A Novel Image Analysis Assay for Determining Programmed Death-Ligand (PD-L1 [22C3]) in Non-small Cell Lung Cancer that Accounts for Tissue and Alveolar Macrophages

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Background

The Dako PD-L1 (22C3) antibody is used to measure tumor PD-L1 levels in non-small cell lung cancer (NSCLC), serving as a companion diagnostic to the check point inhibitor drug pembrolizumab. However, interpretation of the PD-L1 (22C3) immunohistochemistry (IHC) assay is cumbersome and time-consuming, resulting in poor intra- and inter-pathologist precision. We developed a clinically validated PD-L1 (22C3) image analysis (IA) assay that was previously shown to perform equivalently to manual pathologist scoring, require less labor, and produce standardized and reproducible results with greater precision than manual scoring across repeated assessment. Nonetheless, there is growing literature on the significance of tumor associated macrophage (TAM) PD-L1 positivity for predicting treatment response, highlighting the need to differentiate PD-L1 positivity in tumor cells vs. macrophages. This need is underscored by the tendency of manual and digital reads to confuse alveolar macrophages as tumor cells. In this study, we applied a novel machine learning solution for tissue and alveolar macrophage detection to our PD-L1 IA assay to improve its digital scoring accuracy in NSCLC as well as generate complementary data on the presence and PD-L1 positivity status of tissue and alveolar macrophages.

Materials & Methods

98 NSCLC samples (24 whole tissue [WT] and 74 tissue microarray [TMA] cores) were sectioned and IHC-stained for PD-L1 (22C3). Slides were scanned at 20X magnification and analyzed using Flagship's clinically validated PD-L1 (22C3) IA assay (Fig. 1) that separates tumor and stromal compartments, quantifies PD-L1 expression, and calculates a digital Tumor Proportion Score (TPS, %; Fig. 4C) within the tumor compartment of each sample (Fig. 3A, 3B). The digital images were also manually scored for TPS by three board-certified MD pathologists. To account for tissue and alveolar macrophages in the digital TPS, we applied a machine learning macrophage solution (Fig. 2) to the validated PD-L1 (22C3) IA assay. For the solution development, image markups of macrophage detection and PD-L1 expression were reviewed by a board-certified MD pathologist for acceptance (Fig. 3C). The macrophage solution was applied to the PD-L1 (22C3) IA assay to calculate an updated digital TPS (excluding TAMs: tissue and alveolar macrophages in the tumor compartment) for the same 98 NSCLC samples. The concordance of the original and updated digital TPS with manual pathology TPS were examined (Fig. 4). To verify the accuracy of the IA macrophage solution, additional serial sections from the same NSCLC samples were taken and stained for the CD68 macrophage marker and PD-L1 (22C3). The PD-L1 (22C3) IA assay with the macrophage solution was applied to the PD-L1 images to calculate TPS, the number of TAMs, and the number of PD-L1+ TAMs for each sample (Fig. 5). Concordance of the macrophages identified by the IA macrophage solution with those identified by CD68 positivity were examined (Fig. 6).

Results & Conclusions

Retains TPS Integrity: By removing the confounding effects of PD-L1+ TAMs, application of the macrophage solution resulted in similar or reduced TPS values but the correlation between the digital and manual TPS remained high (Fig. 4).

Provides Complementary TAM Data with Potential Clinical Relevance: The % of PD-L1+ TAMs stratified samples binned for treatment via TPS into additional subgroups that were identified in the literature as treatment high-responders: high TPS and high % of PD-L1+ TAMS (Fig. 5). Macrophages identified by the solution significantly correlated with the CD68 macrophage marker, supporting its accuracy (Fig. 6).

The PD-L1 (22C3) Image Analysis Assay was Clinically Validated According to CLIA Guidelines

Performance Parameter	Acceptance Criteria	Cohort Pass/Fail	Cohort Percentage Pass
SPECIFICITY	**Sample Criteria:** Appropriate cell recognition in ≥ 90% of cells evaluated, and the staining classification false positive rate is ≤ 10% (as determined by the pathologist). **Cohort Criteria:** ≥ 90% of the tissue image cohort must pass the sample criteria.	Pass	97% (64/66)
SENSITIVITY	**Sample Criteria:** Appropriate cell identification in ≥ 90% of cells evaluated, and the staining classification false negative rate is ≤ 10% (as determined by the pathologist). **Cohort Criteria:** ≥ 90% of the tissue image cohort must pass the sample criteria.	Pass	100 % (66/66)
ACCURACY	**Sample Criteria:** Concordance in non-treatment (TPS < 1%) vs. treatment (TPS ≥ 1%) binning according to the digital and manual pathology TPS evaluation. **Cohort Criteria:** ≥ 90% of the tissue image cohort must pass the sample criteria.	Pass	91% (60/66)
PRECISION	**Sample Criteria:** Concordance in non-treatment (TPS < 1%) vs. treatment (TPS ≥ 1%) binning according to the digital TPS evaluation of the same samples stained on different days in the same lab (endpoint precision: Day 2 vs. Day 3) and in different labs (inter-lab precision: Day 1 vs. Day 2, Day 1 vs. Day 3). **Cohort Criteria:** ≥ 80% of the tissue image cohort must pass the sample criteria.	Pass	100% (4/4) [1]

[1] Endpoint precision and inter-lab precision

Fig. 1 | A Prior Clinical Validation Study Demonstrated the Sensitivity, Specificity, Accuracy, and Precision of the PD-L1 (22C3) Image Analysis Assay. A different set of 66 NSCLC samples (20 WT and 46 TMA cores) were IHC-stained for PD-L1 (22C3); 4 of the WT samples had serial sections stained on 3 separate days (Day 1 in an outside lab, Day 2 and Day 3 in Flagship Biosciences' CAP/CLIA lab). Slides were scanned at 20X magnification and analyzed using Flagship's IA solutions that quantify PD-L1 expression and separate tumor and stromal compartments. Resulting image markups of cell detection and PD-L1 expression were reviewed by an MD pathologist for acceptance. PD-L1 staining was evaluated by digital IA in the tumor compartment for TPS (%; Fig. 3B). The image analysis assay passed the acceptance criteria for all validation performance parameters.

Macrophage Solution: Tissue and Alveolar Macrophage Recognition Algorithms Approved by Pathologists

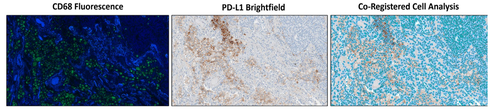
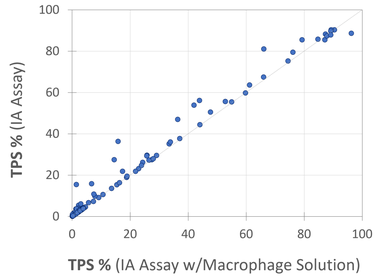


Fig. 2| Macrophage Algorithm Development Workflow Integrates CD68 Positivity for Verification and Pathologist Review for Clinical Oversight. The macrophage solution was developed using an alternate set of 23 NSCLC WT sections that were co-stained for PD-L1 (brown: brightfield) and the macrophage associated CD68 marker (green: fluorescence). Tissue and alveolar macrophages were identified by their CD68 profile using Flagship's proprietary IA software to quantify hundreds of defining cellular characteristics and leveraging machine learning algorithms to provide robust decision tree models for their classification. Cell metrics were generated using features derived from the PD-L1 image, while the CD68 status was applied as a label for supervised model training. An iterated random forest classifier was trained on blocks of total cell data (>12,000,000 cells) at intervals of 500,000 observations and separated into a training set (475,000 cells) and a test set (25,000 cells) at each block to estimate out-of-sample accuracy. As a quality control, checkpoints for pathology review were built into the algorithm development workflow at multiple steps: accuracy of cell detection, stain thresholding for positivity, tumor/stroma separation, and macrophage detection. The trained model was implemented in tissues outside of the training set. Test tissues were separated into tumor/stroma compartments to differentiate macrophages from tumor cells within the tumor compartment (Fig. 3B and 3C).

Digital TPS (with and without the Macrophage Solution) Significantly Correlated with Manual Pathology TPS from Three Different Pathologists

A. IA Assay Digital TPS (without & with Macrophage Solution)



B. Manual Pathology TPS vs. IA Assay Digital TPS (without and with Macrophage Solution)

Pathologist	Manual TPS vs. Digital TPS (IA Assay)	Manual TPS vs. Digital TPS (IA Assay w/Macrophage Solution)
#1 (Flagship)	r = 0.900 (p < 0.00001)	r = 0.901 (p < 0.00001)
#2 (Outside)	r = 0.884 (p < 0.00001)	r = 0.859 (p < 0.00001)
#3 (Outside)	r = 0.858 (p < 0.00001)	r = 0.838 (p < 0.00001)

C. Tumor Proportion Score (TPS, %) =

$$\frac{\text{\# viable tumor cells with 1+ or greater membrane IHC PD-L1 positivity}}{\text{total \# of tumor cells}} \times 100$$

PD-L1 Binned Expression Level for FDA-Approved Therapy
No Expression (Non-treatment): TPS < 1%
Expression (Treatment): TPS ≥ 1%
High Expression (Treatment): TPS ≥ 50%

Fig. 4| Applying the Macrophage Solution to the Clinically Validated PD-L1 (22C3) IA Assay Removes the Contribution of PD-L1+ Macrophages from the Digital TPS Without Affecting its Integrity.

A) Comparing the digital TPS from the same samples calculated with the IA assay with versus without the macrophage solution reveals very similar values. However, without applying the macrophage solution, there was a consistent shift towards higher TPS values. Overall, by removing the confounding contribution of macrophages in calculating the TPS, application of the macrophage solution to the IA assay resulted in similar or reduced TPS values.

B) Digital-to-manual PD-L1 (22C3) scoring agreement was assessed by Pearson's correlation analysis of the TPS from the clinically validated IA assay and from each pathologist. All three pathologists (1 internal pathologist [FLG], two outside pathologists) demonstrated a significant correlation with the digital TPS from the IA assay without and with the macrophage solution.

Image Analysis Algorithms Stratify Tumor Cells from Macrophages and Quantify PD-L1 Expression

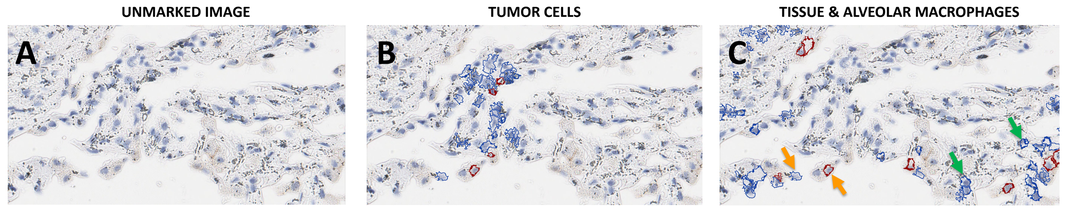


Fig. 3| Digital Analysis of PD-L1-Stained NSCLC Samples. A) DAB-conjugated PD-L1 stained NSCLC tissue (brown) without IA markups. **B)** The clinically validated PD-L1 (22C3) IA assay detected and quantified PD-L1 cellular expression within the tumor compartment to calculate the Tumor Proportion Score (Blue IA markups: PD-L1 negative cells. Red IA markups: PD-L1 positive cells). **C)** Application of the macrophage solution to the PD-L1 (22C3) IA assay differentiates tissue macrophages (green arrows) and alveolar macrophages (orange arrows) from tumor cells and excludes these macrophages from the Tumor Proportion Score calculation.

Complementary TAM Data: % of PD-L1+ Tissue & Alveolar Macrophages in the Tumor Compartment

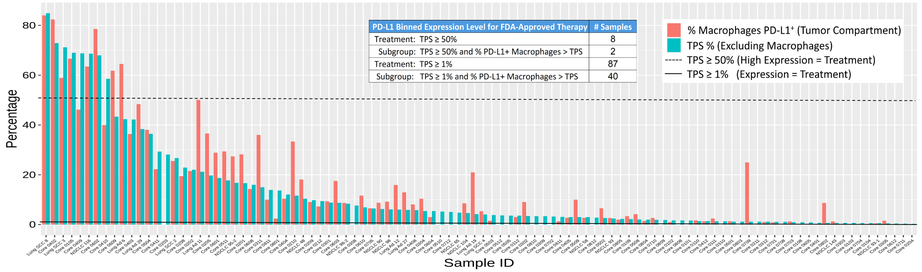


Fig. 5| The TPS and % of PD-L1+ Macrophages (Tissue & Alveolar) in the Tumor Compartment is Reported for Each Sample. Application of the macrophage solution to the PD-L1 (22C3) IA assay provides a more accurate TPS (teal bars) by accounting for and excluding macrophages in the calculation. The macrophage solution also provides complementary data on the % of PD-L1+ macrophages in the tumor compartment (TAMs: pink bars) which stratifies samples binned for treatment (via TPS) into subgroups that were identified in the literature as treatment high-responders (high TPS and high %PD-L1+ TAMs). An outcomes study is planned to test the clinical relevance of this differential metric.

Number of Macrophages Identified by the IA Macrophage Solution Significantly Correlated with the Number of CD68+ Macrophages

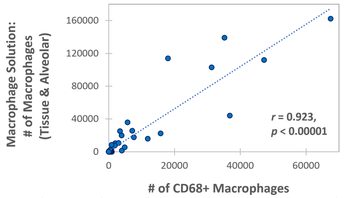


Fig. 6| Concordance Between the # of Macrophages (Tissue & Alveolar) Identified by the IA Macrophage Solution and the # of CD68+ Macrophages in Serial Sections of the Same Samples Support the Accuracy of the Solution. The IA macrophage solution-to-CD68 macrophage marker agreement was assessed by Pearson's correlation analysis of the number of macrophages identified in the PD-L1-stained sections by the macrophage solution with the number of macrophages that were identified by CD68 positivity in the CD68-stained sections. While the number of macrophages identified by the macrophage solution was greater than those identified in the CD68-stained images, the two measurements were significantly correlated. The number discrepancy suggests there are additional macrophages in the tissues that were not detected with the CD68 marker. An additional verification study comparing the macrophage solution with the TAM specific marker CD163 and the CD68 macrophage marker is ongoing.