: Frequency of Pre-Study PD-L1 Expression in All Randomized Subjects with UC – CHECKMATE-274

Population PD-L1 Expression Category	Placebo	Nivolumab	Total
Overall	356	353	709
Baseline PD-L1 Expression ≥ 1% (N (%))	141 (39.6)	139 (39.4)	280 (39.5)
Baseline PD-L1 Expression < 1% (N (%))	209 (58.7)	210 (59.5)	419 (59.1)
Baseline PD-L1 Expression - Indeterminate (N (%))	2 (0.6)	0	2 (0.3)
Baseline PD-L1 Expression - Not Evaluable (N (%))	3 (0.8)	3 (0.8)	6 (0.8)
Baseline PD-L1 Expression - Not Reported (N (%))	1 (0.3)	1 (0.3)	2 (0.3)

Nivolumab treatment resulted in a statistically significant and clinically meaningful improvement in DFS compared to placebo in all randomized subjects: median of 20.76 months (95% CI: 16.49, 27.63) vs 10.84 months (95% CI: 8.25, 13.86) with nivolumab vs placebo, respectively (HR = 0.70 [98.22% CI: 0.55, 0.90]; p = 0.0008) (Table 20).

Table 20: Disease Free Survival in Randomized Subjects with UC – CHECKMATE-274

Disease Free Survival	Placebo (n=356)	Nivolumab (n=353)
All Randomized Subjects		
Events N (%)	204 (57.3%)	170 (48.2%)
Median DFS (mo) (95% CI mo) [a]	10.84 (8.3, 13.9)	20.76 (16.5, 27.6)
Hazard ratio (% CI)[b]	0.70 (98.2% CI: 0.55, 0.90)	
Stratified log-rank p-value[c]	0.0008[d]	
All Randomized Subjects PD-L1 ≥ 1%		
Events N (%)	81 (57)	55 (39)
Median DFS (mo.) (95% CI mo.) [a]	8.4 (5.6, 21.2)	N.R. (21.2, N.E.)
Hazard ratio (% CI)[b]	0.55 (98.7% CI: 0.35, 0.85)	
Stratified log-rank p-value[c]	0.0005[e]	
All Randomized Subjects PD-L1 < 1%[f]		
Events N (%)	120 (57)	114 (54)
Median DFS (mo.) (95% CI mo.) [a]	11.07 (8.3, 16.7)	16.49 (13.8, 20.8)
Hazard ratio (% CI)[b]	0.82 (95% CI: 0.63, 1.06)	
Stratified log-rank p-value[c]	N.A.	

N.R. Not reached, N.E. Not estimable
[a] Based on Kaplan-Meier Estimates.
[b] Stratified Cox proportional hazard model. Hazard Ratio is Nivolumab over Placebo.
[c] 2 sided p values from stratified regular log-rank test.
[d] Log-rank test stratified by prior neo-adjuvant cisplatin, pathological nodal status, PD-L1 status (>=1% versus <1%/indeterminate) as entered in the IRT.
[e] Log-rank test stratified by prior neoadjuvant cisplatin, pathological nodal status as entered in the IRT. Boundary for statistical significance in all randomized patients with PD-L1 ≥1%: p-value <0.01282.
[f] Results are based on the PDL1 negative clinical database, not from the IRT.

18. Troubleshooting

Table 21: Troubleshooting

Problem	Probable Cause	Suggested Action
1. No staining of control or specimen slides	1a. Programming error.	1a. Verify that the SK005 PD-L1 IHC 28-8 pharmDx program was selected for programming of slides.
	1b. Lack of reaction with DAB+ Substrate-Chromogen Solution (DAB)	1b. Verify that DAB+ Substrate-Chromogen Solution was prepared properly.
	1c. Sodium azide in wash buffer.	1c. Use only EnVision FLEX Wash Buffer, Code K8007.
	1d. Degradation of Control Slide	1d. Check kit expiration date and kit storage conditions on outside of package.
2. Weak staining of specimen slides.	2a. Inappropriate fixation method used.	2a. Ensure that only neutral buffered formalin fixative and approved fixation methods are used.
	2b. Insufficient reagent volume applied.	2b. Check size of tissue section and reagent volume applied.
	2c. Inappropriate wash buffer used.	2c. Use only EnVision FLEX Wash Buffer, Code K8007.
3. Weak staining of specimen slides or the positive cell line on the Agilent-supplied Control Slide.	3a. Inadequate target retrieval.	3a. Verify that the 3-in-1 pre-treatment procedure was correctly performed.
	3b. Inappropriate wash buffer used.	3b. Use only EnVision FLEX Wash Buffer, Code K8007
4. Excessive background staining of slides.	4a. Paraffin incompletely removed.	4a. Verify that the 3-in-1 pre-treatment procedure was correctly performed.
	4b. Slides dried while loading onto the Autostainer Link 48.	4b. Ensure slides remain wet with buffer while loading and prior to initiating run.
	4c. Nonspecific binding of reagents to tissue section.	4c. Check for proper fixation of the specimen and/or the presence of necrosis.

Problem	Probable Cause	Suggested Action
	4d. Inappropriate fixation method used.	4d. Ensure that only neutral buffered formalin fixative and approved fixation methods are used.
	4e. Inadequate mixing of wash buffer.	4e. Ensure wash buffer is properly mixed.
5. Tissue detached from slides.	5a. Use of incorrect microscope slides.	5a. Use FLEX IHC Microscope Slides, (Code K8020), or Superfrost Plus slides.
	5b. Inadequate preparation of specimens	5b. Cut sections should be placed in a 58 ± 2 °C oven for 1 hour prior to staining.
6. Excessively strong specific staining.	6a. Inappropriate fixation method used.	6a. Ensure that only approved fixatives and fixation methods are used.
	6b. Inappropriate wash buffer used.	6b. Use only EnVision FLEX Wash Buffer, Code K8007.
7. 1x EnVision FLEX Target Retrieval Solution is cloudy in appearance when heated.	7. When heated the 1x EnVision FLEX Target Retrieval Solution turns cloudy in appearance.	7. This is normal and does not influence staining.
8. 1x EnVision FLEX Target Retrieval Solution does not meet pH specifications.	8a. pH meter is not calibrated correctly.	8a. Ensure pH meter is calibrated per manufacturer's recommendations. After re-calibration, re-test the pH of 1x EnVision FLEX Target Retrieval Solution. Do not modify the pH of 1x EnVision FLEX Target Retrieval Solution. If the pH is outside the acceptable range (6.1 ± 0.2), discard 1x EnVision FLEX Target Retrieval Solution. Prepare new 1x EnVision FLEX Target Retrieval Solution. Check the pH of the new 1x EnVision FLEX Target Retrieval Solution.
	8b. Inferior quality water is used to dilute the EnVision FLEX Target Retrieval Solution concentrate.	8b. Ensure that distilled or deionized water is used to prepare 1x EnVision FLEX Target Retrieval Solution.
	8c. Incorrect Target Retrieval Solution is used.	8c. Ensure that the correct EnVision FLEX Target Retrieval Solution specified in 'Materials Provided' Section 4 and 'Reagent Preparation' Section 9 is used.

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 contact 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).*[14]

19. References

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Explanation of symbols

REF	Catalogue number		Temperature limitation	IVD	In vitro diagnostic medical device
	Manufacturer	LOT	Batch code		Contains sufficient for <n> tests
	Use by		Consult instructions for use	EC REP	Authorized representative in the European Community


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