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Introduction

Intended Use in Melanoma

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-squamous non-small cell lung cancer (NSCLC) and melanoma tissue using EnVision FLEX visualization system on Autostainer Link 48. PD-L1 protein expression is defined as the percentage of tumor cells exhibiting positive membrane staining at any intensity.

PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in non-squamous NSCLC may be associated with enhanced survival from OPDIVO® (nivolumab).

PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in melanoma may be used as an aid in the assessment of patients for whom OPDIVO (nivolumab) and YERVOY® (ipilimumab) combination treatment is being considered..

How to Use the PD-L1 IHC 28-8 pharmDx Interpretation Manual

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

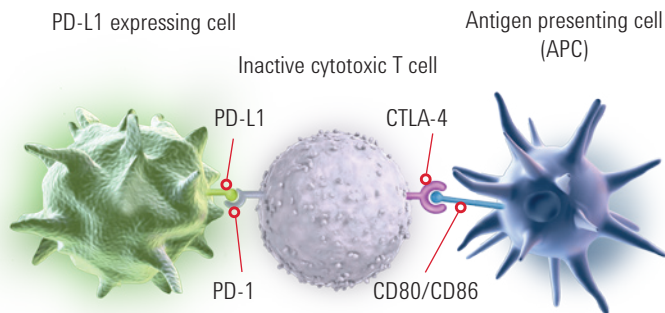
The PD-L1 IHC 28-8 pharmDx Instructions for Use contains guidelines and technical tips for ensuring high-quality staining in your laboratory.

Review of this PD-L1 IHC 28-8 pharmDx Interpretation Manual will provide a solid foundation for evaluating melanoma specimens stained with PD-L1 IHC 28-8 pharmDx. For more details, please refer to the current version of the PD-L1 IHC 28-8 pharmDx Instructions for Use provided or visit www.dako.com.

The included photomicrographs are melanoma unless otherwise noted.

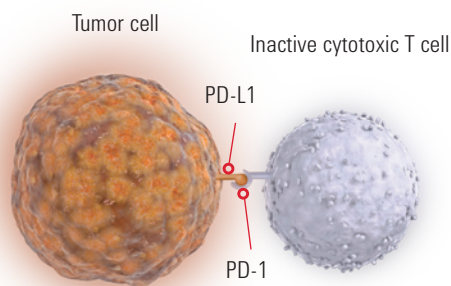
OPDIVO and YERVOY are trademarks of Bristol-Myers Squibb Company.

The Role of CTLA-4 and PD-1/PD-L1 Pathways in Cancer



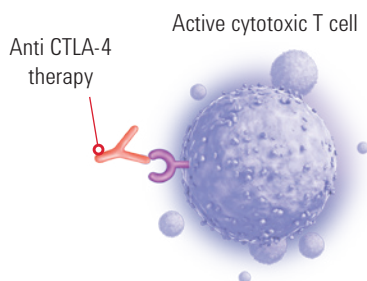
Normal cells limit damage to healthy tissue

Inactivation of T cells limits damage to healthy tissue.



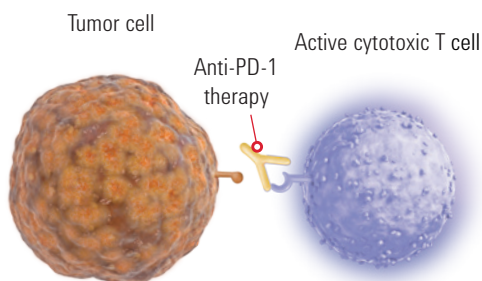
Tumor cells escape detection

Inactivation of T cells reduces tumor cell killing.



CTLA-4 therapy augments T cell activation and proliferation

Blocking CTLA-4 contributes to an increase in anti-tumor immune response.



PD-1 therapies harness the immune response to fight tumors

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

Clinical Interpretation of PD-L1 IHC 28-8 pharmDx Results in Melanoma Patients

PD-L1 IHC 28-8 pharmDx assay demonstrated clinical results in the phase 3 CheckMate 067 clinical trial.

- **The CheckMate 067 study was a three armed study of OPDIVO (nivolumab) monotherapy or OPDIVO in combination with YERVOY (ipilimumab) versus YERVOY monotherapy**
- **Progression free survival (PFS) was evaluated across PD-L1 subgroups**
- **In patients with <5 % PD-L1 expression levels, combination of OPDIVO (nivolumab) and YERVOY (ipilimumab) demonstrated improved PFS relative to OPDIVO (nivolumab) treatment**

PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx may aid the assessment of patients for whom OPDIVO (nivolumab) and YERVOY (ipilimumab) combination treatment is being considered in melanoma.

PFS at PD-L1 expression level $\geq 5\%$ and $< 5\%$

Treatment	PD-L1 expression level	N of events/ N of patients	Median PFS in month (95% CI)	HR vs. ipilimumab (95% CI)	HR vs. nivolumab monotherapy (95% CI)
nivolumab +ipilimumab	$\geq 5\%$	28/68	13.96 (9.72, NR)	0.39 (0.25, 0.62)	0.96 (0.58, 1.58)
	$< 5\%$	103/210	11.24 (7.98, NR)	0.42 (0.32, 0.54)	0.70 (0.54, 0.91)
nivolumab	$\geq 5\%$	33/80	14.00 (9.07, NR)	0.41 (0.26, 0.63)	-
	$< 5\%$	122/208	5.32 (2.83, 7.06)	0.59 (0.47, 0.75)	-
ipilimumab	$\geq 5\%$	53/75	3.94 (2.79, 4.21)	-	-
	$< 5\%$	154/202	2.83 (2.76, 3.09)	-	-

PFS values at PD-L1 expression level $\geq 1\%$ and $< 1\%$

Treatment	PD-L1 expression level	N of events/ N of patients	Median PFS in month (95% CI)	HR vs. ipilimumab (95% CI)	HR vs. nivolumab monotherapy (95% CI)
nivolumab +ipilimumab	$\geq 1\%$	72/155	12.35 (8.51, NR)	0.44 (0.32, 0.58)	0.95 (0.69, 1.31)
	$< 1\%$	59/123	11.17 (6.93, NR)	0.38 (0.27, 0.53)	0.56 (0.40, 0.79)
nivolumab	$\geq 1\%$	79/171	12.39 (8.11, NR)	0.46 (0.34, 0.61)	-
	$< 1\%$	76/117	2.83 (2.76, 5.13)	0.67 (0.49, 0.92)	-
ipilimumab	$\geq 1\%$	122/164	3.91 (2.83, 4.17)	-	-
	$< 1\%$	85/113	2.79 (2.66, 2.96)	-	-

Abbreviations: CI = confidence interval, NR = not reached, PFS = progression-free survival

HR = Hazard ratio for treatment effect based on Cox proportional hazard model with treatment, PD-L1 status, and treatment by PD-L1 status interaction

Table 1: Guidelines for scoring and reporting of PD-L1 IHC 28-8 pharmDx

PD-L1 IHC 28-8 pharmDx Kit Overview - Code SK005

PD-L1 IHC 28-8 pharmDx contains optimized reagents and the protocol required to complete an IHC staining procedure of FFPE tissue sections using Autostainer Link 48 and the PT Link Pretreatment Module (see Figure 1). Following incubation with the primary monoclonal antibody PD-L1 or the Negative Control Reagent, the specimens are incubated with a PD-L1 IHC 28-8 pharmDx purified Rabbit 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 is then counterstained and coverslipped. Results are interpreted using a light microscope. Control Slides containing two formalin-fixed, paraffin-embedded human cell lines are provided to aid in validating staining runs.

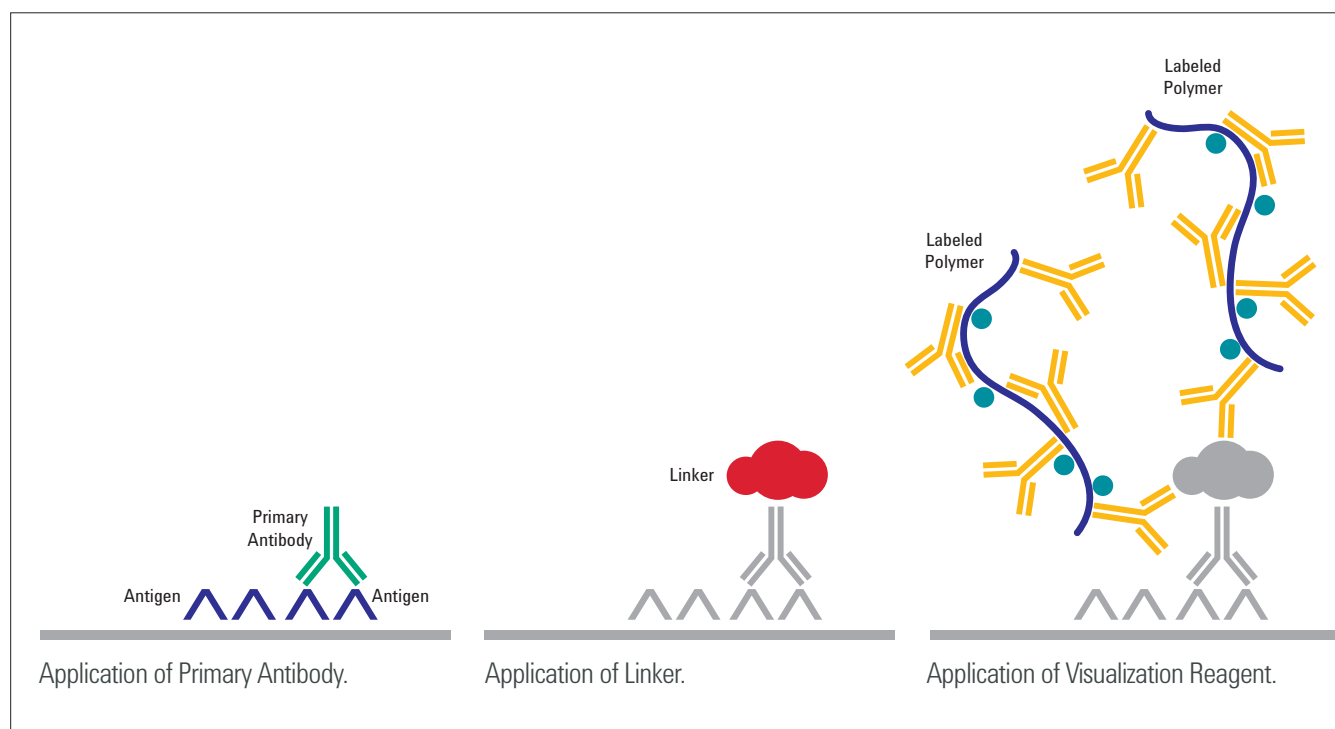


Figure 1: PD-L1 IHC 28-8 pharmDx staining procedure

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

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

NOTE: All PD-L1 IHC 28-8 pharmDx reagents are to be performed on the Autostainer Link 48.

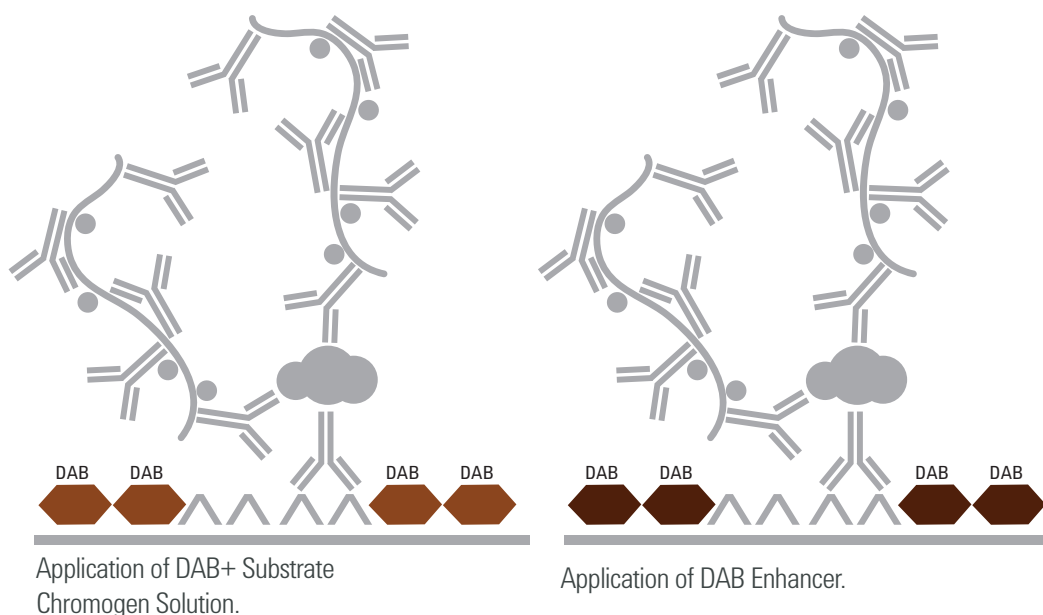
All reagents must be used as indicated in the Instructions for Use in order for the test to perform as specified.

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

Refer to Instructions for Use for a complete list of required materials and equipment.



Figure 2: PD-L1 IHC 28-8 pharmDx components



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

Optimal staining performance is achieved by adhering to the recommended PD-L1 IHC 28-8 pharmDx protocol in the Instructions for Use. The following are tips for optimizing staining performance. Technical problems relating to the performance of PD-L1 IHC 28-8 pharmDx may arise in two areas; those involving specimen collection and specimen preparation prior to performing the test, as well as problems involving the actual performance of the test itself. Technical problems of the test can be minimized through a thorough understanding of the product instructions by the user.

Specimen Collection and Processing

Specimens must be handled in a way which preserves the tissue for immunohistochemical staining. Tissue should be stained and interpreted as close to time of biopsy as possible. Stability of PD-L1 immunoreactivity in tissue blocks has not been assessed, and tissue may be susceptible to loss of PD-L1 immunoreactivity with age. Confirm appropriate intact tumor morphology and the presence of sufficient tumor cells for evaluation. Use recommended methods of tissue processing for all specimens.

Control Tissue

Differences in processing and embedding in the user's laboratory may produce significant variability in results. Include positive and negative control tissue in each staining run, in addition to the PD-L1 IHC 28-8 pharmDx Control Slides (See Figure 3).

Select positive and negative control tissue from fresh melanoma specimens. Fix, process, and embed the control tissue in the same manner as patient specimens. Control tissue processed differently from the patient specimen validates reagent performance only and does not verify tissue preparation. The ideal positive control tissue gives weak

to moderate positive staining. The variety of different cell types present in most tissue sections offers internal negative control sites; this should be verified by the user. A suggested melanoma-negative control tissue is one that shows no staining in tumor cells but possesses stained immune cells such as macrophages and lymphocytes.

Tissue Processing

Formalin-fixed, paraffin-embedded tissues are suitable for use.

Block specimens into a thickness of 3 mm or 4 mm, fix in 10% Neutral Buffered Formalin (NBF), and dehydrate and clear in a series of alcohols and xylene, followed by infiltration with melted paraffin. An ischemia time from excision to fixation start time of less than 30 minutes followed by immersion in 10% neutral buffered formalin for 24-48 hours is recommended. The paraffin temperature should not exceed 60 °C. Decalcified tissue has not been validated on PD-L1 IHC 28-8 pharmDx and is not recommended.

Cut tissue specimens into sections of 4-5 µm. After sectioning, mount tissues on FLEX IHC microscope slides, Code K8020, or Fisherbrand Superfrost Plus charged slides. 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. Slide storage and handling conditions should not exceed 25 °C at any point post-mounting to ensure tissue integrity and antigenicity.

PD-L1 IHC 28-8 pharmDx Staining Procedure

The PD-L1 IHC 28-8 pharmDx reagents and instructions have been designed for optimal performance. Further dilution of the reagents, alteration of incubation times, temperatures, or materials may give erroneous results. All of the required steps and incubation times for staining are preprogrammed in the DakoLink software.

Reagent Storage

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

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 package.

EnVision FLEX Target Retrieval Solution, Low pH

Dilute EnVision FLEX Target Retrieval Solution, Low pH (50x) 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 Pretreatment Module tank and will treat up to 24 slides per use. The pH of the working solution should be 6.1 ± 0.2 . Discard Low pH working solution after three uses. Do not use after 5 days following dilution.

EnVision FLEX Wash Buffer, Code K8007

Dilute EnVision FLEX Wash Buffer (20x) 1:20 using distilled or deionized water (reagent-quality water). Store unused working solution at 2-8 °C for no more than one 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 Solution is stable for 5 days if stored in the dark at 2-8 °C. Mix thoroughly prior to use. Any precipitate developing in the solution does not affect staining quality.

- Add 9 drops of DAB+ Chromogen to a full bottle of DAB+ Substrate Buffer. Although the DAB+ Substrate Buffer label states 7.2mL, 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

Control slides are recommended to determine that the PD-L1 IHC 28-8 pharmDx results (generated by the system containing reagents, instrument hardware and software) are functioning properly. For each staining run include the following control slides:

- One PD-L1 IHC 28-8 pharmDx Control Slide stained with the Primary Antibody in each staining run.
- Two positive control tissue slides (one stained with Primary Antibody and another stained with Negative Control Reagent) for each set of test conditions.
- Two negative control tissue slides (one stained with Primary Antibody and another stained with Negative Control Reagent).
- Lastly, for each patient specimen stained with Primary Antibody, include a sequential section of patient specimen stained with Negative Control Reagent.

Deparaffinization, Rehydration and Target Retrieval

Use PT Link Pretreatment Module 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 prepared 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 pre-heated Target Retrieval Solution, Low pH in PT Link tank. Start the PT Link program and 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 rack with slides into a tank (e.g., PT Link Rinse Station, Code PT109) containing room temperature EnVision FLEX Wash Buffer working solution.
- Leave Autostainer rack with slides in room temperature EnVision FLEX Wash Buffer for 5 minutes.

Staining and Counterstaining

Place the Autostainer rack with slides on the Autostainer Link 48. Ensure slides remain wet with buffer while loading and prior to initiating the run. Dried tissue sections may display increased non-specific staining.

Select the PD-L1 IHC 28-8 pharmDx protocol. The instrument performs the staining and counterstaining procedures by applying the appropriate reagent, monitoring the incubation time and rinsing slides between reagents. Counterstaining of slides using EnVision FLEX Hematoxylin, Code K8008, is included in the staining protocol.

Mounting

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

PD-L1 IHC 28-8 pharmDx Technical Checklist

Customer Name / Institution _____

Name and Title _____

Autostainer Link 48 Serial Number _____

Software Version _____

	Yes	No
Regular preventive maintenance is performed on the Autostainer Link 48 and PT Link?	<input type="checkbox"/>	<input type="checkbox"/>
All the necessary equipment is available to perform the PD-L1 IHC 28-8 pharmDx according to protocol?	<input type="checkbox"/>	<input type="checkbox"/>
PD-L1 IHC 28-8 pharmDx is used before the expiration date printed on the outside of the box?	<input type="checkbox"/>	<input type="checkbox"/>
All PD-L1 IHC 28-8 pharmDx components, including Control Slides, are stored in the dark at 2-8 °C?	<input type="checkbox"/>	<input type="checkbox"/>
All PD-L1 IHC 28-8 pharmDx components, including Control Slides, are equilibrated to room temperature (20-25 °C) prior to immunostaining?	<input type="checkbox"/>	<input type="checkbox"/>
Appropriate positive and negative control tissue from melanoma are identified?	<input type="checkbox"/>	<input type="checkbox"/>
Tissues are fixed in neutral buffered formalin?	<input type="checkbox"/>	<input type="checkbox"/>
Tissues are infiltrated with melted paraffin, at or below 60 °C?	<input type="checkbox"/>	<input type="checkbox"/>
Tissue sections of 4-5 µm are mounted on FLEX IHC Microscope Slides or Fisherbrand Superfrost Plus charged slides?	<input type="checkbox"/>	<input type="checkbox"/>
Specimens are stained within 4 months of sectioning when stored in the dark at 2-8 °C or room temperature up to 25 °C?	<input type="checkbox"/>	<input type="checkbox"/>
EnVision FLEX Target Retrieval Solution, Low pH is prepared properly?	<input type="checkbox"/>	<input type="checkbox"/>
EnVision FLEX Wash Buffer is prepared properly?	<input type="checkbox"/>	<input type="checkbox"/>
DAB+ Substrate-Chromogen Solution is prepared properly?	<input type="checkbox"/>	<input type="checkbox"/>
The Deparaffinization, Rehydration and Target Retrieval 3-in-1 procedure is followed, using PT Link?	<input type="checkbox"/>	<input type="checkbox"/>
Slides remain wet with buffer while loading and prior to initiating run on Autostainer Link 48?	<input type="checkbox"/>	<input type="checkbox"/>
The PD-L1 IHC 28-8 pharmDx protocol is selected on Autostainer Link 48?	<input type="checkbox"/>	<input type="checkbox"/>
Slides are counterstained with EnVision FLEX Hematoxylin?	<input type="checkbox"/>	<input type="checkbox"/>

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

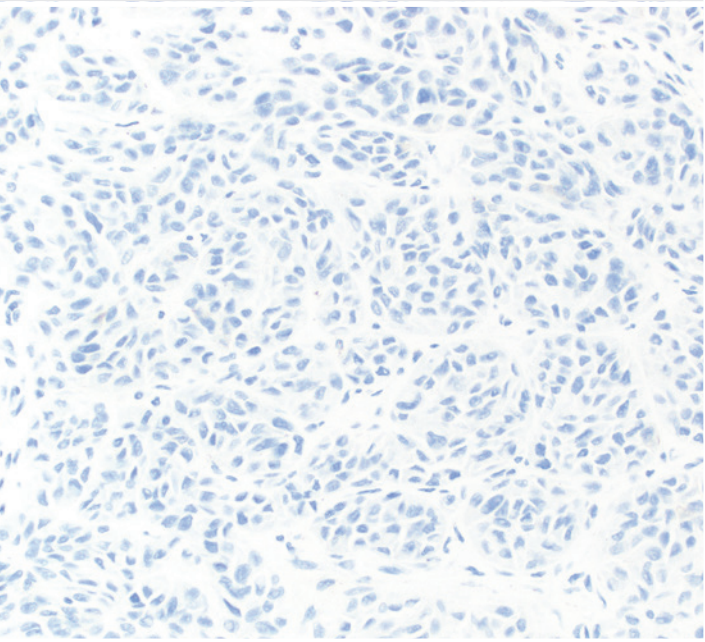
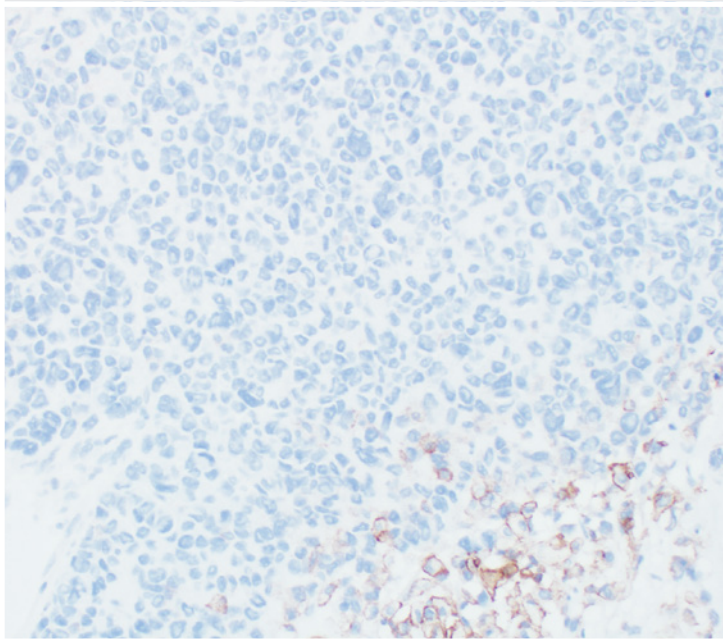
Additional observations or comments:

Guidelines for Scoring PD-L1 IHC 28-8 pharmDx in Melanoma

Dako emphasizes that scoring of PD-L1 IHC 28-8 pharmDx must be performed in accordance with the guidelines established in the Instructions for Use, within the context of best practices and the pathologist’s experience.

The percentage of stained viable tumor cells in the specimen determines the PD-L1 IHC 28-8 pharmDx result. Scoring guidelines and reporting recommendations are presented in Table 2. See page 22 for example pathology report for PD-L1 IHC 28-8 pharmDx.

Clinical interpretation of PD-L1 IHC 28-8 pharmDx results in patients with melanoma based on the phase 3 CheckMate 067 clinical trial.

<p>< 1% of the viable tumor cells exhibit complete circumferential or partial linear plasma membrane staining at any intensity.</p>	<p>≥ 1% of the viable tumor cells exhibit complete circumferential or partial linear plasma membrane staining at any intensity.</p>
	
<p>PD-L1 expression < 1%</p>	<p>PD-L1 expression ≥ 1%</p>

PD-L1 IHC 28-8 pharmDx Result Report to treating physician

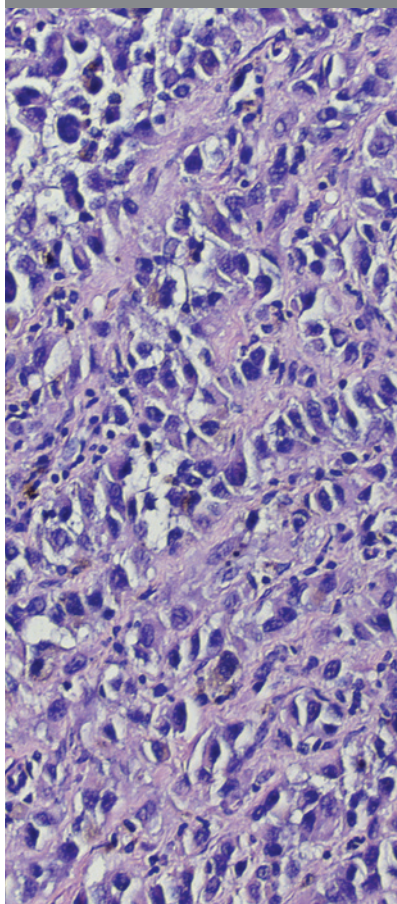
Table 2: Guidelines for scoring and reporting of PD-L1 IHC 28-8 pharmDx

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

The following flow of slide review is recommended when conducting interpretation of PD-L1 IHC 28-8 pharmDx. **Refer to detailed description on pages 18-21.**

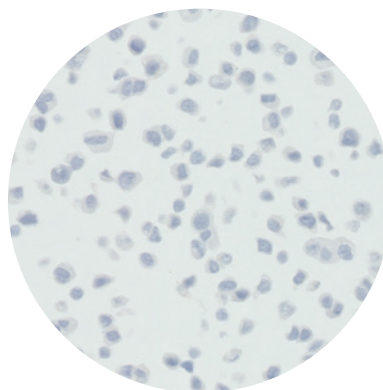
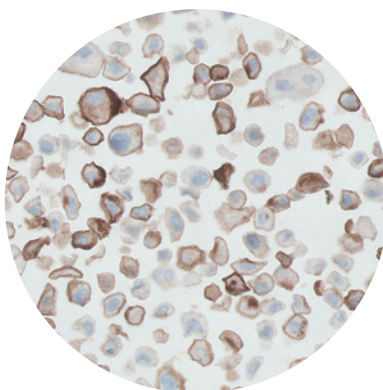
1

Patient Specimen stained with H&E
Histology and preservation quality
Acceptable



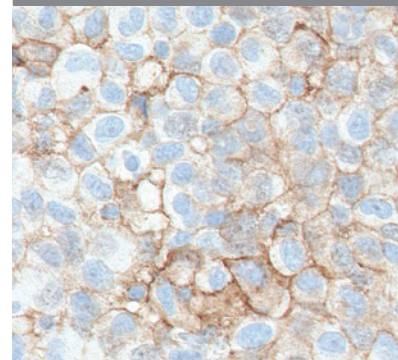
2

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



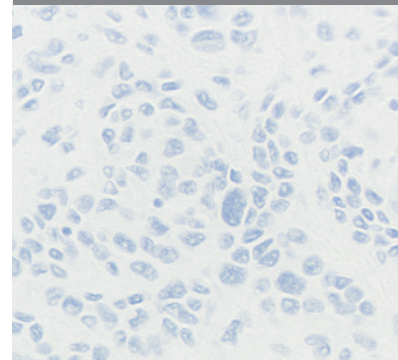
3a

Positive Control Tissue
Stained with PD-L1 Primary Antibody
Acceptable



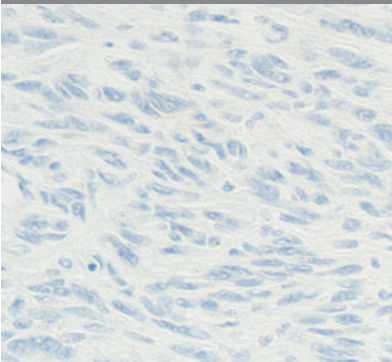
3b

Positive Control Tissue
Stained with Negative Control Reagent
Acceptable



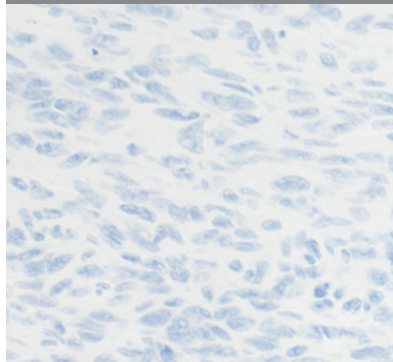
4a

Negative Control Tissue
Stained with PD-L1 Primary Antibody
Acceptable



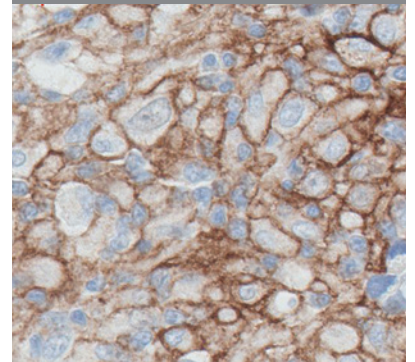
5

Patient Specimen
Stained with Negative Control Reagent
Acceptable



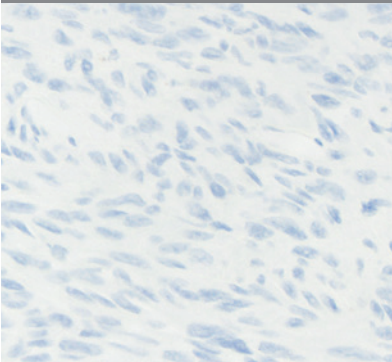
6

Patient Specimen
Stained with PD-L1 Primary Antibody
Acceptable



4b

Negative Control Tissue
Stained with Negative Control Reagent
Acceptable



Include when scoring:

- ≥ 100 viable tumor cells.
Score viable tumor cells exhibiting complete circumferential or partial linear plasma membrane staining at any intensity.
- Determine the percentage of stained viable tumor cells in the entire specimen.

Exclude from scoring:

Cytoplasmic staining
Immune cells
Normal cells
Necrotic cells

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

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

PD-L1 staining is defined as complete circumferential or partial linear plasma membrane staining at any intensity.

Only the PD-L1 IHC 28-8 pharmDx Control Slide is provided in the PD-L1 IHC 28-8 pharmDx kit. Positive control tissue slides and negative control tissue slides should be supplied by the laboratory. Laboratory provided positive and negative control tissue may be included on the same slide as the patient specimen.

1 Patient Specimen Stained with H&E

An H&E stained section is required for the evaluation of histology and preservation quality. PD-L1 IHC 28-8 pharmDx and the H&E staining should be performed on serial sections from the same paraffin block of the specimen.

2 PD-L1 IHC 28-8 pharmDx Control Slide

Examine the PD-L1 IHC 28-8 pharmDx Control Slide to ascertain that reagents are functioning properly. Each slide contains sections of cell pellets with positive and negative PD-L1 expression, see Figure 3. If any staining of the Control Slide is not satisfactory, all results with the patient specimens should be considered invalid. Do not use the Control Slide as an aid in interpretation of patient results.

For the PD-L1 **positive cell pellet on the Control Slide**, the following staining is acceptable, see Figure 4:

- At least 80% of the cells contain plasma membrane staining of at least 2+ average staining intensity
- Any background staining is of less than 1+ staining intensity

For the PD-L1 **negative cell pellet on the Control Slide**, the following staining is acceptable, see Figure 5:

- No plasma membrane staining
- Any background staining is of less than 1+ staining intensity

Note: Staining of a few cells in the negative pellet on the Control Slide may occasionally be observed. The presence of 10 or fewer cells with distinct plasma membrane staining, or cytoplasmic staining with $\geq 1+$ intensity within the boundaries of the cell pellet are acceptable.

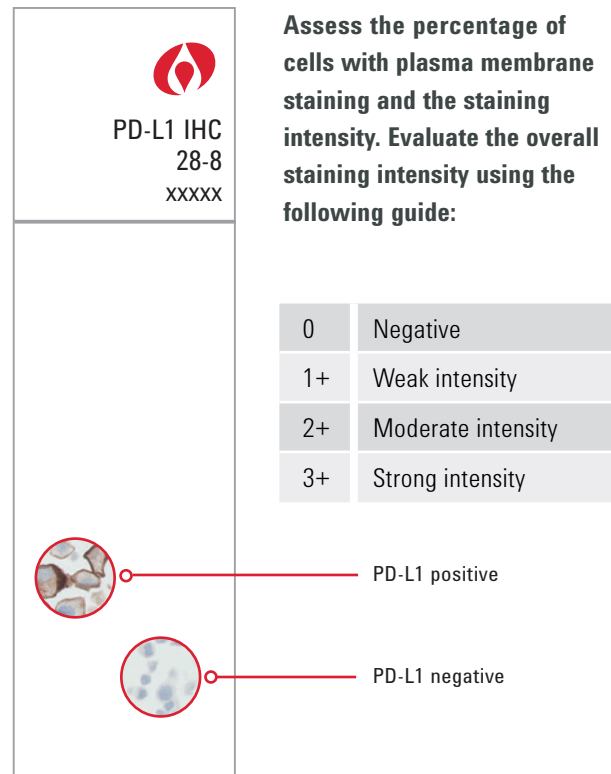


Figure 3: Each Control Slide contains sections of cell pellets with positive and negative PD-L1 expression.

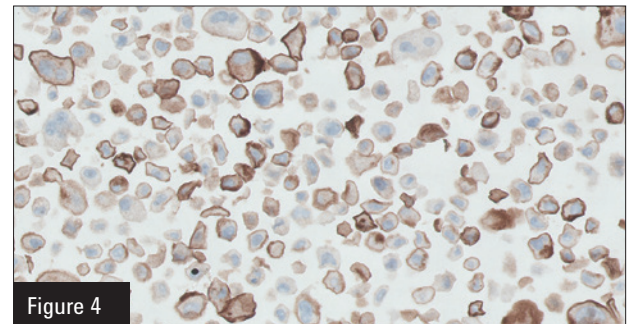


Figure 4

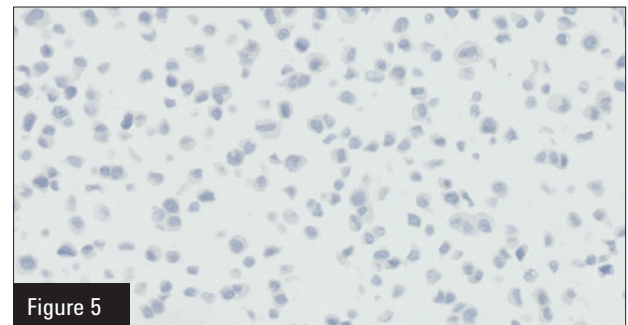


Figure 5

Figure 4: Acceptable staining of positive pellet.

Figure 5: Acceptable staining of negative pellet.

3 Positive Control Tissue Slides

Examine the positive melanoma control tissue slides (one stained with Primary Antibody and other with Negative Control Reagent) to ascertain if tissues are correctly prepared and reagents are functioning properly. Any background staining should be of $\leq 1+$ staining intensity. Exclude necrotic or degenerated cells from evaluation. If staining of positive control tissues is not satisfactory, all results with the patient specimens should be considered invalid. Do not use control tissue as an aid in interpretation of patient results.

4 Negative Control Tissue Slides

Examine the negative melanoma control tissue slides (one stained with Primary Antibody and other with Negative Control Reagent) to confirm that there is no unintended staining. Any background staining should be of $\leq 1+$ staining intensity. If unwanted specific plasma membrane staining of malignant cells occurs in the negative control tissue, all results with the patient specimens should be considered invalid. Do not use control tissue as an aid in interpretation of patient results.

5 Patient Specimen Stained with Negative Control Reagent (NCR)

Examine the patient specimen stained with Negative Control Reagent to identify non-specific background staining or to determine patient melanin content that may interfere with PD-L1 staining interpretation. If any staining is not satisfactory, results with the patient specimen should be considered invalid.

The Negative Control Reagent indicates non-specific background staining and allows better interpretation of patient specimen stained with the Primary Antibody.

6 Patient Specimen Stained with Primary Antibody

Staining should be assessed within the context of any non-specific background staining of the patient specimen stained with Negative Control Reagent.

Assess a minimum of 100 viable tumor cells in the PD-L1-stained patient slide to determine the percentage of stained cells.

1	At 4x objective magnification, carefully examine the tumor areas of the entire specimen. Well-preserved and well-stained areas of the specimen should be used to evaluate PD-L1 staining. When determining the percentage of stained tumor cells in the entire specimen, the numerator is the total stained viable tumor cells in the entire specimen and the denominator is the total number of tumor cells in the specimen.
2	At 10-40x objective magnification score PD-L1 positive staining defined as complete circumferential and/or partial linear plasma membrane staining of tumor cells at any intensity. The entire specimen must be evaluated. All viable tumor cells on the entire PDL1 stained patient slide must be evaluated and included in the PDL1 scoring assessment.
3	Exclude cytoplasmic staining from scoring. Exclude immune cells, normal cells and necrotic cells from scoring. An indeterminate specimens is when the tumor cell membrane staining is hampered for reasons attributed to the biology of tumor tissue sample rather than improper sample preparation. For example high level of melanin pigment.

See Figures 6-17 for examples of melanoma stained with Primary Antibody.

Tips and Special Considerations

Include the entire specimen for evaluation of PD-L1 expression. Use higher magnifications to confirm cell types and areas absent of staining. Be careful not to overlook weak 1+ staining, which can be missed at 4x and 10x.

Melanin: The presence of melanin may hamper scoring of plasma membrane staining of viable tumor cells. In certain specimens, elevated melanin content may impede scoring making the specimen non evaluable. See Figure 13 for an example. Tissue from a deeper level of the block or potentially another block could present tumor with less melanin, making it suitable for use. In such cases, the patient specimen stained with Negative Control Reagent may be useful to identify the pattern of melanin when interpreting the Patient specimen stained with PD-L1 Primary Antibody. See Figure 13 and Figure 14 for an example.

Non-evaluable Specimens: The specimen should be considered non-evaluable if there are fewer than 100 Viable Tumor Cells or the presence of melanin prohibits scoring. A different section from the same block or another block from the same patient may be required to present sufficient viable tumor cells or less melanin to support PD-L1 IHC 28-8 pharmDx evaluation.

Reporting Results

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

PD-L1 IHC 28-8 pharmDx, Code SK005 Summary of Sample Tested:

Date of Run: _____

PD-L1 IHC 28-8 pharmDx Lot: _____

Staining Run Log ID: _____

Specimen ID: _____

Patient Identifier: _____

Type of Service: IHC Stain with Manual Interpretation

Other: _____

Type of Tissue Skin or other: _____

PD-L1 Included in melanoma Comprehensive Panel: Yes No

Additional Tests Performed with PD-L1 IHC 28-8 pharmDx: _____

PD-L1 IHC 28-8 pharmDx Controls Results:

PD-L1 IHC 28-8 Control Slides: Pass Fail

Positive Control Tissue Slides: Pass Fail

Negative Control Tissue Slides: Pass Fail

PD-L1 Results:

Based on the PD-L1 expression analyses from this study, the greatest PFS benefit for the combination of nivolumab + ipilimumab vs. nivolumab alone may occur in the setting of <5% PD-L1 expression

Viable Tumor Cells Present (≥ 100 cells)

PD-L1 expression < 5%

Percent of melanoma cells with complete linear circumferential or partial membrane PD-L1 staining is < 5%

PD-L1 expression $\geq 5\%$

Percent of melanoma cells with complete linear circumferential or partial membrane PD-L1 staining is $\geq 5\%$

% Percent Expression PD-L1 Tumor Cells _____ %

Other Comments to Treating Physician _____

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

The following images present examples of melanoma tumor samples stained with PD-L1 IHC 28-8 pharmDx.

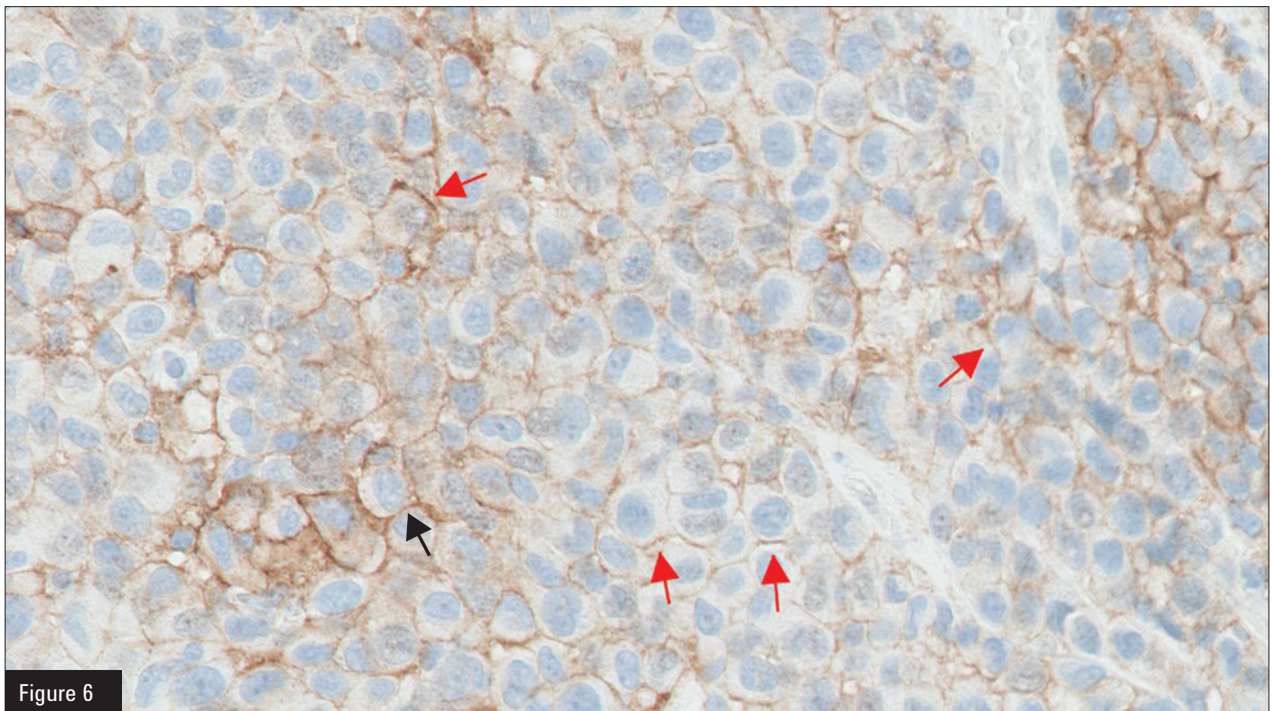


Figure 6: Red arrows show partial linear plasma membrane staining of viable tumor cells. Black arrow shows complete circumferential membrane staining of viable tumor cells.

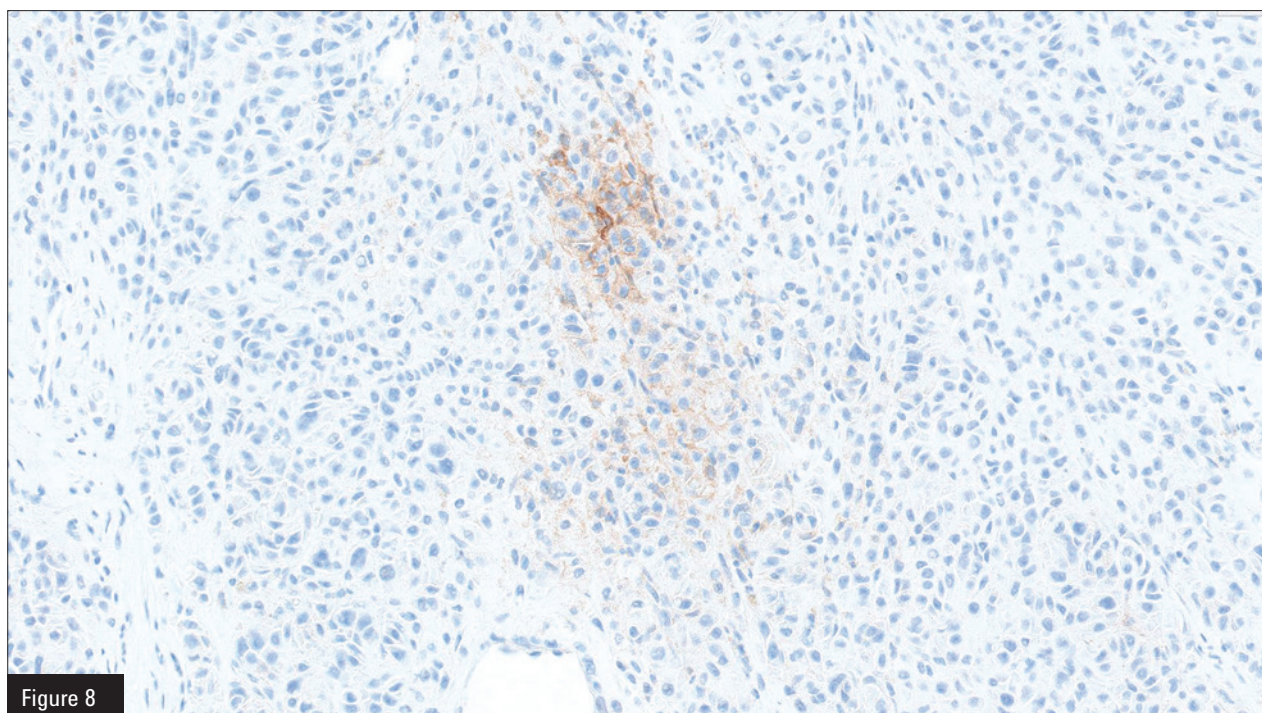
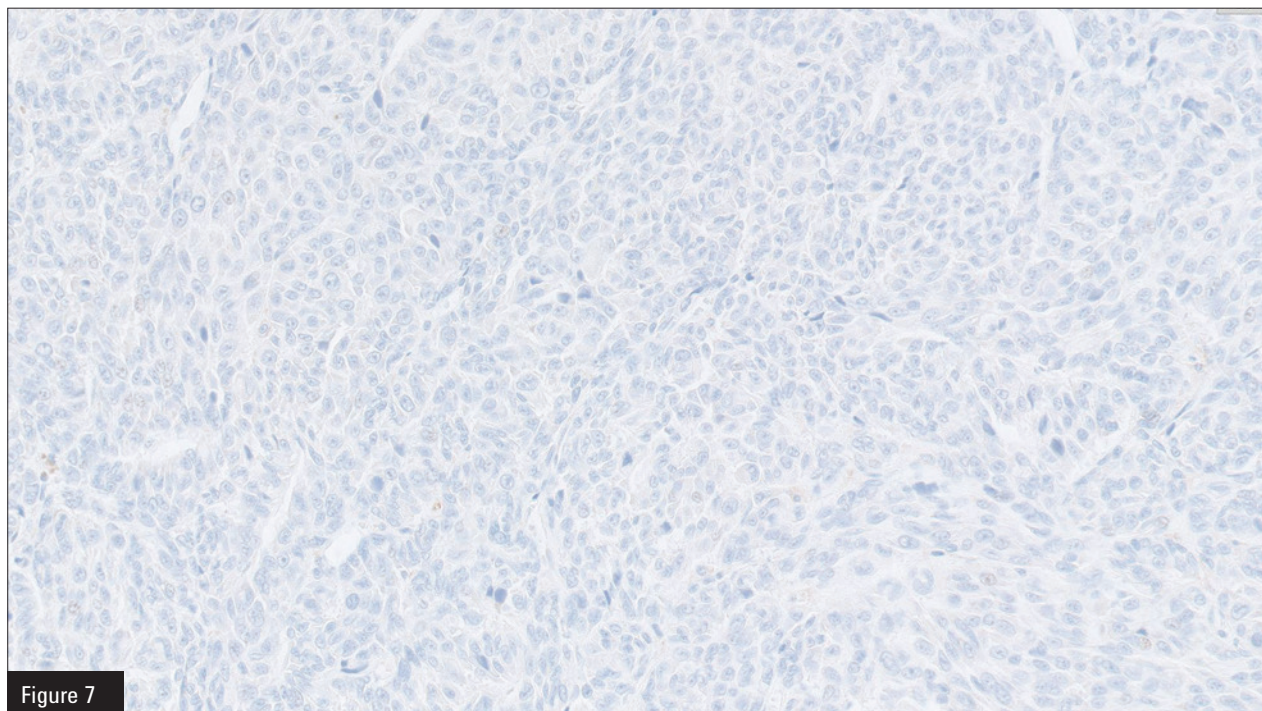


Figure 7: PD-L1 expression < 1%.

Figure 8: PD-L1 expression ≥ 1%.

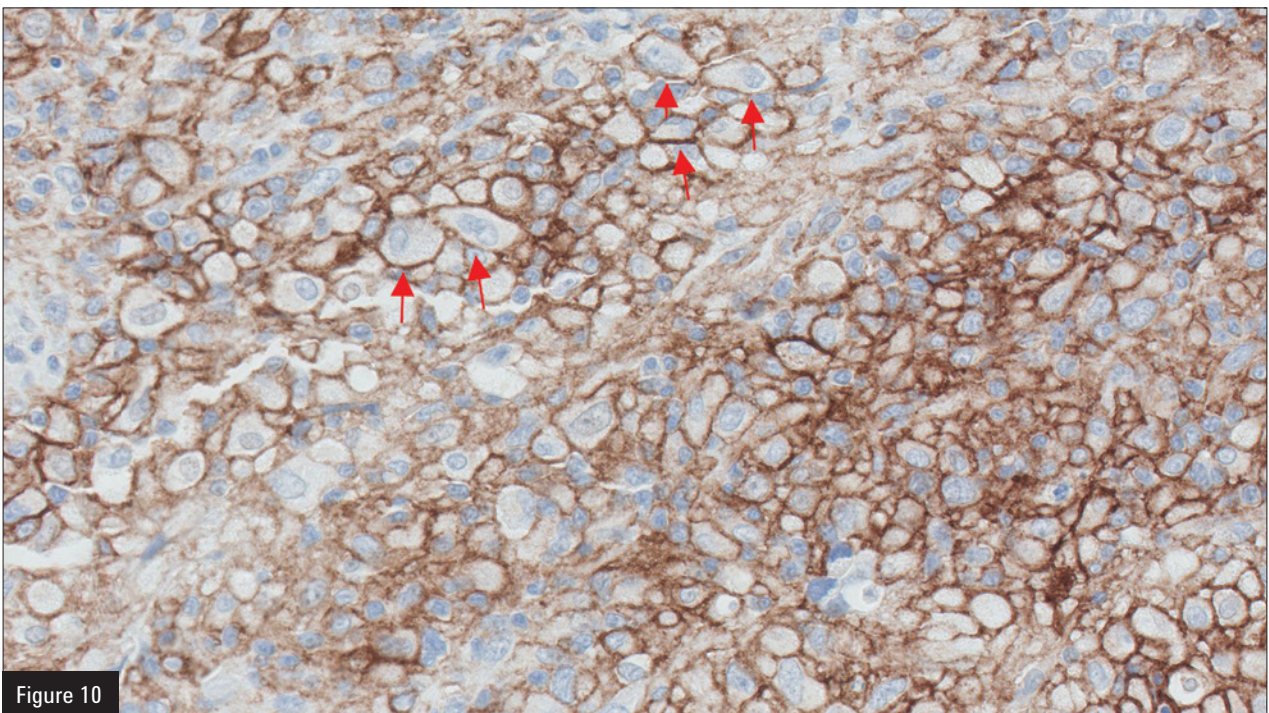
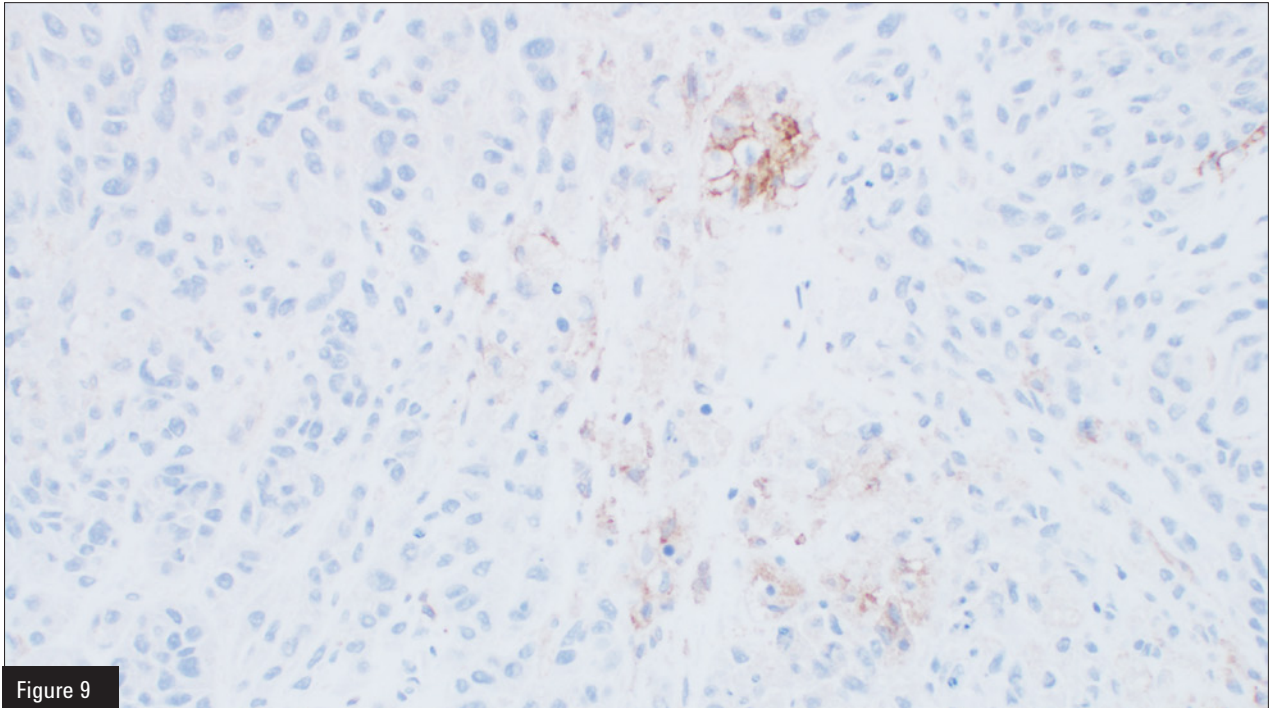


Figure 9: PD-L1 expression < 5%.

Figure 10: PD-L1 expression ≥ 5%. Red arrows show complete circumferential membrane staining.

Challenging Cases for Melanoma

PD-L1 IHC 28-8 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 sections, and incomplete rinsing of slides.

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

Possible Cause of Background

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

The non-specific background staining of the negative test specimen is useful in determining the level of background staining in the positive test specimen. All specimens must have $\leq 1+$ non-specific background staining.

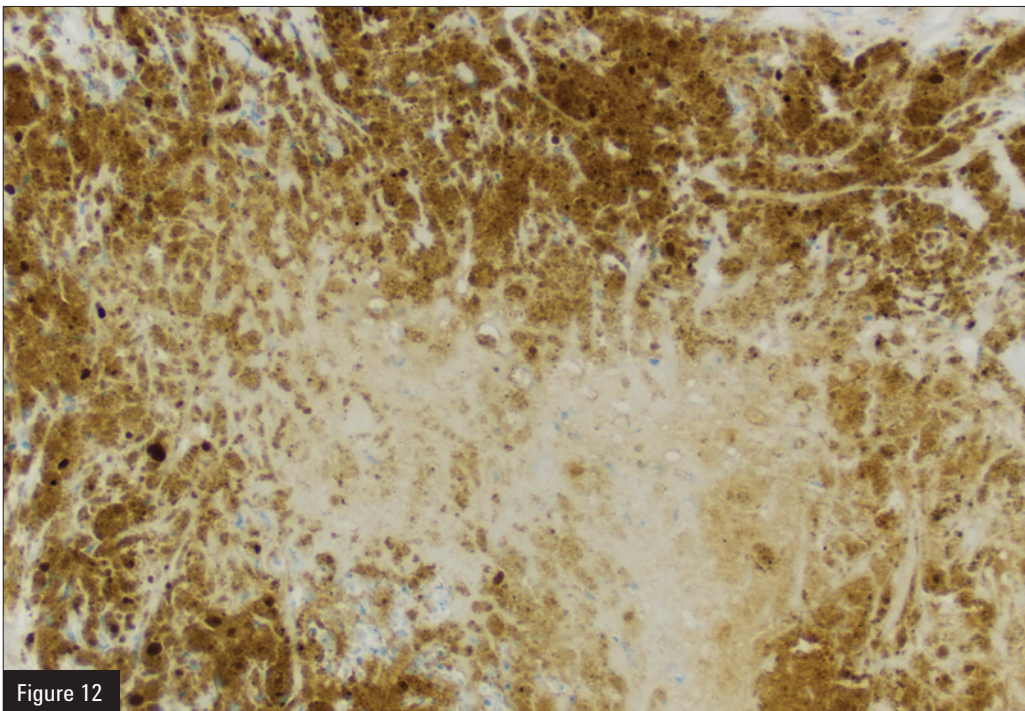
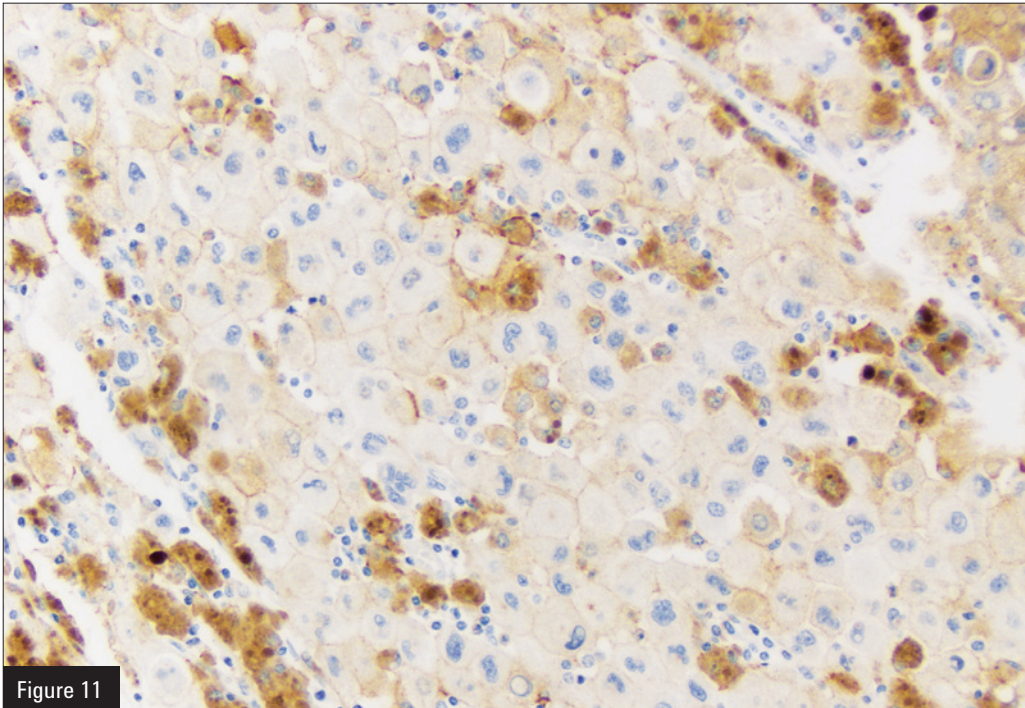


Figure 11: Positive PD-L1 specimen showing background staining and cytoplasmic melanin pigment.

Figure 12: Necrotic area stained with PD-L1 IHC 28-8 pharmDx.

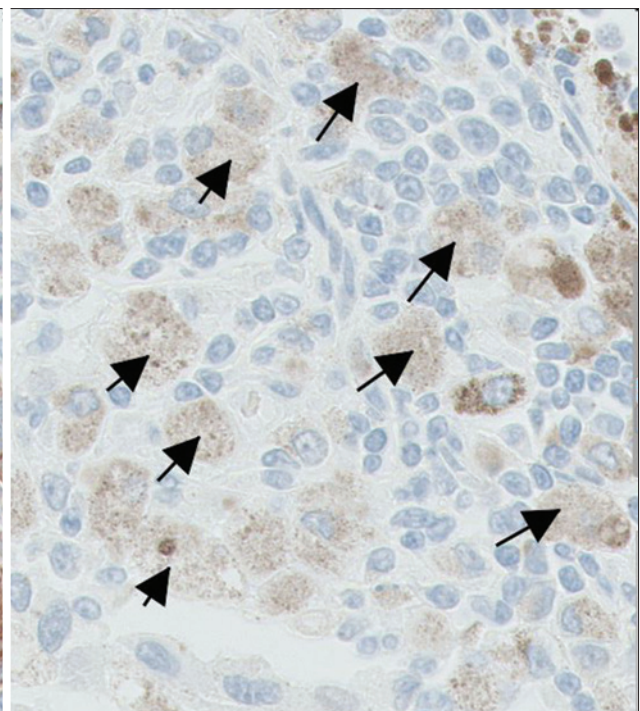
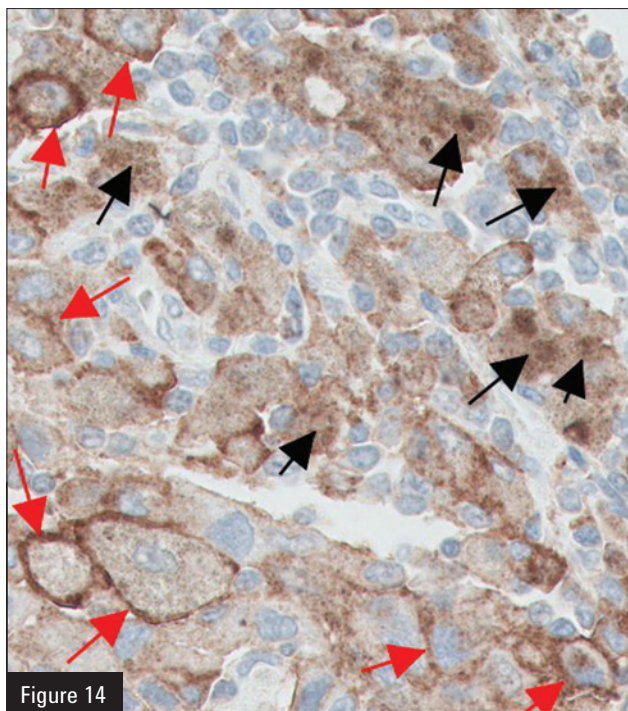
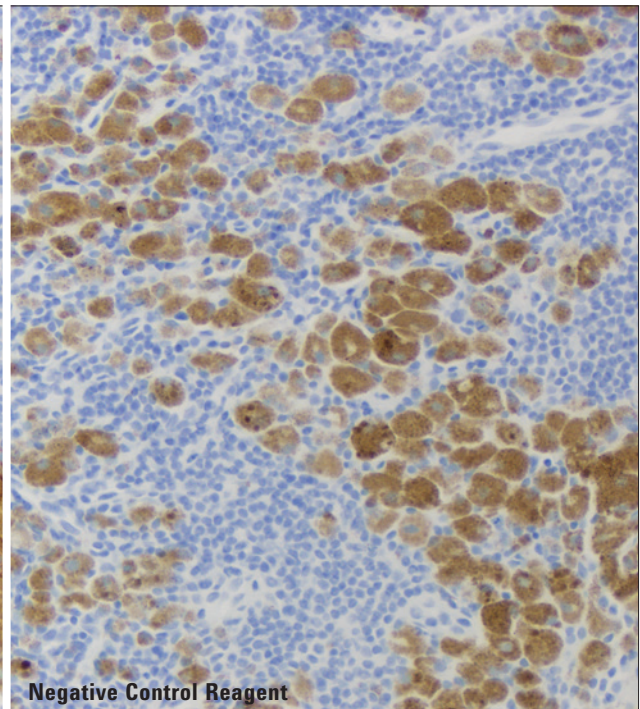
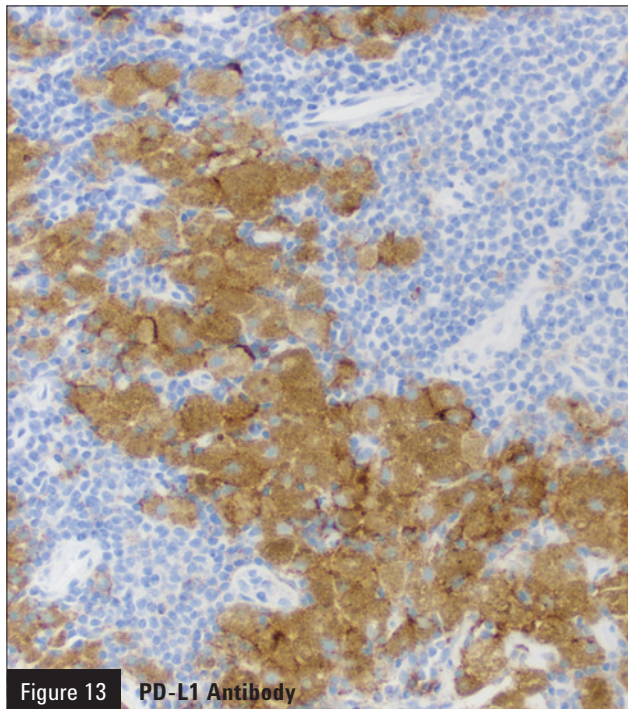


Figure 13: Elevated melanin content in a specimen may be excluded from interpretation. Left panel shows viable tumor cells stained with Primary Antibody. Right panel shows viable tumor cells stained with Negative Control Reagent.

Figure 14: Example of indeterminate case due to excess cytoplasmic staining. Red arrows show complete circumferential plasma membrane staining of viable tumor cells. Black arrows indicate cytoplasmic staining.

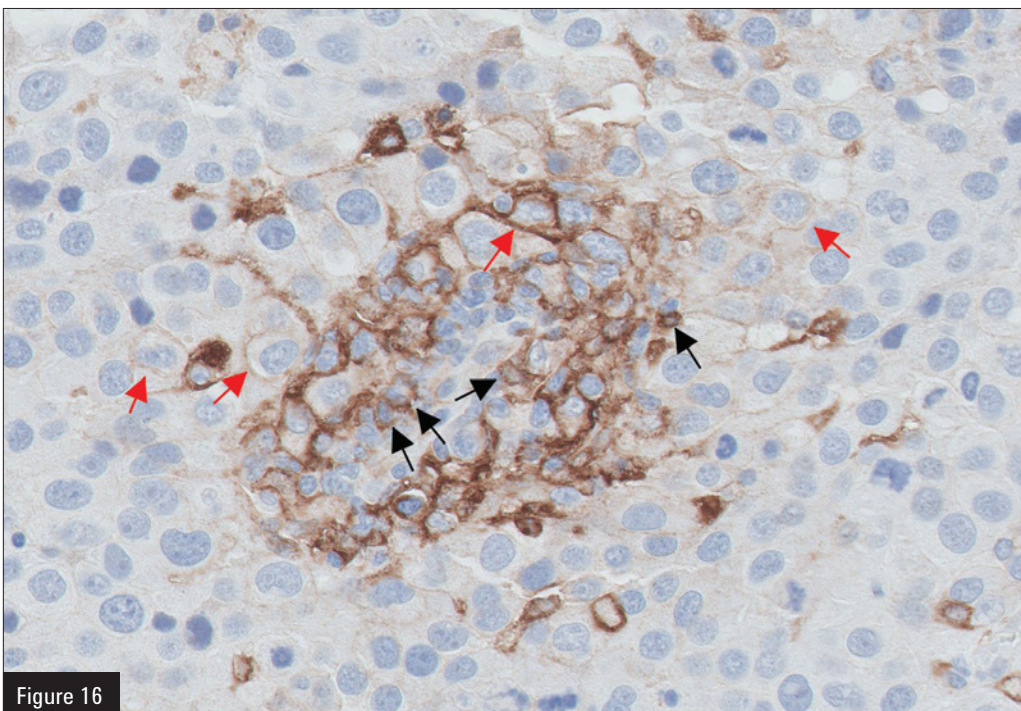
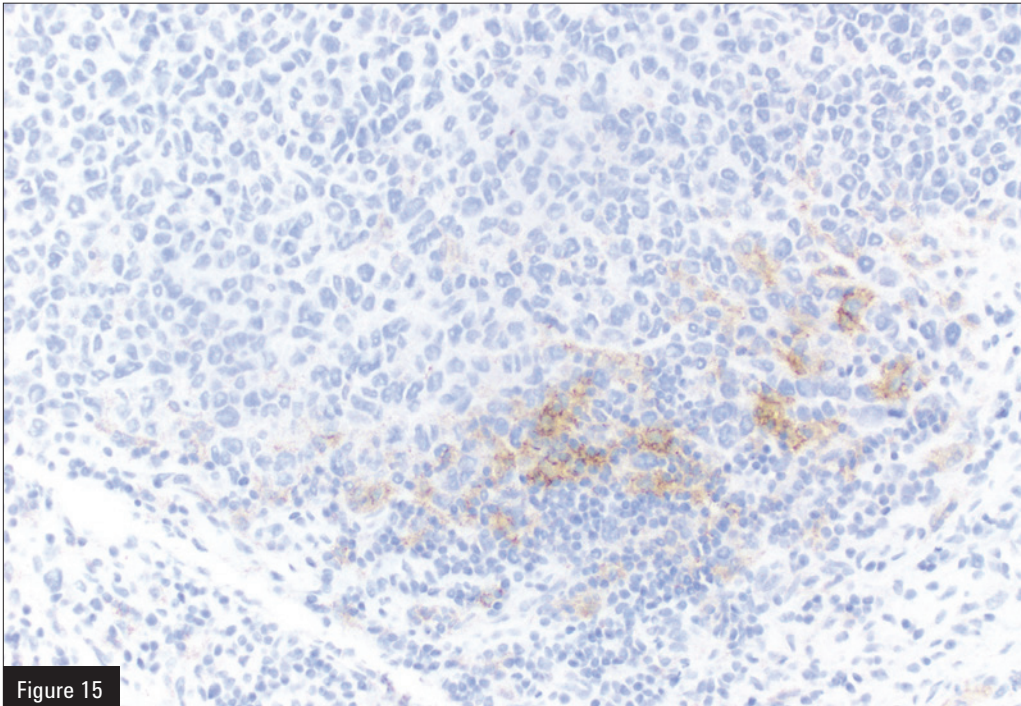


Figure 15: Immune cells staining with PD-L1 IHC 28-8 pharmDx are excluded from scoring. The tumor cells are negative in this example.

Figure 16: Immune cells staining with PD-L1 IHC 28-8 pharmDx. Red arrows show plasma membrane staining of viable tumor cells. Black arrows show staining of immune cells. Exclude immune cells from scoring.

Troubleshooting Guide for PD-L1 IHC 28-8 pharmDx

Problem	Probable Cause	Suggested Action
1. No staining of control or specimen slides.	1a. Programming error.	1a. Verify that the SK005 PD-L1 IHC 28-8 pharmDx protocol was selected for programming of slides.
	1b. Lack of reaction with DAB+ Substrate-Chromogen Solution.	1b. Verify that DAB+ Substrate-Chromogen Solution was prepared properly.
	1c. Sodium azide in wash buffer.	1c. Use only Dako Wash Buffer, Code K8007.
	1d. Degradation of Control Slide.	1d. Check kit expiration date and kit storage conditions on outside of package.
2a. Weak staining of specimen slides.	2a. Inappropriate fixation method used.	2a. Ensure that only approved fixatives and fixation methods are used.
2b. Weak staining of specimen slides or of the positive cell line on the Dako-provided Control Slide.	2b. Inadequate target retrieval.	2b. Verify that the 3-in-1 pre-treatment procedure was correctly performed.
3. Excessive background staining of slides.	3a. Paraffin incompletely removed.	3a. Verify that the 3-in-1 pre-treatment procedure was correctly performed.
	3b. Slides dried while loading onto the Autostainer Link 48.	3b. Ensure slides remain wet with buffer while loading and prior to initiating run.
	3c. Nonspecific binding of reagents to tissue section.	3c. Check for proper fixation of the specimen and/or the presence of necrosis.
4. Tissue detached from slides.	4a. Use of incorrect microscope slides.	4a. Use Dako FLEX IHC Microscope Slides, Code K8020, or Fisherbrand Superfrost Plus charged slides.
	4b. Inadequate preparation of specimens.	4b. Cut sections should be placed in a 58 ± 2 °C oven for one hour prior to staining.
5. Excessively strong specific staining.	5a. Inappropriate fixation method used.	5a. Ensure that only approved fixatives and fixation methods are used.
	5b. Inappropriate wash buffer used.	5b. Use only Dako Wash Buffer, Code K8007.
6. The Target Retrieval Solution is cloudy in appearance when heated.	6. Components in the Target Retrieval Solution cause the reagent to appear cloudy when heated.	6. No action required. This is normal and does not affect staining.

Study Data for PD-L1 IHC 28-8 pharmDx Results in Melanoma Patients

PD-L1 expression as detected by PD-L1 IHC 28-8 pharmDx in melanoma may be used as an aid in the assessment of patients for whom OPDIVO (nivolumab) and YERVOY (ipilimumab) combination treatment is being considered.

PD-L1 IHC 28-8 pharmDx was evaluated using specimen from patients enrolled in clinical trial CA209067, a Phase 3, randomized, double-blind study of nivolumab monotherapy or nivolumab in combination with ipilimumab versus ipilimumab monotherapy in patients with previously untreated metastatic melanoma. Of the 1296 patients enrolled, 945 patients were randomized to one of the three treatment arms in a 1:1:1 ratio and stratified by PD-L1 status ($\geq 5\%$ by a clinical trial assay).

PD-L1 expression status was ascertained for 843 (89%) study patients. The proportion of patients with tumor PD-L1 expression at $\geq 1\%$ and $< 1\%$, levels were balanced between the treatment groups. PD-L1 expression status for study patients with PD-L1 IHC pharmDx test results in CA209067 are presented in Table 3.

Progression free survival (PFS) was evaluated across PD-L1 subgroups defined as $< 1\%$ and $\geq 1\%$, $< 5\%$ and $\geq 5\%$, in all three arms of the study. The PFS Hazard Ratios (HRs) and median PFS by PD-L1 expression level are presented in Table 4.

Patients treated with nivolumab and nivolumab + ipilimumab who had $\geq 1\%$ or $\geq 5\%$ tumor cell membrane expression had a numerically higher Overall response rate (ORR) compared to those who had $< 1\%$ or $< 5\%$ tumor cell membrane expression respectively. In addition, a higher ORR was observed with nivolumab + ipilimumab than with nivolumab monotherapy regardless of PD-L1 expression level. The investigator-assessed ORR by PD L1 expression level is presented in Table 5.

	Number of subjects, n (%)		
	Nivolumab	Nivolumab + ipilimumab	Ipilimumab
PD-L1 quantifiable subjects ^a	288	278	277
PD-L1 expression level:			
$\geq 5\%$	80 (27.8)	68 (24.5)	75 (27.1)
$< 5\%$	208 (72.2)	210 (75.5)	202 (72.9)
$\geq 1\%$	171 (59.4)	155 (55.8)	164 (59.2)
$< 1\%$	117 (40.6)	123 (44.2)	113 (40.8)

^a Number of quantifiable PD-L1 results only; does not include the number indeterminate PD-L1 results.

Table 3: Frequency of PD-L1 Expression in All Randomized Subjects with Melanoma - CA209067

PFS values at PD-L1 expression level $\geq 5\%$ and $< 5\%$

Treatment	PD-L1 expression level	N of events/ N of patients	Median PFS in month (95% CI)	HR vs. ipilimumab (95% CI)	HR vs. nivolumab monotherapy (95% CI)
nivolumab +ipilimumab	$\geq 5\%$	28/68	13.96 (9.72, NR)	0.39 (0.25, 0.62)	0.96 (0.58, 1.58)
	$< 5\%$	103/210	11.24 (7.98, NR)	0.42 (0.32, 0.54)	0.70 (0.54, 0.91)
nivolumab	$\geq 5\%$	33/80	14.00 (9.07, NR)	0.41 (0.26, 0.63)	-
	$< 5\%$	122/208	5.32 (2.83, 7.06)	0.59 (0.47, 0.75)	-
ipilimumab	$\geq 5\%$	53/75	3.94 (2.79, 4.21)	-	-
	$< 5\%$	154/202	2.83 (2.76, 3.09)	-	-

PFS values at PD-L1 expression level $\geq 1\%$ and $< 1\%$

Treatment	PD-L1 expression level	N of events/ N of patients	Median PFS in month (95% CI)	HR vs. ipilimumab (95% CI)	HR vs. nivolumab monotherapy (95% CI)
nivolumab +ipilimumab	$\geq 1\%$	72/155	12.35 (8.51, NR)	0.44 (0.32, 0.58)	0.95 (0.69, 1.31)
	$< 1\%$	59/123	11.17 (6.93, NR)	0.38 (0.27, 0.53)	0.56 (0.40, 0.79)
nivolumab	$\geq 1\%$	79/171	12.39 (8.11, NR)	0.46 (0.34, 0.61)	-
	$< 1\%$	76/117	2.83 (2.76, 5.13)	0.67 (0.49, 0.92)	-
ipilimumab	$\geq 1\%$	122/164	3.91 (2.83, 4.17)	-	-
	$< 1\%$	85/113	2.79 (2.66, 2.96)	-	-

Abbreviations: CI = confidence interval, NR = not reached, PFS = progression-free survival

HR = Hazard ratio for treatment effect based on Cox proportional hazard model with treatment, PD-L1 status, and treatment by PD-L1 status interaction

Table 4: Summary of Progression-free Survival by PD-L1 Level and Treatment Group - All Randomized Subjects with Melanoma - CA209067

Treatment	PD-L1 expression level	Number of Patients	ORR	
			%	[95% CI]
nivolumab +ipilimumab	$\geq 5\%$	68	72%	(59.9, 82.3)
	$< 5\%$	210	55%	(47.8, 61.6)
	$\geq 1\%$	155	65%	(56.4, 72.0)
	$< 1\%$	123	52%	(42.8, 61.1)
nivolumab	$\geq 5\%$	80	58%	(45.9, 68.5)
	$< 5\%$	208	41%	(34.6, 48.4)
	$\geq 1\%$	171	54%	(46.6, 62.0)
	$< 1\%$	117	33%	(24.9, 42.6)
ipilimumab	$\geq 5\%$	75	21%	(12.7, 32.3)
	$< 5\%$	202	18%	(12.8, 23.8)
	$\geq 1\%$	164	19%	(13.2, 25.7)
	$< 1\%$	113	19%	(11.9, 27.0)

* ORR = Overall Response Rate

Table 5: Investigator-assessed ORR: Melanoma Patients by PD-L1 Expression - PD-L1 Tested Subjects - CA209067

Bibliography

- Clinical and Laboratory Standards Institute (formerly NCCLS). Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline – Third Edition. CLSI document M29-A3 [ISBN 1-56238-567-4]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087–1898 USA, 2000
- Clinical and Laboratory Standards Institute (formerly NCCCLS). Quality assurance for Immunocytochemistry; Approved guideline. CLSI document MM4-A (1-56238-396-5) CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA; 1999
- Department of Health, Education and Welfare, National Institutes for Occupational Safety and Health, Rockville, MD. “Procedures for the decontamination of plumbing systems containing copper and/or lead azides.” DHHS (NIOSH) Publ. No. 78-127, Current 13. August 16, 1976
- Herman GE, Elfont EA. The taming of immuno-histochemistry: the new era of quality control. *Biotech & Histochem* 1991; 66:194
- Larkin J1, Chiarion-Sileni V, Gonzalez R et al. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med* 2015;373(1):23-34
- Omata M, Liew C-T, Ashcavai M, Peters RL. Nonimmunologic binding of horseradish peroxidase to hepatitis B s surface antigen: a possible source of error in immuno-histochemistry. *Am J Clin Pathol* 1980; 73:626
- Postow M, Chesney J, Pavlick A, et al. Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. *N Engl J Med* 2015;372(21):2006-17
- Taylor CR, Rudbeck L. Education Guide: Immunohistochemical Staining Methods. Sixth Edition. Dako, Carpinteria, California; 2013
- Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. *Curr Opin Immunol* 2012;24(2):207-212
- Topalian SL, Hodi FS, Brahmer JR, et. al. Safety, Activity, and Immune Correlates of Anti-PD-1 Antibody in Cancer. *New Eng J Med* 2012; 366(26):2443-2454
- Wang C, Thudium KB, Han M, et al. In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human primates. *Cancer Immunol Res* 2014;2(9):846-56
- Weber JS, D’Angelo SP, Minor D, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. *Lancet Oncol* 2015; 16: 375–84
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