Modern Pathology (2018) 31:1630–1644
https://doi.org/10.1038/s41379-018-0071-1

ARTICLE

Optimization and validation of PD-L1 immunohistochemistry staining protocols using the antibody clone 28-8 on different staining platforms

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Received: 17 November 2017 / Revised: 5 April 2018 / Accepted: 8 April 2018 / Published online: 26 June 2018
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Abstract

Several immunohistochemistry (IHC) assays have been developed to assess tumor programmed death-ligand 1 (PD-L1) expression levels in patients who are candidates for programmed death-1 (PD-1)/PD-L1 inhibitor therapy. The PD-L1 IHC 28-8 pharmDx kit is FDA-approved as a complementary diagnostic and CE-marked as an in vitro diagnostic device for nivolumab therapy in melanoma and specific lung cancer subtypes (and for squamous cell carcinoma of the head and neck/urothelial carcinoma in Europe only). Kit availability is limited outside the United States, and its use requires the Dako Autostainer Link 48 platform, which is unavailable in many laboratories. Validated laboratory-developed tests based on 28-8 concentrated antibody outside the kit are needed. This study compared the results from PD-L1 expression level analysis across four immunohistochemistry platforms (Dako Autostainer Link 48, Dako Omnis, Leica Bond-III, and Ventana BenchMark ULTRA) with the 28-8 pharmDx kit in lung cancer (multiple histologies), melanoma, and head and neck cancer (multiple histologies). Samples were prepared per protocol for each platform and stained using PD-L1 IHC 28-8 pharmDx kit on Dako Autostainer Link 48, and per protocol for each platform. The control samples (tonsil and placenta tissue; cell lines with prespecified PD-L1 expression levels) were tested to evaluate the specificity and the sensitivity of test assays. An agreement level of 0.90 with the pharmDx kit was set for each platform. Inter- and intra-assay reliability were assessed. Evaluable samples were lung cancer = 29; melanoma = 31; head and neck cancer = 30. Mean agreement was calculated for PD-L1 expression levels of ≥1%, ≥5%, ≥10%, and ≥50%. Mean overall agreement for all indications was 0.87–0.99. Inter- and intra-assay of scoring/classification repeatability was 100%. Analysis of PD-L1 expression levels using laboratory-developed immunohistochemistry assays with 28-8 antibody may be permissible if the platform is validated using reference samples with defined expression levels.

Introduction

Programmed cell death-1 (PD-1) receptor and its ligands, programmed cell death-ligands 1 and 2 (PD-L1, PD-L2), are important checkpoint regulators in immune cells [1–3]. PD-L1 and PD-L2 are frequently expressed in many cancer types, including non-small cell lung cancer, melanoma, and squamous cell carcinoma of the head and neck [4–6]. Binding of these ligands to PD-1 has been shown to impair the activity of cytotoxic T cells [1, 2, 4, 7]. This phenomenon enables tumor cells to evade the body's natural immune defense mechanism, thereby allowing cancers to progress. Studies have shown that blockade of PD-1 restores the immune function of T cells [8]. Assessment of PD-L1 expression using validated diagnostic antibodies may therefore be useful in predicting the response to anti-PD-1/PD-L1 treatment.

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Nivolumab was the first PD-1 immune checkpoint inhibitor to be approved worldwide; it was licensed in Japan in 2014 for the treatment of unresectable malignant melanoma [9]. Since then, nivolumab and other PD-1/PD-L1 inhibitors, including pembrolizumab and atezolizumab, have been approved for use in patients with previously treated locally advanced and/or metastatic non-small cell lung cancer [10–15]. Pembrolizumab is also approved for previously untreated metastatic non-small cell lung cancer. These PD-1/PD-L1 inhibitors, along with the PD-L1 inhibitor durvalumab, have also been approved for the treatment of various other tumor types [10–16]. In metastatic non-small cell lung cancer, treatment with pembrolizumab currently requires assessment of tumor PD-L1 expression, either with the companion diagnostic the PD-L1 IHC 22C3 pharmDx kit (Agilent Technologies, Santa Clara, CA) (U.S. requirement) [11] or with any other validated immunohistochemistry (IHC) assay [14]. For the treatment of non-small cell lung cancer with pembrolizumab, PD-L1 levels must be ≥50% in treatment-naive patients and ≥1% in previously treated patients [11, 14]. There is no companion diagnostic required for treatment with nivolumab, although a U.S. Food and Drug Administration (FDA)-approved complementary diagnostic is available for non-squamous non-small cell lung cancer and melanoma, the PD-L1 IHC 28-8 pharmDx kit (Agilent Technologies, Santa Clara, CA) [10, 17, 18]. This diagnostic assay is also available in Europe as a CE-marked in vitro diagnostic device for the same lung and melanoma indications, and for patients with squamous cell carcinoma of the head and neck or urothelial carcinoma who may benefit from nivolumab [18, 19].

Extensive research has examined the value of tumor PD-L1 expression levels, both in assessing the disease prognosis and in predicting the response to anti-PD-1/PD-L1 immunotherapies. Higher PD-L1 expression levels are associated with poor prognosis, independently of treatment, in some tumor types, including non-small cell lung cancer [20–23]. However, other studies have found no association with prognosis in PD-L1-expressing non-small cell lung cancer or melanoma [6, 23, 24]. The association between PD-L1 expression levels and treatment response may be related to tumor histology (e.g., squamous cell vs. adenocarcinoma) [21, 25–30]. The value of PD-L1 expression as a predictor of response to anti-PD-1/PD-L1 treatments may also differ depending on whether the patients with advanced or metastatic disease receive these treatments as first-line therapy, or after previous therapy. In first-line treatment of patients with advanced squamous and non-squamous non-small cell lung cancer with high PD-L1 expression levels (≥50% of tumor cells), treatment with the PD-1 inhibitor pembrolizumab was associated with longer progression-free and overall survival, compared with chemotherapy [31]. In contrast, another study with lower prespecified PD-L1 expression levels (≥5% of tumor cells) found that PD-1 inhibitor therapy showed similar outcomes to chemotherapy [32].

Several factors contribute to difficulties in the assessment of PD-L1 expression. PD-L1 induction in tumors results from either oncogenic signaling, leading to constitutive widespread expression, or in response to IFN-γ release by effector T cells during their immune response to the tumor, leading to variable expression of inducible PD-L1 [33, 34]. Prior therapy may therefore alter the expression of inducible PD-L1, as well as other biomarkers that form part of the dynamic immune landscape of the tumor and tumor microenvironment and interact with PD-L1 [26, 35–37]. Levels of constitutive versus inducible PD-L1 may vary across tumor types, with predominant expression of IFN-γ-induced PD-L1 in melanoma, and a lower level of constitutive PD-L1 in melanoma compared with lung tumors [37]. The spatial and temporal heterogeneity of induced PD-L1 also means that the location and timing of the biopsy and type of sample (for example, biopsy vs. surgical resection) can influence PD-L1 expression levels [26, 38].

At the epitope level, there may also be difficulties in assessing PD-L1 expression as a biomarker of response to anti-PD-1/PD-L1 treatments. Currently, only PD-L1 in the cell membrane, rather than intracellular protein or mRNA, is assessed [23]. However, in cells with strong cytoplasmic staining, it can be difficult to determine the extent of membranous staining [39]. The structure of PD-L1, with two small linear hydrophilic binding regions, restricts the number of sites available for antibody binding [23, 26, 40], which makes detection using typical immunohistochemistry methods on formalin-fixed, paraffin-embedded specimens difficult [23, 26]. This means that immunohistochemistry antibodies typically bind PD-L1 at sites different from the therapeutic antibodies [26]. Linear epitope mapping of anti-PD-L1 antibodies, including 28-8, has suggested that potential conformational epitopes of PD-L1 targeted by the anti-PD-L1 antibody may affect the staining patterns [41].

The PD-L1 IHC 28-8 pharmDx kit is a validated [42–45], automated immunohistochemistry assay using Monoclonal Rabbit Anti-PD-L1, Clone 28-8 (Agilent Technologies, Santa Clara, CA) in combination with the Dako Autostainer Link 48 platform. Outside the United States, pathologists have expressed an interest in validated staining protocols [46] using the rabbit monoclonal antihuman PD-L1 antibody (clone 28-8) on common staining platforms. These validated protocols may be useful when the PD-L1 IHC 28-8 pharmDx kit or Dako Autostainer Link 48 platform are difficult to access. To this end, we have developed and validated immunohistochemistry staining protocols using the anti-PD-L1 28-8 antibody purchased from Abcam (Cambridge, UK) [47] on four major automated staining

platforms: Dako Autostainer Link 48 (Agilent Technologies, Santa Clara, CA) [48]; Dako Omnis (Agilent Technologies, Santa Clara, CA) [49]; Leica Bond-III (Leica Biosystems, Wetzlar, Germany) [50]; and Ventana BenchMark ULTRA (Ventana Medical Systems, Roche Diagnostics, Tucson, AZ [software version 12.3]) [51]. Results from each of these four staining protocols were compared with those from the PD-L1 IHC 28-8 pharmDx kit on the Dako Autostainer Link 48 applied to PD-L1-expressing and non-PD-L1-expressing samples of lung cancer (various histologies), melanoma, and head and neck cancer (various histologies), together with batch tissue controls (tonsil and placenta) and reference cell lines with defined levels of PD-L1 expression (#HD787, Horizon Discovery Group, Cambridge, UK) [52]. This study was designed to examine whether PD-L1 expression levels assessed using four widely available platforms produced comparable results to the FDA-approved and CE-marked IHC 28-8 pharmDx kit.

Materials and methods

Sample preparation

Formalin-fixed, paraffin-embedded sample specimens from surgical resections were obtained from the archive of the Institute of Pathology Nordhessen (Kassel, Germany) following ethical approval by the Landesärztekammer Hessen. Additional samples were purchased from a commercial biobanking service (Cureline, Inc., San Francisco, CA). Evaluable tumor samples included human lung cancer (total $n = 29$: 3 squamous cell carcinoma, 7 adenocarcinoma, 16 indeterminate, 1 sarcomatoid, 2 large-cell carcinoma); melanoma ($n = 31$); head and neck cancer (total $n = 30$: 21 squamous cell carcinoma, 9 adenocarcinoma). Individual patient informed consent had been obtained for all samples. All samples were stained once with the PD-L1 IHC 28-8 pharmDx kit to determine the reference PD-L1 expression levels.

A comparison of assay results on different staining platforms was performed on tissue microarrays prepared from one 1.5 mm diameter core per donor block. The tissue microarrays were prepared and analyzed at Targos Molecular Pathology GmbH, Kassel, Germany, or the Institute of Pathology, Universitätsmedizin Göttingen, Göttingen, Germany (for Dako Omnis platform). From each tissue microarray, serial sections of 4–5 µm were cut, mounted onto Fisherbrand Superfrost Plus charged slides, and placed in an oven at 58 °C (± 2 °C) for 1 h (except for samples prepared for the Dako Omnis platform, where the slides were placed in the oven at 65 °C for 20 min, according to local protocol). The sections were deparaffinized in xylene, then rehydrated in a standard series of descending alcohol immersions and stained with the immunohistochemistry protocols described in Table 1. After each run, on all platforms, slides were dehydrated in ascending alcohol, ending in xylene, and coverslipped using a non-aqueous mounting medium and standard glass coverslips. Tissue microarray cores were excluded from the analysis if they were washed off, did not contain tumor, or the sample was not evaluable for other reasons.

Staining of control samples

Tonsil epithelium and placental syncytiotrophoblast were prepared as above and stained as batch control samples, one with Abcam anti-PD-L1 28-8 antibody and a second specimen with negative control reagent (Table 1). A PD-L1 standard microarray (#HD787, Horizon Discovery Group, Cambridge, UK) [52] comprising cell lines with controlled PD-L1 expression (negative [−], low positive [+], intermediate positive [++], and strong positive [+++]) provided an additional control. The samples were pre-analyzed for PD-L1 expression using the PD-L1 IHC 28-8 pharmDx kit to verify the immunohistochemistry performance. PD-L1 expression levels were then determined by immunohistochemistry using the Abcam anti-PD-L1 28-8 antibody on each of the four platforms detailed below. Validation was performed according to all relevant College of American Pathologists/Clinical Laboratory Improvement Amendments criteria for immunohistochemistry assays.

Dako PD-L1 28-8 pharmDx kit

The immunohistochemistry staining protocol using the PD-L1 IHC 28-8 pharmDx kit was performed as previously described [43] and as specified in the pharmDx kit label [18]. Slides were processed on the Autostainer Link 48 (Agilent Technologies, Santa Clara, CA) alongside control slides provided with the kit, using an automated staining protocol validated for the PD-L1 immunohistochemistry assay, and prepared using EnVision FLEX Target Retrieval Solution, Low pH (Dako, Glostrup, Denmark).

Assay establishment and validation procedure

For each of the staining platforms, protocols were established starting as closely similar as possible to the original PD-L1 28-8 pharmDx kit protocol. For the application of the 28-8 antibody on the Dako Autostainer Link 48, this included the use of all reagents (Detection Kit, Wash Buffer, Antibody Diluent) from Dako and application of the same incubation conditions as in the pharmDx assay (Table 1). The concentration of the primary antibody was then adjusted by titration series (23.84–2.98 µg/mL). The same procedure was used to adapt the protocol for the Dako

Table 1 Final validated staining protocols and reagents

Platform	Heat-induced epitope retrieval	Block	Wash buffer	Antibody diluent	Positive primary antibody	Negative primary antibody	Secondary antibody (linker)	HRP polymer	Detection	Counterstain
IHC 28-8 pharmDx kit (SK005, Dako, Glostrup, Denmark) on Dako Autostainer Link 48	EnVision Flex Target Retrieval Solution, pH 6.1 (K8005) Preheat to 65 °C 97 °C 20 min, cool to 65 °C (PT Link) Leave 5 min in wash buffer at room temperature	Peroxidase block (from kit) 5 min Wash x1 in wash buffer	Wash buffer (K8007) (diluted 1:20)	EnVision Flex Antibody Diluent (K800621)	28-8 (from kit) 11.92 µg/mL 60 min Wash x1 in wash buffer	Negative control reagent (from kit; prediluted) 11.92 µg/mL 60 min Wash x1 in wash buffer	Anti-rabbit IgG linker (from kit) 30 min Wash x1 in wash buffer	Visualization reagent (from kit) 30 min Wash x1 in wash buffer, leave 5 min in wash buffer	Liquid DAB+ substrate buffer and chromogen (from kit) 2 × 5 min Wash x1 in wash buffer DAB Enhancer (from kit) 5 min Wash x1 in wash buffer	Hematoxylin (K8008) 7 min Wash x1 in water, leave 5 min in wash buffer, wash x1 in water
Abcam 28-8 antibody on Dako Autostainer Link 48	EnVision Flex Target Retrieval Solution, pH 6.1 (K8005) Preheat to 65 °C 97 °C 20 min, cool to 65 °C (PT Link) Leave 5 min in wash buffer at room temperature	K4010/K4011 or SM801/K8000 5 min Wash x1 in wash buffer	Wash buffer (K8007) (diluted 1:10)	Dako Flex Antibody Diluent (K800621)	28-8 (Abcam ab205921) 10.09 µg/mL (1:100) 60 min Wash x1 in wash buffer	DA1E IgG (Cell Signaling 3900S) 11.90 µg/mL (1:210) 60 min Wash x1 in wash buffer	Anti-rabbit IgG linker (SM805 or K8009) 30 min Wash x1 in wash buffer	EnVision Flex HRP (K4010 or K4011 or K8000) 30 min Wash x1 in wash buffer, leave 5 min in wash buffer	Liquid DAB+ Substrate-Chromogen system (K3468) 2 × 5 min Wash x1 in wash buffer DAB Enhancer (S196130-2) 5 min Wash x1 in wash buffer	Hematoxylin (SM806 or K8008) 7 min Wash x1 in water, leave 5 min in wash buffer, wash x1 in water
Dako Omnis	EnVision Flex Target Retrieval Solution, pH 6.1 (GV805) 97 °C 30 min Leave 2 min 40 s in wash buffer, 2 cycles	Peroxidase-blocking reagent (DM841, from EnVision Flex High pH kit GV823 Dako Omnis) 3 min Leave 2 min in wash buffer, 10 cycles	Wash buffer (Dako Omnis GC 807) (diluted 1:50)	Dako Flex Antibody Diluent (K800621)	28-8 (ab205921 Abcam) 10.09 µg/mL (1:100) 30 min Leave 2 min in wash buffer, 10 cycles	DA1E IgG (Cell Signaling 3900S) 11.90 µg/mL (1:210) 30 min Leave 2 min in wash buffer, 10 cycles	EnVision Flex+ Rabbit LINKER (Dako Omnis GV809) 10 min Leave 2 min in wash buffer, 10 cycles	EnVision Flex HRP (DM842 Dako Omnis) 20 min Leave 2 min in wash buffer, 10 cycles Leave 31 s in water Leave 2 min in wash buffer, 10 cycles	EnVision Flex DAB+ substrate/ buffer (DM847/ DM843 Dako Omnis) 5 min Leave 2 min in wash buffer, 10 cycles Leave 31 s in water Leave 2 min in wash buffer, 10 cycles	Hematoxylin (GC808 Dako Omnis) 3 min Leave 2 min in water, 10 cycles Leave 2 min in wash buffer, 10 cycles

Table 1 (continued)

Platform	Heat-induced epitope retrieval	Block	Wash buffer	Antibody diluent	Positive primary antibody	Negative primary antibody	Secondary antibody (linker)	HRP polymer	Detection	Counterstain
Leica Bond-III and Bond Polymer Refine Detection (DS9800, Leica Biosystems, Wetzlar, Germany)[a]	EnVision Flex Target Retrieval Solution, pH 6.1 (K8005) 95–98 °C 40 min approx (steamer) Cool 20 min at room temp to approx. 60 °C Wash in wash buffer Wash in water	Peroxide block 10 min Wash x3 in wash buffer	Bond™ Wash Solution (Leica AR9590) (diluted 1:10)	Dako Flex Antibody Diluent (K800621)	28-8 (ab205921 Abcam) 2.52 µg/mL (1:400) 60 min Wash x3 in wash buffer	DA1E IgG (Cell Signaling 3900S) 2.53 µg/mL (1:990) 60 min Wash x3 in wash buffer	Post-primary 30 min Leave 2 min in wash buffer, 3 cycles Polymer anti-rabbit Poly-HRP-IgG 30 min Leave 2 min in wash buffer, 2 cycles Wash x1 in wash buffer	DAB Refine substrate 10 min Wash x5 in water	Hematoxylin 8 min Wash x5 in water	
Ventana Benchmark ULTRA with OptiView DAB IHC Detection Kit (760-700 Ventana Medical Systems/ Roche Diagnostics, Basel, Switzerland)[b]	Ultra CC1 (950-224 Ventana) 100 °C, 4 min, then change buffer every 8 min for 64 min	OptiView peroxidase inhibitor	EZ Prep solution (950-102 Ventana)/ saline sodium citrate SSC (950-110) (diluted 1:10)	Ventana antibody diluent (251-018 Ventana)	28-8 (ab205921 Abcam) 5.05 µg/mL (1:200) 37 °C 24 min	Rabbit Monoclonal Negative Control Ig (790-4795 Ventana) pre-diluted at approx. 10 µg/mL 37 °C 24 min	OptiView HQ Universal Linker 8 min	OptiView HRP Multimer 8 min	OptiView DAB IHC Detection Kit (760-700) + OptiView Amplification Kit (860-099 Ventana) 4 min peroxide + 4 min multimer	Hematoxylin II (790-2208 Ventana) 8 min Bluing Reagent (760-2037 Ventana) 8 min

IHC immunohistochemistry; *HRP* horseradish peroxidase.

Unless otherwise specified, reagents were obtained from Dako, Glostrup, Denmark.

Unless otherwise specified, incubations were carried out at room temperature.

[a]Reagents used in this row are from the Leica Bond™ Polymer Refine Detection Kit, unless otherwise specified.

[b]Reagents used in this row are from the Ventana OptiView DAB IHC Detection Kit, unless otherwise specified.

Omnis platform, with antibody concentrations ranging from 11.92 to 2.98 µg/mL and Dako FLEX and FLEX+ detection systems being tested. Most importantly, the antigen retrieval conditions were not changed from the original pharmDx protocol. For the remaining staining platforms, the pretreatment buffers and detection systems selected differed from the Dako reagents. Originally, similar pretreatment conditions were used on Leica Bond-III; however, neither Leica ER2 nor ER1 solutions yielded satisfactory results, and were replaced by external heat-induced epitope retrieval using EnVision FLEX Target Retrieval Solution, Low pH in a steamer (Table 1). Tested primary antibody concentrations on the Leica Bond-III ranged from 47.68 to 2.52 µg/mL and consisted of double incubation of the antibody and a signal amplification step using FLEX+ Rabbit Linker antibody from Dako (final protocol is specified in Table 1). While establishing a protocol for the Ventana BenchMark ULTRA, we investigated a range of incubation times for CC1 pretreatment (8–64 min), primary antibody concentrations (15.89–5.96 µg/mL), and incubation times (30–60 min), respectively. The OptiView DAB detection system including an additional signal amplification step was required for the Ventana platform as a substitute for the less sensitive, but more commonly used, UltraView system.

All final protocols were confirmed on full sections of formalin-fixed, paraffin-embedded tonsil and placenta specimens, as well as on the selected lung cancer samples and the defined cell-line control slides (Horizon). Validation of the final protocols listed, specified in Table 1, was conducted, as described below.

Repeatability

Inter- and intra-assay repeatability was determined by repeated staining of the selected lung cancer samples (low, medium, and high levels of PD-L1 expression) in three independently performed assay runs, and by staining the three replicates of those specimens in the same assay run, respectively.

Stability and matrix effect

Antigen stability in slide-mounted sections was checked over a period of 6 weeks in total. The effect of various fixatives (10% neutral buffered formalin, 10% non-buffered formalin, AFA, Pen-Fix, Prefer, and Z-5) and fixation times were tested at room temperature using tissue microarrays prepared from specimens derived from the same tumor, but fixed differently.

Sample evaluation and statistical analysis

Semi-quantitative determination of PD-L1 expression levels using bright-field microscopy was performed by senior pathologists experienced and trained in PD-L1 immunohistochemistry evaluation and involved either in clinical trials with PD-L1 as a biomarker (BJ, JR) or in a worldwide training program for 28-8 as a predictive biomarker (CD'A, H-US). Specimens in each tumor category were read by a single pathologist. Three samples (one exhibiting high, one medium, and one low biomarker concentration) were tested in a single assay run. All samples were blinded and analyzed singly. Raw data were documented on customized forms, 2×2 contingency tables were generated, and positive, negative, and overall agreement were calculated using Microsoft Excel 2007.

Interpretation

Staining interpretation (PD-L1 on tumor cells) was performed on the basis of the official interpretation manual of Dako for Monoclonal Rabbit Anti-PD-L1, Clone 28-8 pharmDx kit (Agilent Technologies, Santa Clara, CA). The percentage of tumor cells with PD-L1-membranous staining, regardless of intensity, was determined. Non-malignant and immune cells, cytoplasmic staining, necrotic cells, and debris were excluded. A sample was considered evaluable if at least 100 viable tumor cells were present.

PD-L1-positive tumor cells were quantified by evaluating the ratio of area proportion of stained to unstained tumor cells according to custom scoring criteria of the assay [17, 18]. Prespecified PD-L1 expression levels of ≥1%, ≥5%, ≥10%, and ≥50% tumor cell staining were used for final classification of all samples into positive or negative categories for each threshold level. This scoring algorithm was applied to all platforms and the scores obtained for each platform were compared with the respective scores obtained using the PD-L1 IHC 28-8 pharmDx kit as the reference assay.

An agreement level of 0.90, based on the final PD-L1-positive/negative classifications with respect to the expression thresholds, was used to decide whether each platform provided acceptable results in comparison with the PD-L1 IHC 28-8 pharmDx kit.

Results

All staining protocols used resulted in the characteristic tumor cell pattern of membranous staining with an accompanying cytoplasmic component. The membranous staining obtained with the Leica Bond-III and Ventana BenchMark ULTRA platforms was less clearly defined, compared with the Dako platforms (Autostainer Link 48 and Omnis). The observed percentage, intensity, and distribution of the stained cells covered a wide dynamic range from negative (0%) to strongly positive staining and from focal to homogenous staining of the tumor cells (Fig. 1),

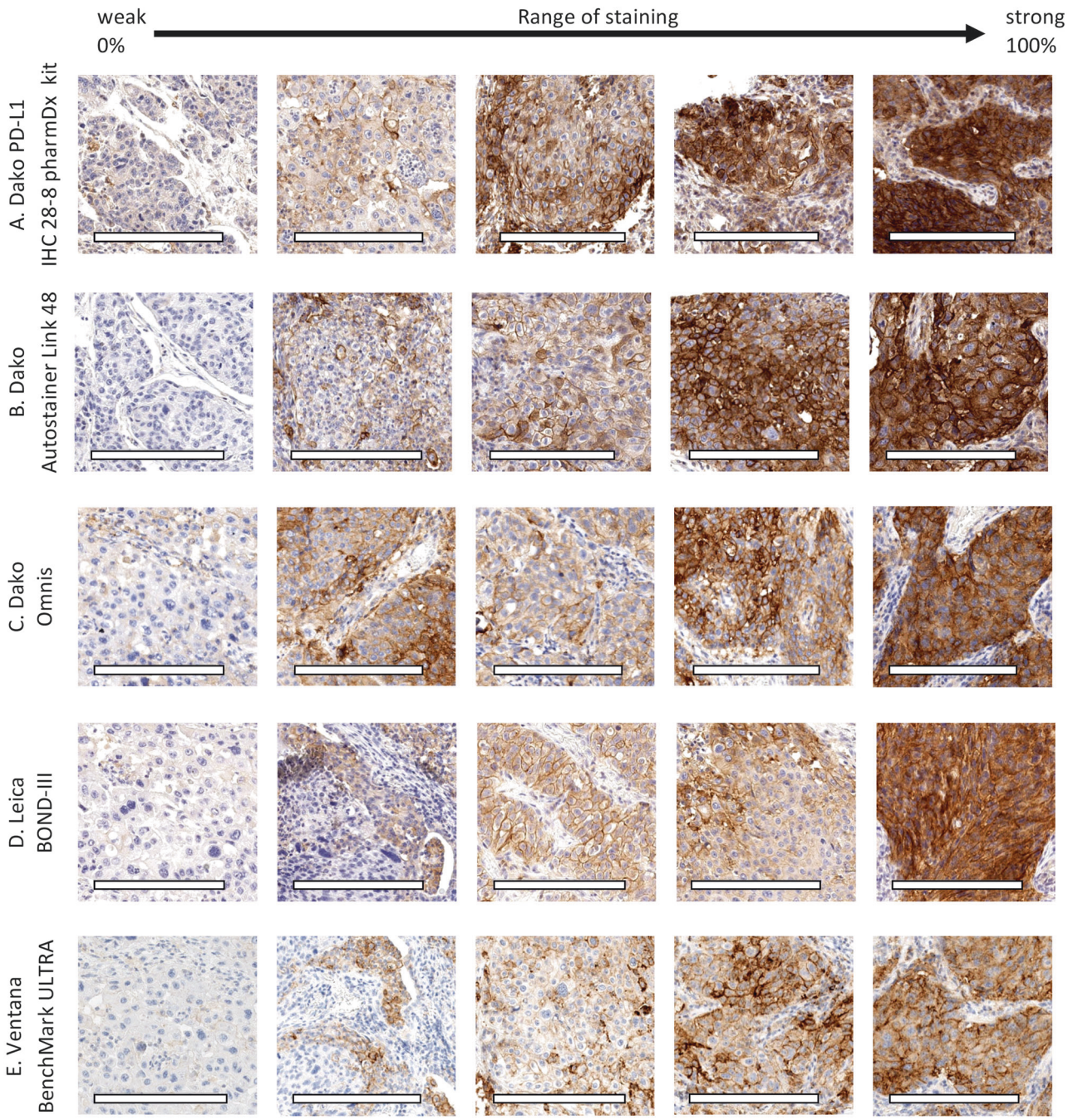

Fig. 1 PD-L1 immunohistochemistry staining in the lung cancer tumor microarray samples displaying different staining intensity (non-matched sections), using antibody clone 28-8 within the pharmDx kit and outside the kit different automated staining platforms. **a** Dako PD-L1 IHC 28-8 pharmDx kit. **b** Dako Autostainer Link 48. **c** Dako Omnis. **d** Leica Bond-III. **e** Ventana BenchMark ULTRA (All images 20×, bar = 200 µm, Aperio Scanscope AT2)

and was found to be comparable among the different platforms, compared with the PD-L1 IHC 28-8 pharmDx kit (Table 2).

Repeatability

For the three selected lung cancer samples, there was 100% inter- and intra-assay repeatability scoring/classification (Table 3), with acceptable coefficient of variation in percentages of positively stained target cells.

Specificity of immunohistochemistry staining across platforms

All protocols adequately stained the tonsil epithelium (Fig. 2) and placental syncytiotrophoblast (Fig. 3), used as

Table 2 Positive, negative, and total agreement for the different immunohistochemistry platforms across different tumor types using Abcam anti-PD-L1 28-8 antibody, compared with Dako PD-L1 IHC 28-8 pharmDx assay

		Lung cancer[a]					Melanoma					Head and neck cancer[b]					All indications				
		28-8 pharmDx	Dako Autostainer Link 48	Dako Omnis	Leica Bond-III	Ventana BenchMark ULTRA	28-8 pharmDx	Dako Autostainer Link 48	Dako Omnis	Leica Bond-III	Ventana BenchMark ULTRA	28-8 pharmDx	Dako Autostainer Link 48	Dako Omnis	Leica Bond-III	Ventana BenchMark ULTRA	28-8 pharmDx	Dako Autostainer Link 48	Dako Omnis	Leica Bond-III	Ventana BenchMark ULTRA
≥1% expression	Positive agreement	10	0.90 (9/10)	0.70 (7/10)	0.80 (8/10)	0.80 (8/10)	4	0.50 (2/4)	0.50 (2/4)	0.33 (1/3)	0.50 (2/4)	7	1.00 (7/7)	1.00 (7/7)	1.00 (7/7)	1.00 (7/7)	21	0.86 (18/21)	0.76 (16/21)	0.80 (16/20)	0.81 (17/21)
	Negative agreement	19	0.95 (18/19)	0.95 (18/19)	0.89 (17/19)	0.89 (17/19)	27	0.78 (21/27)	0.85 (23/27)	0.96 (26/27)	0.81 (22/27)	23	0.91 (20/22)	1.00 (23/23)	1.00 (22/22)	1.00 (23/23)	69	0.87 (59/68)	0.93 (64/69)	0.96 (65/68)	0.90 (62/69)
	Overall agreement	29	0.93 (27/29)	0.86 (25/29)	0.86 (25/29)	0.86 (25/29)	31	0.74 (23/31)	0.81 (25/31)	0.90 (27/30)	0.77 (24/31)	30	0.93 (27/29)	1.00 (30/30)	1.00 (29/29)	1.00 (30/30)	90	0.87 (77/89)	0.89 (80/90)	0.92 (81/88)	0.88 (79/90)
≥5% expression	Positive agreement	9	0.78 (7/9)	0.67 (6/9)	0.67 (6/9)	0.67 (6/9)	2	0.50 (1/2)	0.50 (1/2)	1.00 (1/1)	0.00 (0/2)	6	0.83 (5/6)	1.00 (6/6)	0.83 (5/6)	1.00 (6/6)	17	0.76 (13/17)	0.76 (13/17)	0.75 (12/16)	0.71 (12/17)
	Negative agreement	20	0.95 (19/20)	0.95 (19/20)	0.90 (18/20)	0.90 (18/20)	29	0.90 (26/29)	0.93 (27/29)	0.97 (28/29)	0.86 (25/29)	24	1.00 (23/23)	1.00 (24/24)	1.00 (23/23)	1.00 (24/24)	73	0.94 (68/72)	0.96 (70/73)	0.96 (69/72)	0.92 (67/73)
	Overall agreement	29	0.90 (26/29)	0.86 (25/29)	0.83 (24/29)	0.83 (24/29)	31	0.87 (27/31)	0.90 (28/31)	0.97 (29/30)	0.81 (25/31)	30	0.97 (28/29)	1.00 (30/30)	0.97 (28/29)	1.00 (30/30)	90	0.91 (81/89)	0.92 (83/90)	0.92 (81/88)	0.88 (79/90)
≥10% expression[c]	Positive agreement	7	0.86 (6/7)	0.86 (6/7)	0.43 (3/7)	0.71 (5/7)	1	1.00 (1/1)	1.00 (1/1)	1.00 (1/1)	0.00 (0/1)	5	1.00 (5/5)	1.00 (5/5)	1.00 (5/5)	1.00 (5/5)	13	0.92 (12/13)	0.92 (12/13)	0.69 (9/13)	0.77 (10/13)
	Negative agreement	22	0.91 (20/22)	0.95 (21/22)	0.91 (20/22)	0.86 (19/22)	30	0.90 (27/30)	0.93 (28/30)	0.97 (28/29)	0.93 (28/30)	25	1.00 (24/24)	1.00 (25/25)	1.00 (24/24)	1.00 (25/25)	77	0.93 (71/76)	0.96 (74/77)	0.96 (72/75)	0.94 (72/77)
	Overall agreement	29	0.90 (26/29)	0.93 (27/29)	0.79 (23/29)	0.83 (24/29)	31	0.90 (28/31)	0.94 (29/31)	0.97 (29/30)	0.90 (28/31)	30	1.00 (29/29)	1.00 (30/30)	1.00 (29/29)	1.00 (30/30)	90	0.93 (83/89)	0.96 (86/90)	0.92 (81/88)	0.91 (82/90)
≥50% expression[d]	Positive agreement	3	1.00 (3/3)	1.00 (3/3)	1.00 (3/3)	0.67 (2/3)	0	–	–	–	–	4	1.00 (4/4)	1.00 (4/4)	0.75 (3/4)	0.75 (3/4)	7	1.00 (7/7)	1.00 (7/7)	0.86 (6/7)	0.71 (5/7)
	Negative agreement	26	0.96 (25/26)	1.00 (26/26)	0.96 (25/26)	1.00 (26/26)	31	1.00 (31/31)	0.97 (30/31)	1.00 (30/30)	1.00 (31/31)	26	0.96 (24/25)	1.00 (26/26)	1.00 (25/25)	0.96 (25/26)	84	0.98 (80/82)	0.99 (82/83)	0.99 (80/81)	0.99 (82/83)
	Overall agreement	29	0.97 (28/29)	1.00 (29/29)	0.97 (28/29)	0.97 (28/29)	31	1.00 (31/31)	0.97 (30/31)	1.00 (30/30)	1.00 (31/31)	30	0.97 (28/29)	1.00 (30/30)	0.97 (28/29)	0.93 (28/30)	90	0.98 (87/89)	0.99 (89/90)	0.98 (86/88)	0.97 (87/90)

IHC immunohistochemistry; *PD-L1* programmed death-ligand 1.

Dashes indicate the data that was not available (no specimens in this range).

[a]Dako PD-L1 IHC 28-8 pharmDx assay approved for analysis of non-squamous non-small cell lung cancer only.

[b]Approved for squamous cell carcinoma of the head and neck in Europe only for analysis by Dako PD-L1 IHC 28-8 pharmDx assay.

[c]Expression level not approved for the analysis of melanoma by Dako PD-L1 IHC 28-8 pharmDx assay.

[d]Expression level not approved for the analysis of any indication by Dako PD-L1 IHC 28-8 pharmDx assay.

Table 3 Inter-assay and intra-assay repeatability for the different immunohistochemistry platforms across different tumor types using Abcam anti-PD-L1 28-8 antibody

	Sample 1				Sample 2				Sample 3[a]			
Intra-assay	Repeatability of classification (pos vs. neg, expression level ≥1%)	Mean PD-L1 (%)	SE	CV (%)	Repeatability of classification (pos vs. neg, expression level ≥1%)	Mean PD-L1 (%)	SE	CV (%)	Repeatability of classification (pos vs. neg, expression level ≥1%)	Mean PD-L1 (%)	SE	CV (%)
Dako Autostainer Link 48	100% (3/3)	25.17	1.64	6.52	100% (3/3)	8.33	1.17	14.00	100% (3/3)	0.00	0.00	0.00
Dako Omnis	100% (3/3)	17.00	1.00	5.88	100% (3/3)	6.17	1.92	31.17	100% (3/3)	0.00	0.00	0.00
Leica Bond-III	100% (3/3)	4.00	0.50	12.56	100% (3/3)	4.33	0.33	7.69	100% (3/3)	0.00	0.00	0.00
Ventana BenchMark ULTRA	100% (3/3)	15.00	2.31	15.40	100% (3/3)	11.75	2.65	22.58	100% (3/3)	0.00	0.00	0.00
Inter-assay	Repeatability of classification (pos vs. neg, expression level ≥1%)	Mean PD-L1 (%)	SE	CV (%)	Repeatability of classification (pos vs. neg, expression level ≥1%)	Mean PD-L1 (%)	SE	CV (%)	Repeatability of classification (pos vs. neg, expression level ≥1%)	Mean PD-L1 (%)	SE	CV (%)
Dako Autostainer Link 48	100% (3/3)	23.17	1.74	7.50	100% (3/3)	7.00	0.50	7.14	100% (3/3)	0.00	0.00	0.00
Dako Omnis	100% (3/3)	19.17	2.68	14.01	100% (3/3)	6.00	2.02	33.68	100% (3/3)	0.00	0.00	0.00
Leica Bond-III	100% (3/3)	2.83	1.09	38.51	100% (3/3)	4.33	0.67	15.38	100% (3/3)	0.00	0.00	0.00
Ventana BenchMark ULTRA	100% (3/3)	14.33	2.33	16.27	100% (3/3)	4.50	0.41	9.07	100% (3/3)	0.00	0.00	0.00

CV coefficient of variation; *PD-L1* programmed death-ligand 1; *SE* standard error of the mean.

[a]Sample had 0% PD-L1 expression.

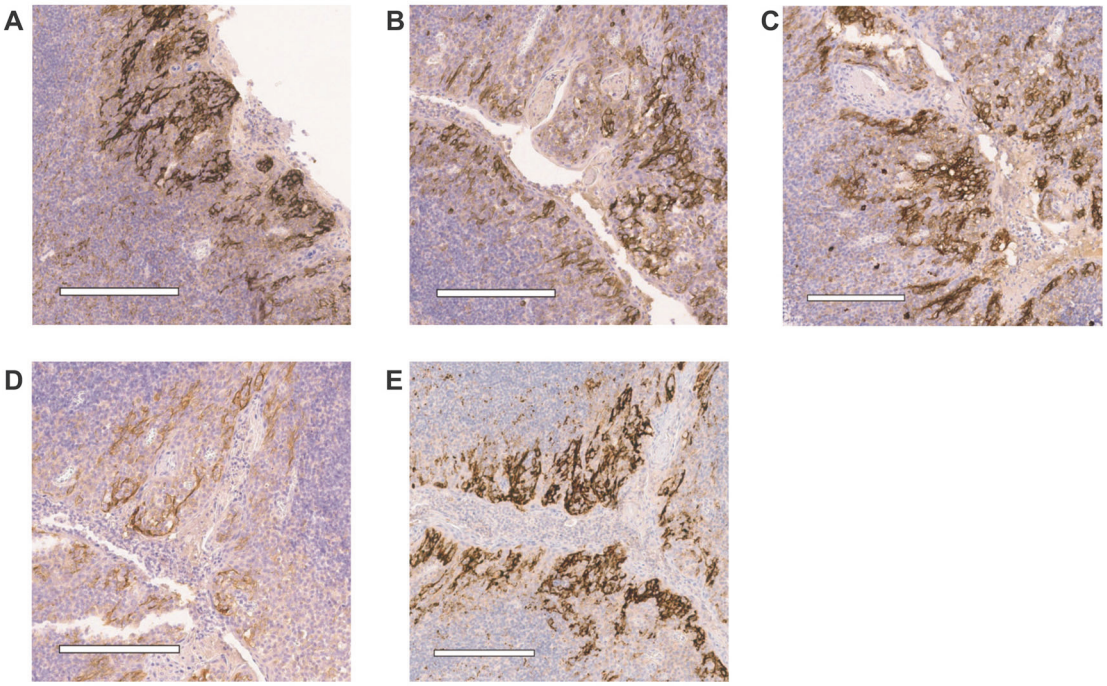

Fig. 2 PD-L1 immunohistochemistry staining in tonsil (positive control tissue) using clone 28-8 within the pharmDx kit and outside the kit on different automated staining platforms. **a** Dako PD-L1 IHC 28-8 pharmDx kit. **b** Dako Autostainer Link 48. **c** Dako Omnis. **d** Leica Bond-III. **e** Ventana BenchMark ULTRA (All images 11×, bar = 200 µm, Aperio Scanscope AT2)

batch positive controls. Thus, the expression of the target antigen could be detected in seven of the seven tonsil and six of the six placenta specimens. A tissue microarray comprising samples from all major human organs (created within Targos Molecular Pathology GmbH) was also tested to verify the specificity of PD-L1 expression in human tissues. Using all immunohistochemistry staining platforms and the pharmDx kit, no physiological presence of PD-L1 expression could be detected in prostate, testis, ovary, fallopian tube, uterus, adrenal gland, colon, stomach, heart, cerebellum, cerebrum, or thyroid gland. Nuclear staining in distal tubules of the kidney was detected in four of the six samples. Liver specimens exhibited non-specific cytoplasmic grossly granular staining in four of the six cases, most likely representative of hemosiderin deposits. The majority of samples across all tumor types had <1% PD-L1 expression, as determined by the PD-L1 IHC 28-8 pharmDx kit (lung cancer 19/29, melanoma 27/30, head and neck cancer 23/30). Depending on the expression level and the tumor indication, negative agreements ranged from 0.74 to 1.00; in most cases, the values for negative agreement were below the specified target value of 0.90 (Table 2).

Sensitivity

All protocols successfully stained a defined PD-L1 standard cell-line microarray, with cell lines negative (−), low positive (+), intermediate positive (++), and strong positive (+++) for PD-L1 expression. Figure 4 shows comparable levels of staining and percentages of PD-L1-expressing cells per cell line [52].

Stability

Despite the observed variations in staining intensity and patterns after storage at room temperature, antigen stability over a 6-week period did not influence the final classification in these samples (PD-L1 expression levels above or below the prespecified expression levels). Compared with the original staining pattern in 10% neutral buffered formalin and non-buffered formalin, all non-formalin-based fixatives showed a marked, but not systematic, change in actual staining patterns in appearance, staining intensity, and number of affected cells (up to and including change to 0% PD-L1 expression, i.e., false-negative staining) (data not shown).

Discussion

A systematic comparison of PD-L1 expression levels in lung cancer, melanoma, and head and neck cancer tissue microarrays assessed by four major automated staining platforms using the Abcam anti-PD-L1 28-8 antibody revealed adequate precision and concordance when compared with the recommended complementary diagnostic kit

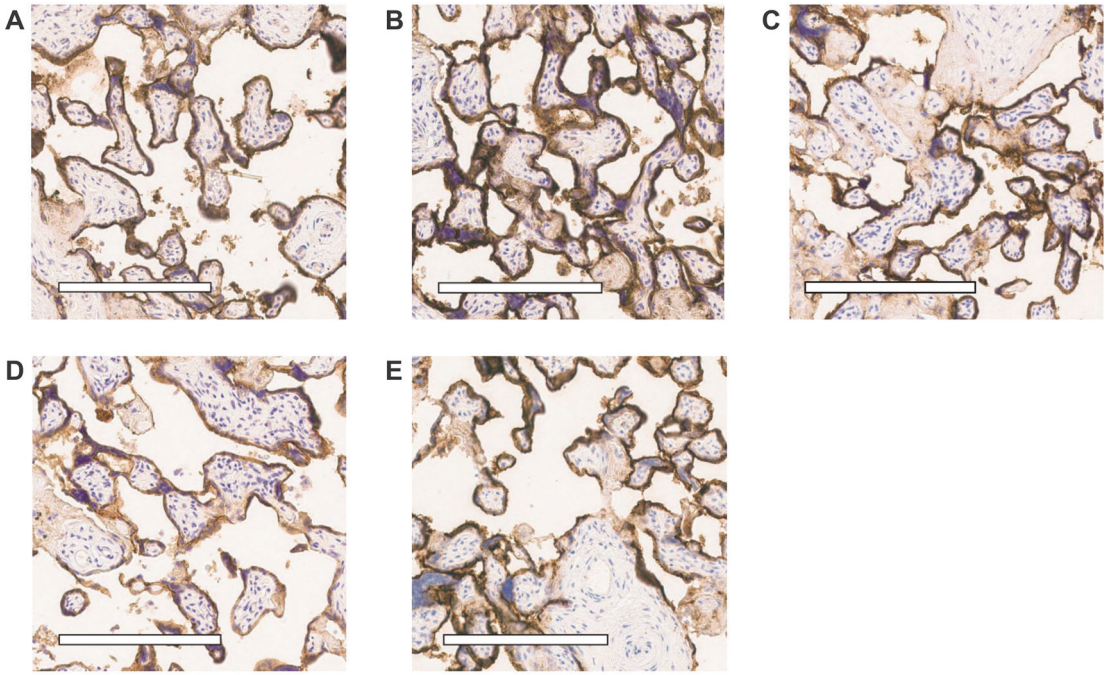

Fig. 3 PD-L1 immunohistochemistry staining in placenta (positive control tissue) using clone 28-8 within the pharmDx kit and outside the kit on different automated staining platforms. **a** Dako PD-L1 IHC 28-8 pharmDx kit. **b** Dako Autostainer Link 48. **c** Dako Omnis. **d** Leica Bond-III. **e** Ventana BenchMark ULTRA (All images 16×, bar = 200 µm, Aperio Scanscope AT2)

for nivolumab, the FDA-approved and CE-marked PD-L1 IHC 28-8 pharmDx kit [10, 17, 18]. The mean overall agreement for all tumor types and all staining protocols was >0.85. Previous comparisons have found mostly good concordance between commercial assays, but variable results when laboratory-developed tests were compared with commercial assays [46, 53–59]. Results from the current study provide encouraging data suggesting that it is possible to validate laboratory-developed PD-L1 immunohistochemistry assays for general use.

We found higher concordance with the IHC 28-8 pharmDx kit for higher PD-L1 expression levels, compared with lower expression levels (0.89 and 0.98 overall for ≥1% and for ≥50%, respectively). However, most tumor samples were PD-L1 negative (<1% expression), particularly melanoma samples. Ideally this study would have been carried out on an equal distribution of prescreened cases with lower and higher PD-L1 expression levels.

Inter- and intra-assay repeatability was excellent, with 100% repeatability of scoring and classification between and within the assays. It was not within the scope of this study to assess the effect of inter-observer variability on scoring. However, previous research has suggested that pathologist training and experience in assessing PD-L1 expression is essential [60], because the pathologist's scoring can provide an intrinsic source of error that should be considered, particularly for lower PD-L1 expression levels [61].

Positive control tonsil and placenta specimens were all found to have high PD-L1 expression levels, suggesting that they may not provide a reliable enough control sample for assessing the relative sensitivity of a given protocol setup. However, cell-line microarrays, which included a range of cell lines showing negative PD-L1 staining to strongly positive PD-L1 staining, provided a more effective control, allowing demonstration of high sensitivity and specificity for assays on all platforms. To validate future protocols, the use of reference tissue specimens and/or cell lines with defined expression levels covering the entire expected range is therefore strongly recommended, in addition to the tonsil and placenta tissue specimens being used, with tonsil as on-slide or run-related batch controls.

The staining of tumor samples remained stable (in terms of maintaining categorization above or below prespecified expression levels) over a period of 6 weeks, although testing with different fixatives and fixation times revealed that non-formalin-containing fixatives resulted in marked changes in staining pattern and intensity, compared with the original staining pattern. The recommended fixatives thus include 10% neutral buffered formalin, as well as non-buffered formalin, applied for 18–24 h. Based on our findings, non-formalin-containing fixatives should be avoided. Outside the current study, however, it has been observed with clinical samples that long-term storage of unstained slides at room temperature leads to a significant reduction of PD-L1 staining intensity. Therefore, the use of archival

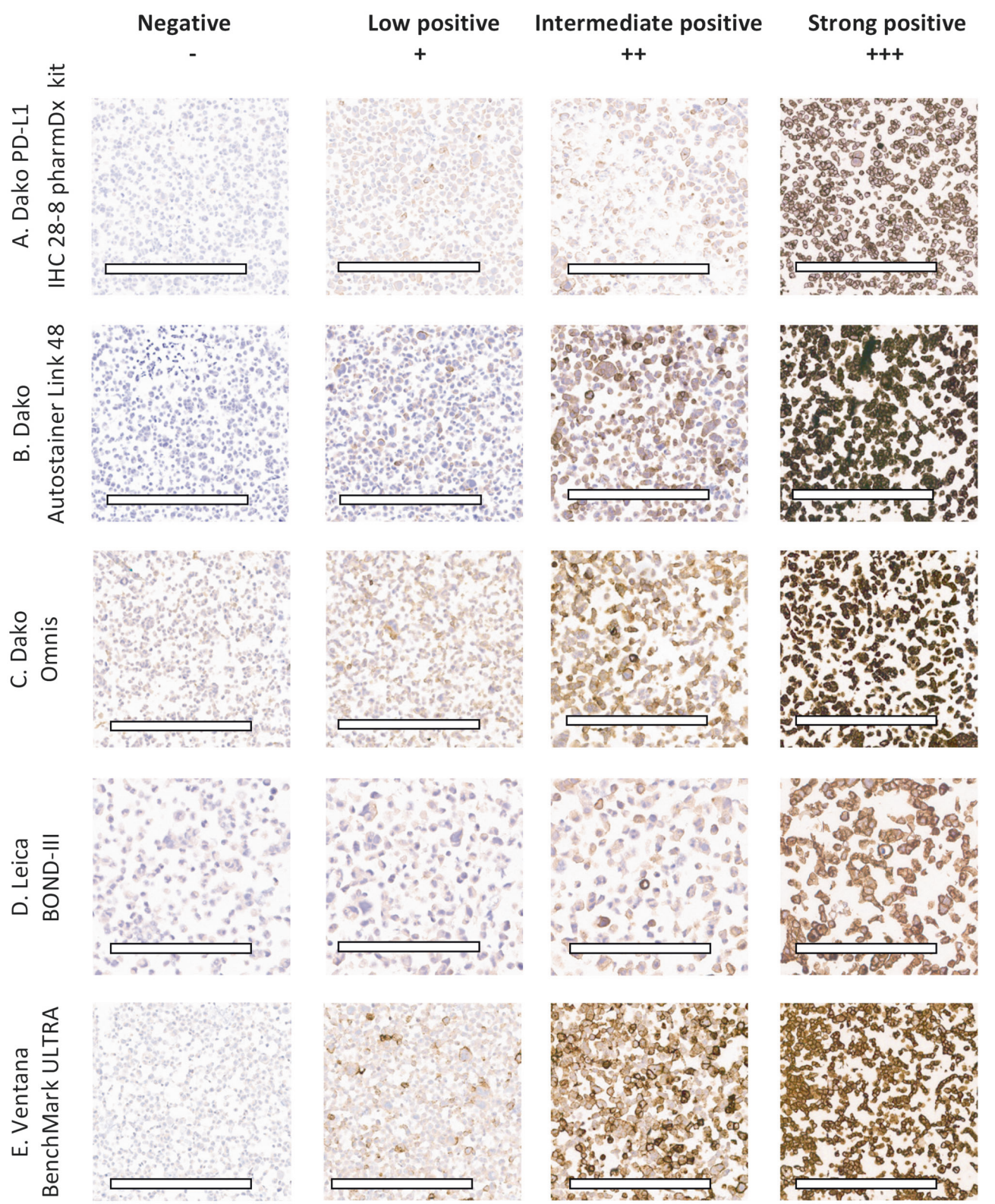

Fig. 4 PD-L1 immunohistochemistry staining of negative (−), low positive (+), intermediate positive (++), and strong positive (+++) protein expressing cell lines. **a** Dako PD-L1 IHC 28-8 pharmDx kit. **b** Dako Autostainer Link 48. **c** Dako Omnis. **d** Leica Bond-III. **e** Ventana BenchMark ULTRA (All images 20×, bar = 200 µm, Aperio Scanscope AT2)

sections that have been stored for longer than 3 months is not recommended.

The small cohort size and the increased likelihood of small tissue specimens such as tissue microarray cores detaching from slides (which reduced the number of evaluable samples) provided some technical limitations. Tissue microarrays were used in the current analysis for an assay-to-assay comparison between staining protocols, and prepared as parallel sections in order to minimize variability. Consideration of intratumoral heterogeneity as a potential confounding factor was not within the scope of this study, though it should be considered as a concern when using tissue microarrays, as it could result in variable levels in PD-L1 expression, depending on the degree of heterogeneity [62–64]. It should be noted that the pharmDx kit has not been validated for tissue microarrays, and can result in lower levels of PD-L1 than found in clinical practice [65]. However, the current study found similar levels and patterns of PD-L1 expression in tissue microarrays across all laboratory-developed tests compared with the pharmDx kit. This supports the applicability of this study to clinical practice, notwithstanding the generally acknowledged limitations of tissue microarray-based analysis. Since cytological specimens may be more easily and less invasively obtained than histological specimens, the performance of assays in different sample types has been examined previously and found to be highly concordant using both the PD-L1 IHC 28-8 and 22C3 pharmDx assays [66]. The analysis of cytological specimens using the PD-L1 IHC 28-8 assay may therefore be possible, although the pharmDx kit has been approved only for histological samples [17, 18], and particular care should be taken with fixative types for cytological specimens [67]. Cytological analysis would require reference samples such as cell-line microarrays, which have also shown utility as a control in analysis of tissue microarrays.

The PD-L1 IHC 28-8 pharmDx kit was not developed to assess immune cells. Only one assay, SP142, has been developed to assess immune cell staining in addition to tumor membrane staining [68]. Although interlaboratory concordance within a single assay was acceptable, most studies have found poor concordance between different assays in immune cell staining, compared with tumor membrane staining [58, 69–72]. This current study therefore did not include immune cell staining in the analysis.

The results of the present analysis demonstrate that protocol optimization by individual laboratories should always be guided by comparison with approved assays, in order to prevent false positives and/or false negatives. If the kit or the staining device is not available (the most likely reason for developing an assay within the laboratory), comparison on a set of reference samples with known results, obtained by a reference laboratory using the approved IHC 28-8 pharmDx kit, is considered a prerequisite. Further studies are needed to validate 28-8 assays on sample types used in clinical practice.

Conclusion

A systematic and comprehensive comparison of PD-L1 immunohistochemistry protocols on the four most commonly used immunohistochemistry platforms using the Abcam anti-PD-L1 28-8 antibody in lung cancer, melanoma, and head and neck cancer, using tissue microarray samples, showed adequate concordance and good reproducibility when compared with the reference PD-L1 IHC 28-8 pharmDx kit. The use of reference samples with known PD-L1 expression levels, including cell-line microarrays, helped to confirm the sensitivity and specificity of each of the laboratory-developed assays.

Further investigation is needed to determine protocols for other tumor and biopsy types. All such laboratory-developed assays should be validated by direct comparison either with the approved protocol in the 28-8 pharmDx kit, or with externally validated reference samples if the kit is not available. Each laboratory should perform its own validation prior to use.

Acknowledgments The authors would like to thank the patients who donated samples, as well as the contribution of Alexander Pieper (Bristol-Myers Squibb). Medical writing and editorial support was provided by Eloise Aston, MSc, and Jay Rathi, MA, of Spark Medica Inc (USA), funded by Bristol-Myers Squibb.

Compliance with ethical standards

Conflict of interest CK, HS, DZ, and BJ are employees of Targos. H-US, SE, and MM-O are employees of Universitätsmedizin Göttingen. CD'A has received honoraria and reimbursements from Roche Pharma, Roche TD, Pfizer and, via Targos, from Bristol-Myers Squibb and MSD. H-US is a consultant for Targos and has received honoraria and reimbursements from Abbott Molecular, Bristol-Myers Squibb, MSD, Novartis, Pfizer, Roche Pharma, ZytoMed, and ZytoVision. JR is a co-founder of Targos and received a fee from Bristol-Myers Squibb for this study.

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