Abstract 156

#### Abstract

**Background:** The detection of circulating nucleic acids using non-invasive blood draws has become highly relevant to clinical testing. In this study, we report on the development of a blood-based PD-L1 test for immunotherapy selection.

**Methods:** We have previously reported on the analytic performance of a droplet digital PCR (<u>ddPCR</u>) assay for circulating cytokeratin 19 and PD-L1. Using a variable threshold based on a logistic regression score for the blood assay and a 1% IHC (immunohistochemistry) tissue cut-off, concordance was 80% (n = 16). Positive calls for the blood-based PD-L1 assay ranged from 2 to 124 copies. In this study, we focused on variables that could impact concordance of the blood assay and tissue results. Criteria included droplet counts for tissue and blood mRNA transcripts and tumor proportion score (TPS) for the tissue IHC assay (22C3 pharmDx).

**Results:** We examined the correlation between PD-L1 in formalin-fixed paraffin-embedded (FFPE) tissue by IHC, mRNA expression in serial cut FFPE sections, and in matched plasma samples collected at the time of tissue resection. Five cases were assayed to confirm PD-L1 positivity by tissue. We successfully recovered RNA from serial tissue sections for each case and detected PD-L1 levels ranging from 6 to 1272 copies. Plasma samples were available for four of the cases for circulating RNA evaluation, and we were successful in detecting PD-L1 in all cases (copy range 32-138). While all four cases contained detectable PD-L1 mRNA in tissue and circulation, we observed little concordance between these levels in tissue and blood. **Conclusions:** We have developed methods to measure the dynamic range of PD-L1 from plasma. We have shown feasibility of these methods by evaluating key immune and cancer-specific RNAs. The current study demonstrates that although the development of quantitative assays for mRNA in blood is possible, concordance with traditional clinical tissue assays such as IHC may not be a useful validation measure. We have initiated prospective validation studies that will continue to evaluate PD-L1 expression by IHC and in the blood-based assay, and will also monitor patient performance measures in response to immunotherapy.

#### **Materials and Methods**

Specimens: Tissue and blood specimens were purchased from a commercial biobank for this study (Indivumed, GmbH, Germany).

Histopathology and Image Capture: Tumor content was estimated using H&E-stained sections. Immunohistochemistry (IHC) of five formalin-fixed paraffin-embedded (FFPE) NSCLC samples was performed. IHC was conducted on the Dako Autostainer Link 48 staining platform (Dako) using the mouse monoclonal anti-PD-L1 antibody clone 22C3 (Dako). Stained slides were scanned with the Axio Scan.Z1 automated slide scanner (Zeiss) using ZEN 2 (blue edition) slidescan software (Zeiss). Representative images shown here were prepared in ZEN 2 lite (blue edition) software at a gamma value of 1.0 after brightness adjustment (white balance), and may deviate slightly from the staining results viewed under a light microscope. The evaluation of immunohistochemically stained samples included the specification of the percentage of stained tumor cells, the staining intensity as well as the predominant staining pattern (homogeneous or heterogeneous). A homogeneous staining pattern implied uniform staining intensity of tumor cells within the tumor region, whereas a heterogeneous staining pattern implied different staining intensities. Further, the predominantly stained subcellular compartment was determined (c: cytoplasm, m: membrane, n: nucleus). If more than one subcellular compartment was stained, all positive compartments were stated in the order of their relative abundance (e.g. m,c.: the predominantly stained compartment was the cell membrane). The Tumor Proportion Score (TPS) of anti-PD-L1 IHC staining was determined according to the Dako interpretation manual. Histopathological evaluation was conducted by a pathologist at Indivumed.

**RNA Extraction and ddPCR**: Tissue samples were extracted at Indivumed according to SOP and shipped to Biodesix as frozen RNA extracts. Concentration of tissue RNA extracts was determined by NanoDrop 2000 Spec, and quality was assessed by Agilent 6000 Nano Assay on the Bioanalyzer 2100. Plasma samples were shipped and extracted at Biodesix according to SOP. Circulating RNA was extracted from frozen plasma samples using the Plasma/Serum Circulating and Exosomal RNA Purification Kit (Norgen Biotek). All RNA samples were purified and concentrated using the RNA Clean-Up and Concentration Micro-Elute Kit (Norgen Biotek). For cDNA synthesis, the entire RNA sample was reverse transcribed using the SuperScript IV First Strand Synthesis System (Thermo Fisher Scientific) and purified using the DNA Clean & Concentrator-5 (Zymo Research). The ddPCR reverse transcriptase (RT) controls were generated using RNA from the NCI-H441 cell line, both with and without enzyme to verify that the PD-L1 assay is specific to RNA. A water control (lacking RNA template) was carried through the RT-ddPCR procedure along side each batch of samples to verify clean **RT-PCR** conditions.

**A**. Tissue Processing and Testing

**B.** Blood Processing and Testing



Figure 1. Methods used for the processing and analysis of tissue and plasma samples. A. IHC was performed using the PD-L1 IHC clone 22C3. As run controls, IHC of PD-L1-positive and PD-L1-negative cells (NCI-H226 and MCF-7 respectively) was executed in each respective run. Isotype controls were prepared for each sample. FFPE tissue samples were sliced into 3-5 µm sections and mounted on SuperFrost Ultra Plus glass slides (Roth) for processing in H&E or IHC. B. Whole blood was spun to recover plasma containing circulating RNA. RT-PCR was conducted, cDNA cleaned up and amplified using ddPCR (QX200, Bio-Rad). RNA recovered from the lung cancer cell line NCI-H441 was used as a positive control and within the no enzyme control. A no template control was also carried through the RT-PCR process. ddPCR reactions were conducted in duplicate wells with summed copies reported for each specimen.

## Performance verification of a plasma-based PD-L1 test that reliably measures mRNA expression from patients with NCSLC

## Hestia Mellert PhD\*, Leisa Jackson and Gary A. Pestano PhD

# Biodesix, Inc., Boulder, CO 80301

Platelet-enriched Plasma (PEP)



Figure 2. Histopathological Evaluation for Donor Cases. Samples were collected from five individual donors previously diagnosed with NSCLC. H&E staining and isotype-matched mouse IgG IHC results are shown in (A) and (B), respectively. All isotype controls were negative, although two samples exhibited several tiny spots of minimal to weak non-specific staining. The expression of PD-L1 was also analyzed by IHC and staining results are shown in (C). Refer to **Table 1** scoring of the tissue results.



Figure 3. 1D Plots showing ddPCR results for RNA Specimens. A. Plasma and B. FFPE Tissue. C. Control samples. Data were processed in QuantaSoft (Bio-Rad) and rendered to show Fluorescence Amplitude (y-axis) for PD-L1 positive droplets. ddPCR Plots for sample 2613 are not shown since a correlative sample from blood was not available. Copy numbers are summarized in **Table 2.** 

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Rebecca Getachew.

#### **Results (continued)**

Tumor	Immune Infiltrating Cells	Macrophages
Several foci with minimal to smic and membrane staining	few foci with weak staining	NA
Several foci with minimal to smic staining	NA	Weak to moderate staining of alveolar macrophages
membrane)	few cells with moderate to weak staining	mainly weak staining of alveolar macrophages
(membrane; also some with staining)	few foci with weak staining	NA
membrane; foci of necrosis fic staining)	several foci with weak staining	numerous macrophages with weak to strong staining

Table 1. PD-L1 IHC Results for Donor Specimens. Histopathological evaluation of PD-L1 staining in tumor, immune infiltrating cells, and macrophages. Weak to strong membranous anti-PD-L1 staining was detected in the tumor cells of all samples. The staining patterns were predominantly heterogeneous. In addition, weak anti-PD-L1 staining of tumor-infiltrating immune cells were detected in four samples, and PD-L1 staining of macrophages was detected in three of the NSCLC samples. By TPS four samples were classified as PD-L1 low expressing and one sample as PD-LI high expressing.

		Tissue PD-L1 mRNA copies	
or Content (%)	PD-L1 IHC (TPS)	(adjusted for % Tumor)	Plasma mRNA copies
35	1	20.4	108
30	1	5.8	138
70	2	172.2	50
40	60	2.4	32
35	4	445.2	N/A

Table 2. Summary Results for Tissue and Blood Expression of PD-L1. PD-L1 levels are shown for matched tissue and plasma samples. mRNA levels were detected by ddPCR and compared to tissue IHC results.



Figure 4. Relative Expression of PD-L1 in Matched Tissue and Plasma Specimens. PD-L1 scores expressed as copies for plasma and adjusted tissue RNA extracts detected by ddPCR or Tumor Proportion

#### **Summary and Conclusions**

Methods were previously developed to reliably measure the dynamic range of PD-L1 mRNA expression in

• The current small retrospective study demonstrates that although the development of PD-L1 assays for mRNA expression in tissue and blood is feasible, high levels of concordance with IHC may not be a useful validation

• mRNA assays may require establishment of their own cut-offs based on clinical utility studies, independent of

• We have initiated a prospective validation study that continues to evaluate PD-L1 expression by both IHC and the blood-based expression assay. The will also monitor patient performance measures (progression) in

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