Validation of an assay to detect programmed death-ligand 1 (PD-L1) expression on circulating tumor cells

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Abstract

Tumor expression of programmed death-ligand 1 (PD-L1) allows evasion of immune surveillance and has been established as a marker of response to anti-PD-1 / PD-L1 checkpoint inhibitors. Clinical response to checkpoint inhibitors has also been observed in PD-L1-negative tumors; this may be due to the difficulty of accurately discerning marker status in tumor biopsies due to spatial and temporal heterogeneity of PD-L1 expression. Cell-based liquid biopsy presents an opportunity to non-invasively assess and monitor PD-L1 expression on circulating tumor cells (CTCs). Here we describe the development and validation of the RarePlex® PD-L1 CTC Panel Kit, an immunofluorescence assay that can be used to enumerate CTCs and characterize their PD-L1 expression.

Methods

We developed an assay for detecting PD-L1 expression on CTCs using the RareCyte platform and validated the assay on model CTC (mCTC) spike-in samples and clinical samples. mCTCs spanned a range of PD-L1 expression and included SW900 (PD-L1 high), H1650 (PD-L1 low), and HAP-1 (PD-L1 negative). For spike-in studies, blood from healthy normal donors was drawn into AccuCyte® Blood Collection Tubes, spiked with mCTCs and processed to 8 slides each using the AccuCyte Sample Preparation System. Slides were then stained with a nuclear dye, and antibodies to (1) cytokeratin and EpCAM (CK/EpCAM) to identify epithelial CTCs, (2) CD45 to exclude white cells, and (3) PD-L1. Slides were scanned using a CyteFinder® Instrument and followed by automated image processing that identifies CTC candidates based on the presence of signal in the CK/EpCAM channel and absence of signal in the CD45 channel, and rank scores them based on several hundred image-based features and a machine learning model trained on >1000 clinical CTCs. Enumeration of CTCs was performed by certified blinded reviewers to confirm true positives and reject potential false positives. Single cell PD-L1 mean fluorescence intensities (MFI) were analyzed to determine PD-L1 expression status. CTC PD-L1 positive/negative status is determined using an MFI threshold, which corresponds to the MFI value that provides a minimum specificity value of 0.9 for the condition-negative analyte (HAP-1) and a high sensitivity value for the condition-positive analyte (SW900). This threshold was then used to categorize PD-L1 expression on individual mCTCs for accuracy and precision studies. PD-L1 expression was verified on clinical liquid biopsy samples obtained from breast, lung, and prostate cancer patients.

Results

mCTC spike-in samples showed the expected trend in per-cell PD-L1 MFI values using the SW900, H1650, and HAP-1 cell lines. We obtained a PD-L1 biomarker expression accuracy of 0.98, sensitivity of 0.99, and specificity of 0.97, as determined by MFI thresholding based on PD-L1-positive and PD-L1-negative mCTC analytes. The assay was then used to study one each of breast, lung, and prostate patient samples, where PD-L1-positive cells were found in each patient.

Conclusions

We have developed and analytically validated an immunofluorescence assay to measure PD-L1 protein expression on CTCs. Test results demonstrated that the assay is highly specific, sensitive, reliable, and repeatable for characterizing PD-L1 expression on CTCs.

Sample preparation and analysis workflow
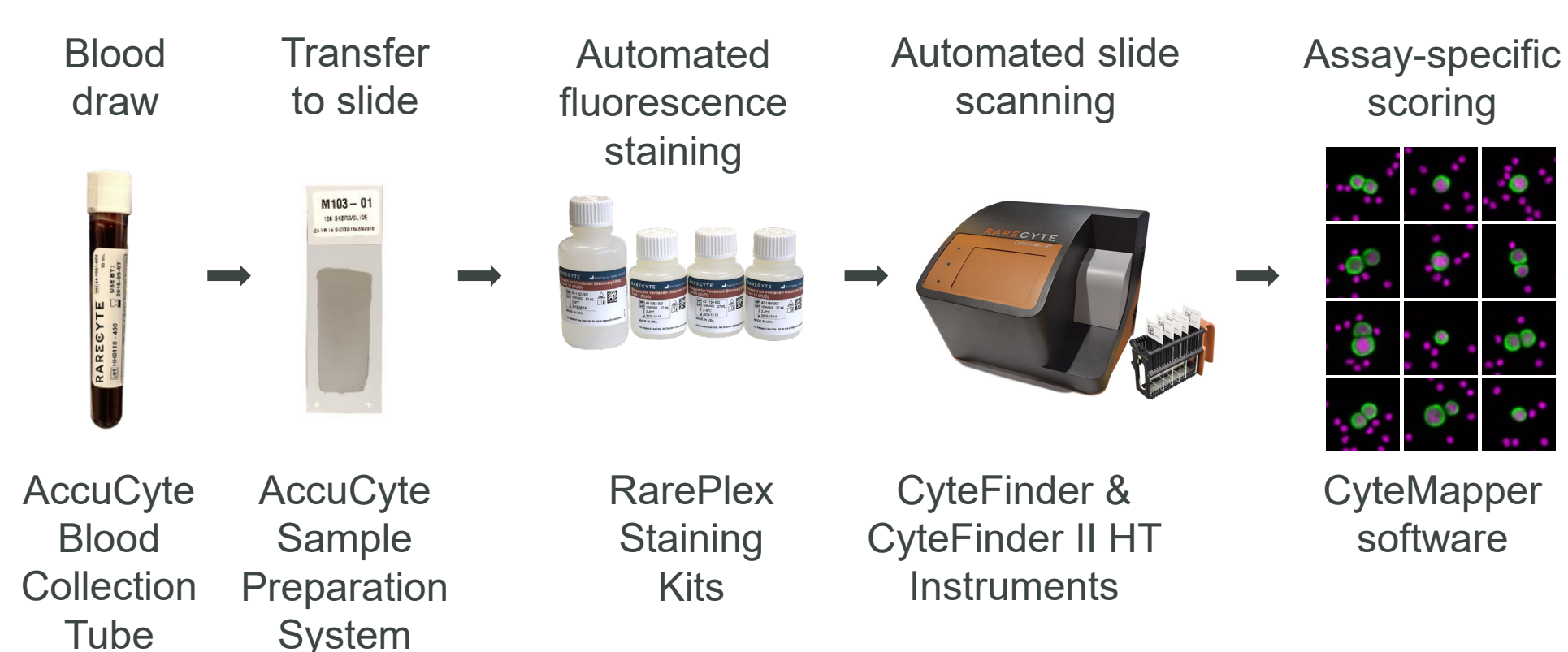
**Figure 1. The RareCyte CTC assay workflow.**
Blood was collected into AccuCyte Blood Collection Tubes. Nucleated blood cells were processed to slides using the density-based AccuCyte Sample Preparation System. Slides were stained with the RarePlex PD-L1 CTC Panel Kit using the Ventana® DISCOVERY® ULTRA automated slide staining system. Slides were scanned using the CyteFinder Instrument and images were analyzed using CyteMapper software and analysis tools. CTCs were analyzed by a trained reviewer and CTC PD-L1 status was determined with a fluorescence intensity threshold.
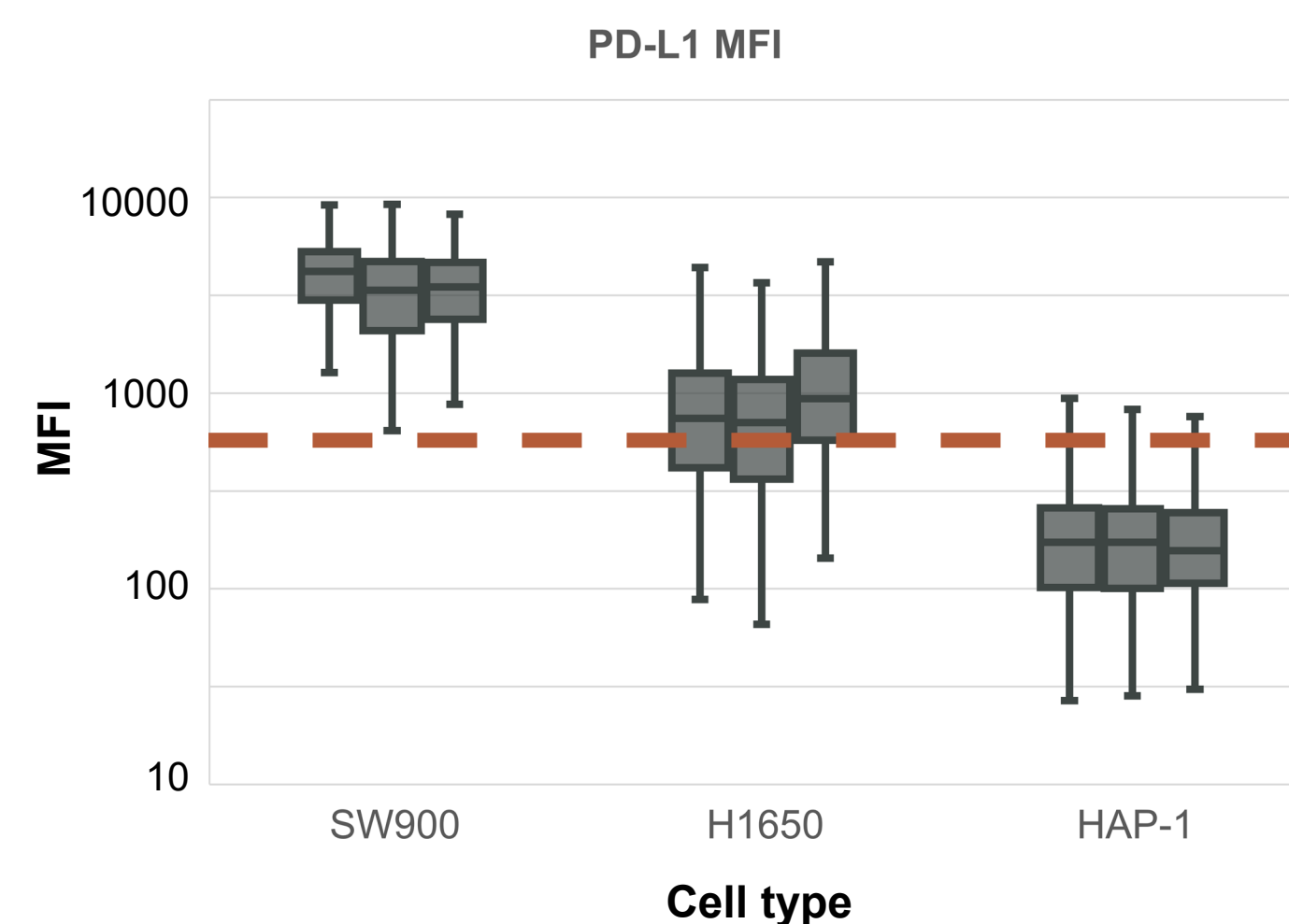
|	PD-L1-	PD-L1+	
	HAP-1	SW900	H1650
Test Positive (MFI > 500)	26	987	694
Test Negative (MFI ≤ 500)	974	13	306
Specificity	0.974		
Sensitivity		0.987	0.694
Accuracy		0.980	0.834

Figure 2. PD-L1 expression on mCTC cell lines using the RarePlex PD-L1 CTC Panel Kit.
The indicated cell lines were spiked into blood and incubated at room temperature for 24 hours followed by sample staining. Data was collected on five slides per mCTC line per stainer run. Positivity threshold (dotted line at MFI=500) is used to determine PD-L1 status on a per-cell basis. The threshold was set by maximizing classification accuracy. Boxes represent the 25th, 50th, 75th percentiles, and whiskers represent min/max values. Test sensitivity, specificity and accuracy are summarized in the table on the right.
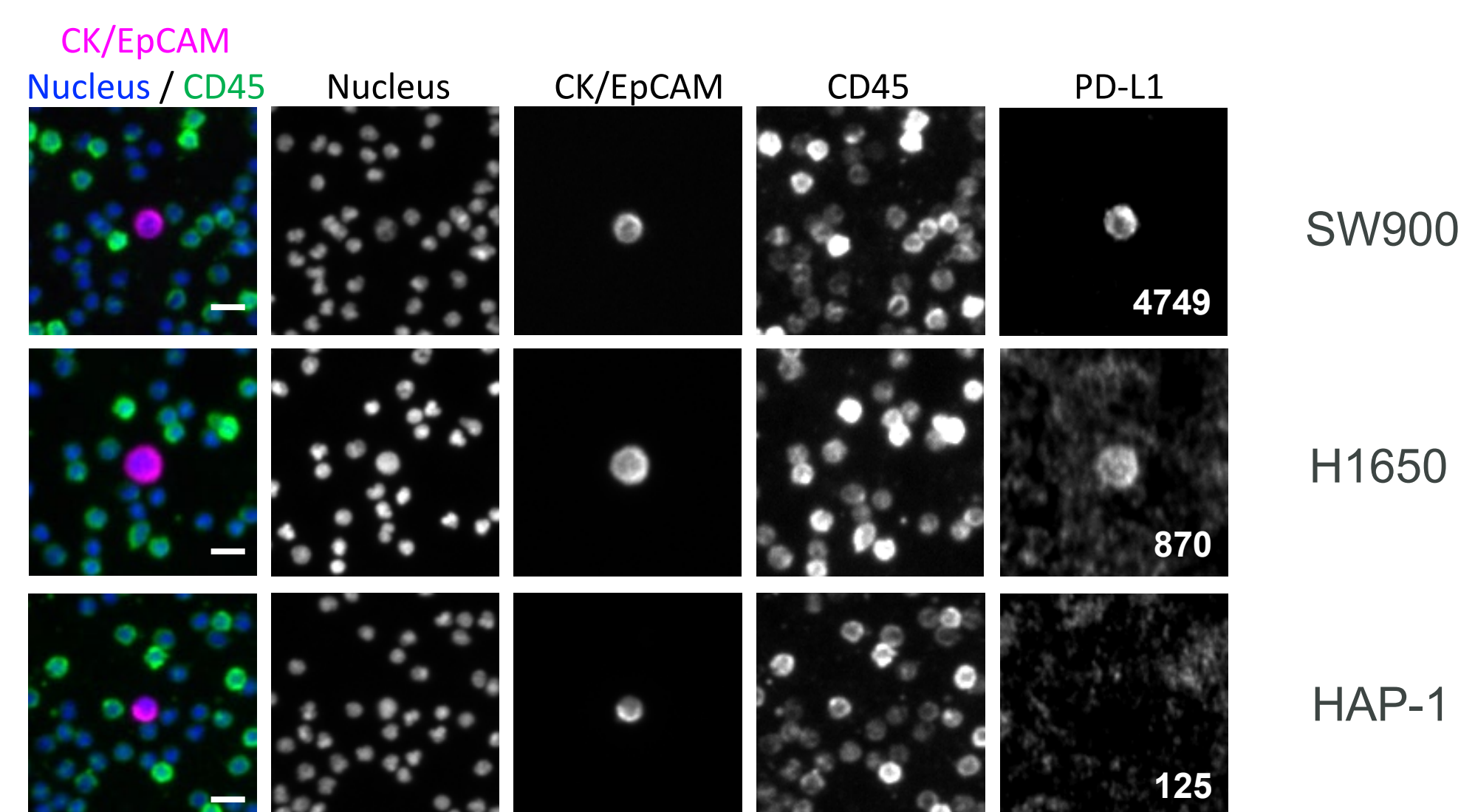
Figure 3. Representative images of mCTCs from tested cell lines.
Each row shows a representative image for a single mCTC of the indicated cell line. Individual channel images for Nucleus, CK/EpCAM, CD45, and PD-L1 are shown, as well as a merged image with CK/EpCAM, Nucleus and CD45. PD-L1 images were individually scaled for display purposes. PD-L1 on mCTCs display the expected membrane localization and the MFI for each cell is displayed in the channel image. Specific PD-L1 staining on platelets is visible on both H1650 and HAP-1 images, but not on SW900 due to the brightness of SW900 cells. Scale bars represent 20 μm.
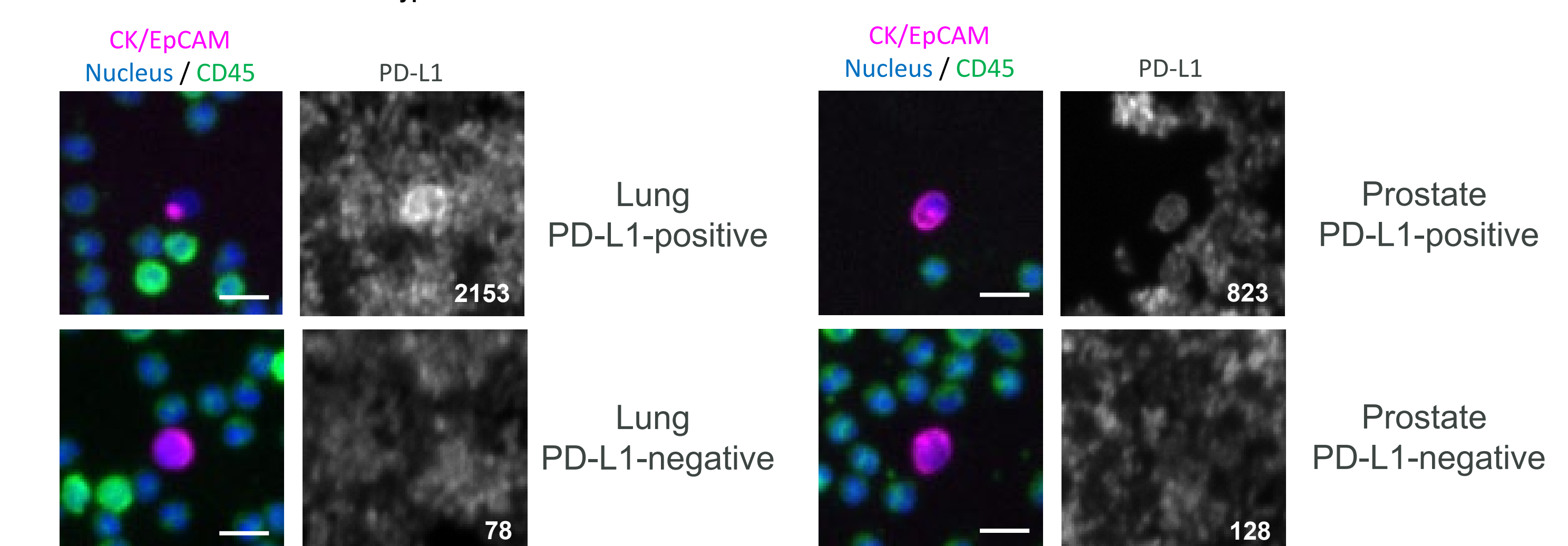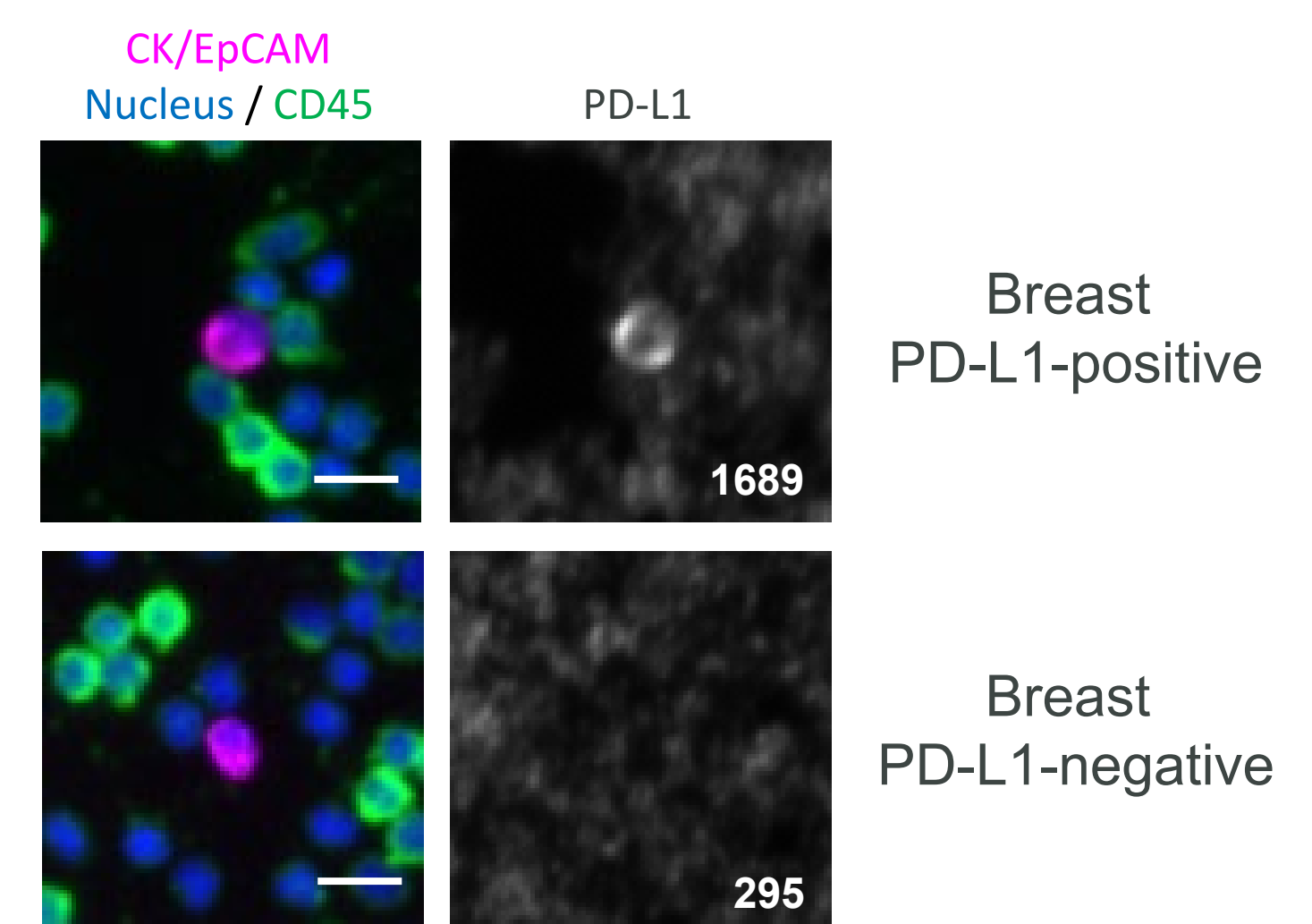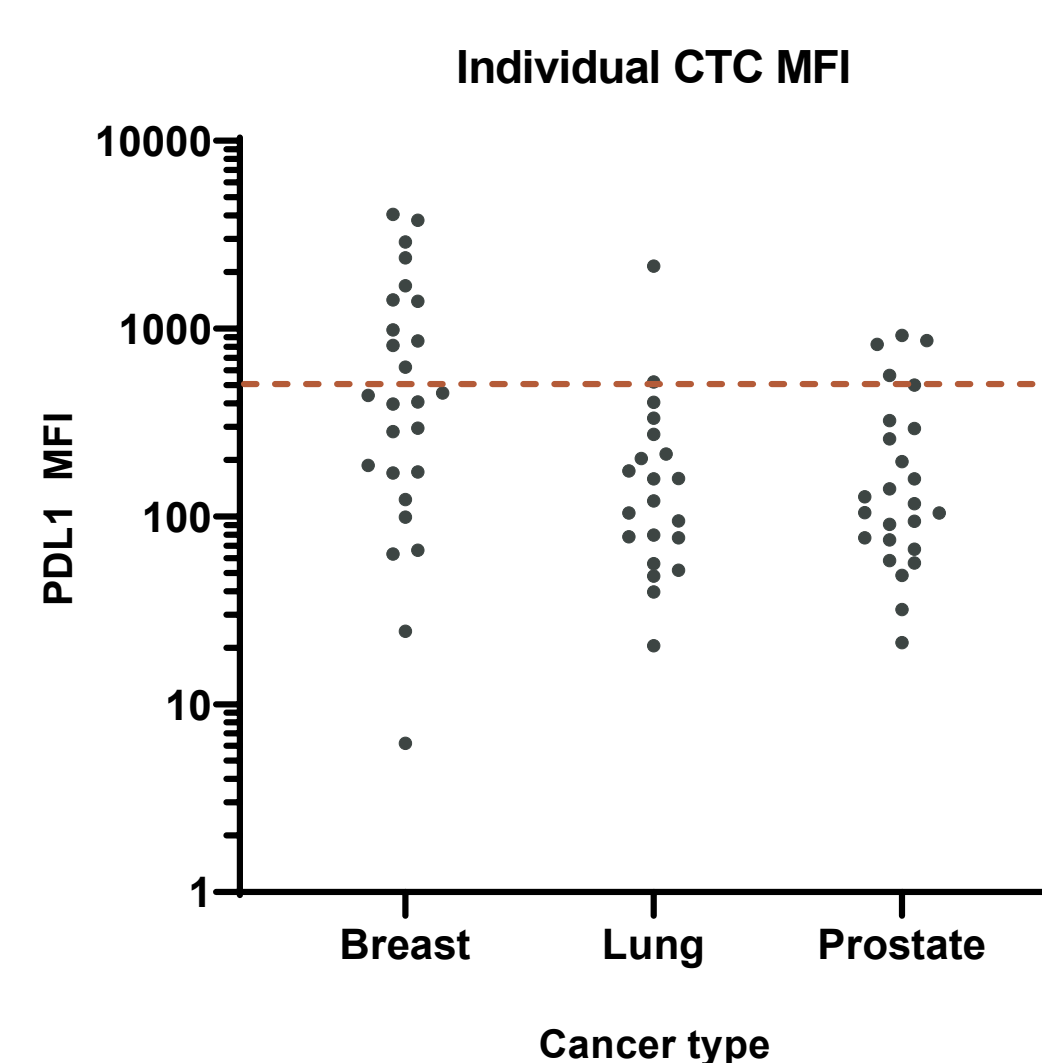
Figure 4. Testing PD-L1 assay on cancer patient samples.
Blood from one breast, one lung, and one prostate cancer patient was collected, processed, and stained. PD-L1 MFI for each CTC was plotted and classified using the MFI threshold of 500, as shown in the dot plot. Two representative CTCs, one PD-L1-positive and one PD-L1-negative, are shown from each patient along with the respective PD-L1 MFI. Display settings for PD-L1-positive cells were set to autoscale which uses linear display scaling with the dimmest pixel displayed as black and the brightest pixel displayed as white. These settings were then applied to the patient-matched PD-L1-negative cell. As with the mCTC samples, platelet PD-L1 staining is visible. Scale bars represent 20 μm.