VALIDATION REPORT

VistaPlex™ Human Fresh Frozen Spatial Immune Profiling Assay Kit

For the CellScape™ Precise Spatial Proteomics platform

Product 531-125000006

Contents

Purpose

VistaPlex Assay Kits contain ready-to-use, reliable reagents and optimized protocols enabling researchers to obtain quick, robust data with the CellScape platform. The objective of this Validation Report is to quantitatively document the performance characteristics of the VistaPlex Human Fresh Frozen (FF) Spatial Immune Profiling Kit to demonstrate the specificity, sensitivity, and reproducibility of the kit. Kit validation is based on experiments performed on human FF tonsil samples. Validation metrics for other tissues are included as a fit-for-use application test and to provide performance considerations for user guidance. This report summarizes the results of the validation testing and the specificity of the markers in the kit.

Note: This assay kit is not compatible with the CellScape XR System.

Validation Metrics and Pass/Fail Criteria

Qualitative suitability and specificity assessment

To determine if 1) fluorescent signal is detected from appropriate tissue locations and 2) antibodies bind only their intended targets, stains are evaluated by a panel of scientists using a numerical scoring system (see Methods). Scores are averaged across all judges and samples of the same tissue type.

Pass: Average score ≥ 1.5 (tonsil) or 1.0 (other tissues)

Fail: Average score < 1.5 (tonsil) or 1.0 (other tissues)

Quantitative sensitivity assessment

To determine if fluorescent signals are strong enough to differentiate positive staining from background fluorescence, signal-to-noise ratios are calculated through two different and commonly used methods (see Methods).

Pass: Average SNR ≥ 2

Fail: Average SNR < 2

Quantitative reproducibility assessment

To verify that antibodies produce consistent results, the density of positive cells is determined from technical replicates on serial sections, measured across different systems, at different physical sites, and by different platform operators (i.e. multi-site experiment). Mean cell density, standard deviations and coefficients of variation (CV) are calculated.

Low Variability: CV of < 25%

Medium Variability: CV of 25 - 50%

High variability: CV of > 50%

Note: Inherent natural variations in cell densities across serial sections (SS) contribute to CV measurements; occasionally, high CV measurements may be due to structural variations rather than differences in antibody performance.

Validation Summary

Table 1. Results summary for specificity, sensitivity, and reproducibility of the Human FF Spatial Immune Profiling Assay Kit. Data were obtained from human fresh frozen tonsil.

Antibody/Stain	Specificity	Sensitivity	Reproducibility
CD163	Pass	Pass	Low Variability
CD19	Pass	Pass	Low Variability
CD20	Pass	Pass	Low Variability
CD274	Pass	Pass	Low Variability
CD279	Pass	Pass	Low Variability
CD3	Pass	Pass	Low Variability
CD38	Pass	Pass	Medium Variability
CD4	Pass	Pass	Low Variability
CD45	Pass	Pass	Low Variability
CD45RA	Pass	Pass	Low Variability
CD45RO	Pass	Pass	Low Variability
CD56	Pass	Pass	Low Variability
CD8	Pass	Pass	Low Variability
FoxP3	Pass	Pass	Low Variability
Ki67	Pass	Pass	Low Variability
panCK	Pass	Pass	Low Variability

Table 2. Results summary for suitability of the Human FF Spatial Immune Profiling Assay Kit.

Tissue	Suitability
Tonsil	Pass
Breast cancer	Variable
Lung cancer	Variable

Validation Data

The following pages detail the validation data for the kit, organized by tissue type:

- Tonsil
- Breast cancer
- Lung cancer

Tonsil

Qualitative Suitability and Specificity Assessment – Scoring

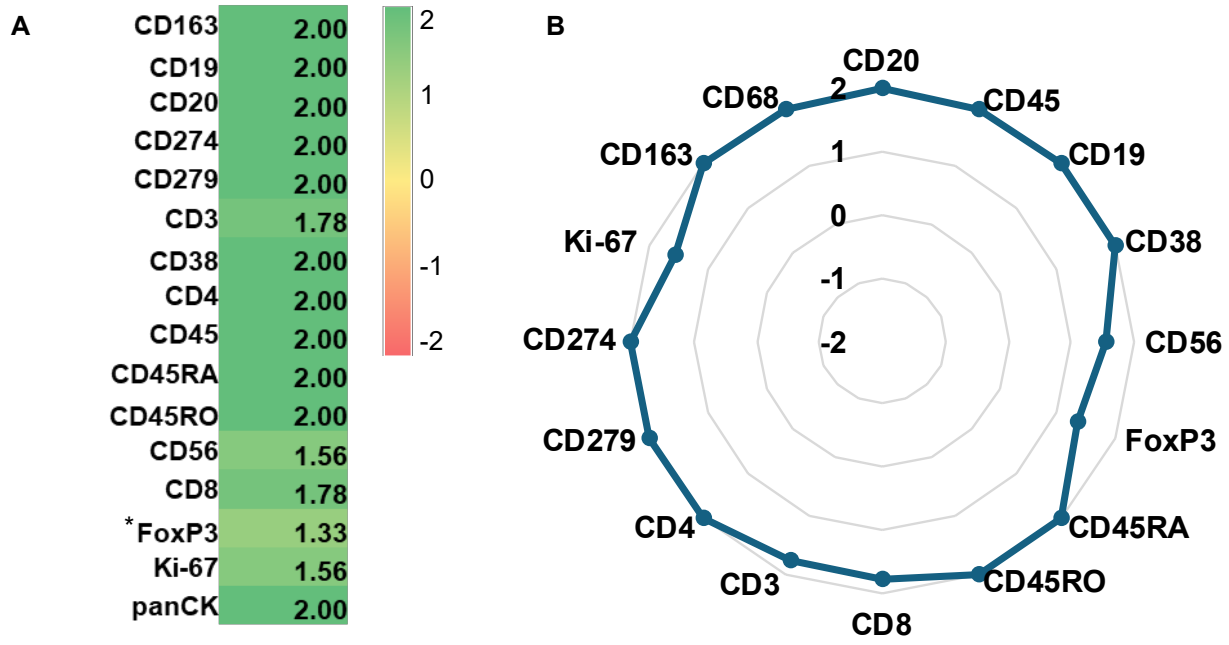


Figure 1. Scoring results of antibodies in the Human FF Spatial Immune Profiling Assay Kit. Average scores from technical replicates of human FF tonsil are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges. *FoxP3 passes since the composite score is comprised of acceptable scores (2, 33% and 1, 67%).

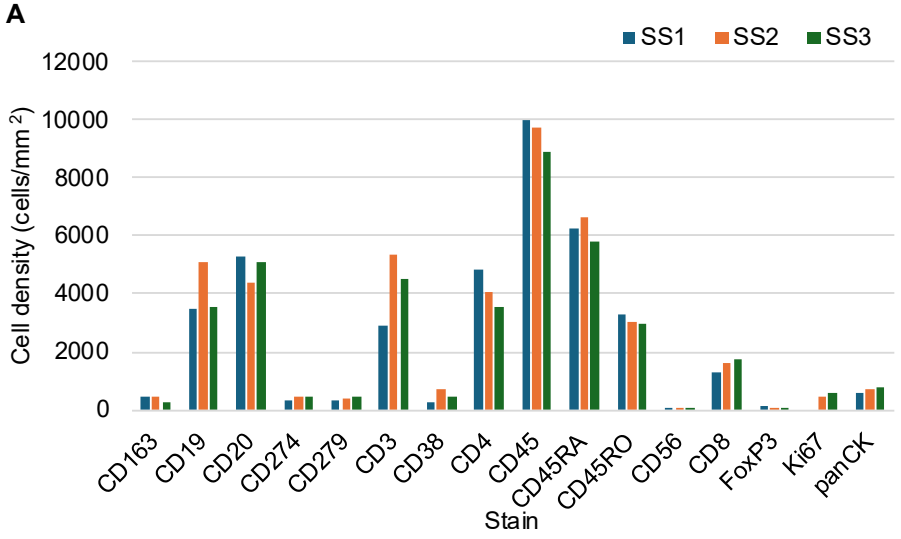
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 3. SNR values for stains in the Human FF Spatial Immune Profiling Assay Kit. Average positive and negative signal intensities and SNR from three technical replicates of human FF tonsil.

	Method 1			Method 2		
	Mean +	**Mean -**	**SNR**	**Mean +**	**Mean -**	**SNR**
CD163	355.32	9.78	36.34	107.89	0.86	125.75
CD19	810.07	38.70	20.93	1281.85	8.62	148.69
CD20	588.56	84.09	7.00	1489.93	12.61	118.12
CD274	530.02	34.36	15.42	1242.68	6.06	204.91
CD279	90.38	2.65	34.09	470.68	0.81	584.25
CD3	262.81	44.83	5.86	555.07	3.29	168.84
CD38	467.84	23.97	19.52	1409.79	3.22	437.55
CD4	491.51	105.74	4.65	1081.51	26.16	41.34
CD45	488.58	183.69	2.66	793.46	178.31	4.45
CD45RA	182.19	43.17	4.22	316.57	22.26	14.22
CD45RO	334.64	34.62	9.67	764.98	3.20	239.41
CD56	91.40	2.37	38.56	177.45	1.21	146.20
CD8	453.67	44.96	10.09	1021.31	0.46	2202.93
FoxP3	23.66	1.50	15.79	109.09	1.42	77.08
Ki67	172.28	1.18	145.72	351.94	0.22	1629.59
panCK	340.38	8.26	41.19	795.44	0.66	1206.70

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
CD163	17.13
CD19	18.10
CD20	8.15
CD274	12.91
CD279	12.39
CD3	23.32
CD38	36.85
CD4	13.02
CD45	4.84
CD45RA	5.45
CD45RO	4.79
CD56	17.40
CD8	12.36
FoxP3	11.07
Ki67	12.15
panCK	11.98

Figure 2. Reproducibility of antibodies in the Human FF Spatial Immune Profiling Assay Kit. Cell density measurements for each stain across technical replicates of human FF tonsil (A) and corresponding CV (B). n = 3 serial sections. *Ki-67 was not stained in SS1, and that datapoint is excluded from the CV calculation accordingly.

Breast cancer

Qualitative Suitability and Specificity Assessment – Scoring

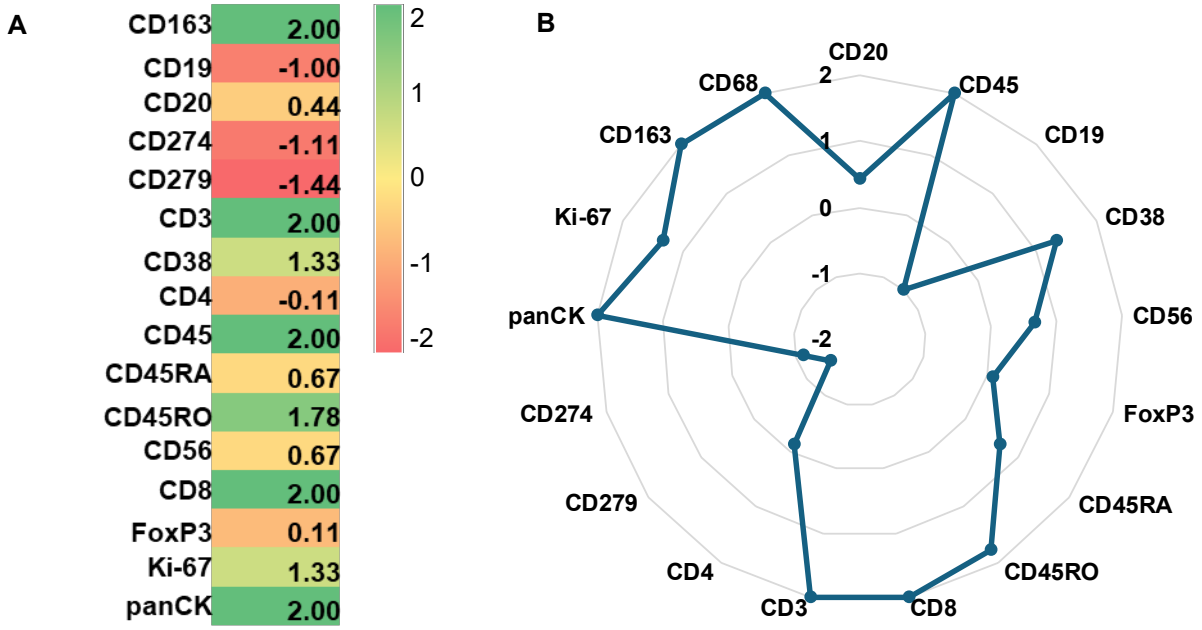


Figure 3. Scoring results of antibodies in the Human FF Spatial Immune Profiling Assay Kit. Average scores from technical replicates of human FF breast cancer are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges.

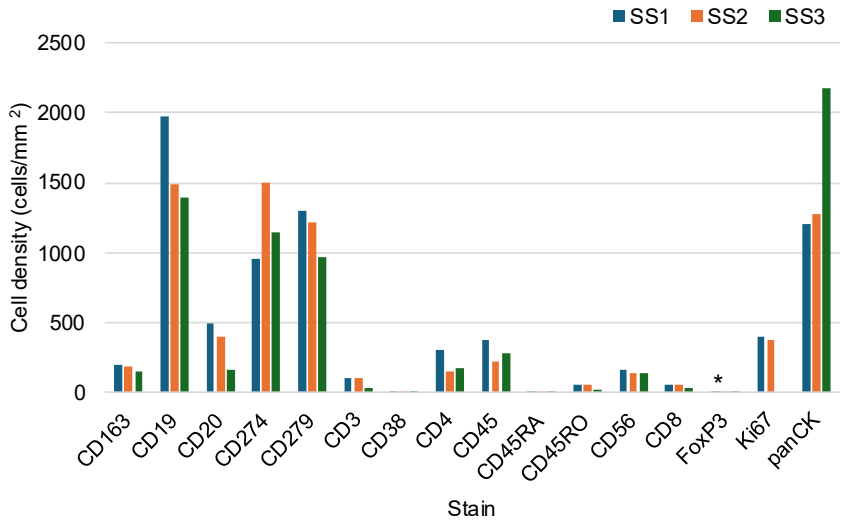
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 4. SNR values for stains in the Human FF Spatial Immune Profiling Assay Kit. Average positive and negative signal intensities and SNR from three technical replicates of human FF breast cancer.

	Method 1			Method 2		
	Mean +	**Mean -**	**SNR**	**Mean +**	**Mean -**	**SNR**
CD163	598.15	18.90	31.64	269.79	1.55	173.99
CD19	44.82	9.83	4.56	92.87	7.16	12.97
CD20	22.59	1.09	20.73	43.75	1.04	42.01
CD274	44.14	10.42	4.24	137.41	9.83	13.98
CD279	153.63	3.33	46.09	50.97	1.99	25.65
CD3	258.05	2.51	102.77	362.73	1.43	253.37
CD38	1147.31	0.45	2573.14	130.15	0.39	336.08
CD4	140.34	14.81	9.48	317.72	6.05	52.52
CD45	357.07	17.53	20.37	1081.50	0.67	1620.22
CD45RA	34.44	1.16	29.66	136.66	1.01	135.14
CD45RO	52.25	1.88	27.80	175.82	0.69	254.30
CD56	63.93	2.15	29.77	97.88	0.81	120.49
CD8	575.39	1.52	379.54	766.26	0.47	1628.27
FoxP3	8.72	0.71	12.29	58.24	1.27	46.01
Ki67	496.16	6.12	81.11	1162.07	0.38	3088.27
panCK	148.82	12.86	11.58	320.57	0.75	426.96

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
CD163	13.39
CD19	15.55
CD20	38.31
CD274	18.66
CD279	14.37
CD3	36.60
CD38	66.24
CD4	32.26
CD45	20.45
CD45RA	26.78
CD45RO	29.88
CD56	7.72
CD8	29.49
FoxP3	13.36
Ki67	3.55
panCK	28.66

Figure 4. Reproducibility of antibodies in the Human FF Spatial Immune Profiling Assay Kit. Cell density measurements for each stain across technical replicates of human FF breast cancer (A) and corresponding CV (B). n = 3 serial sections. *Ki-67 was not stained in SS3. Datapoint is excluded from CV calculation, accordingly.

Lung cancer

Qualitative Suitability and Specificity Assessment – Scoring

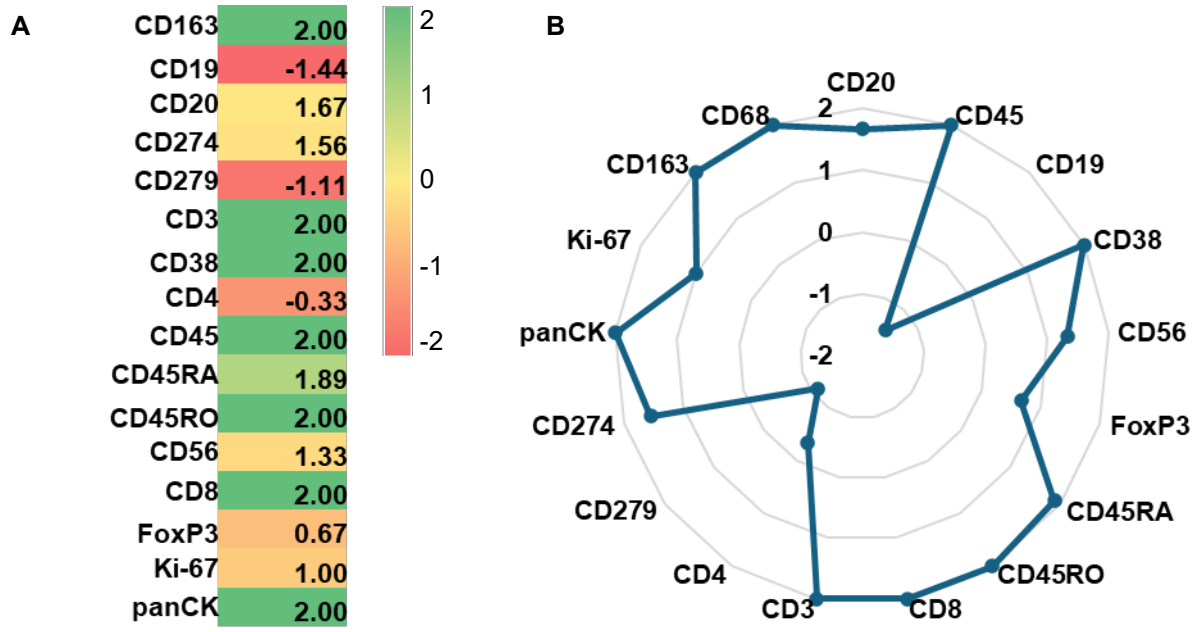


Figure 5. Scoring results of antibodies in the Human FF Spatial Immune Profiling Assay Kit. Average scores from technical replicates of human FF lung cancer are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges.

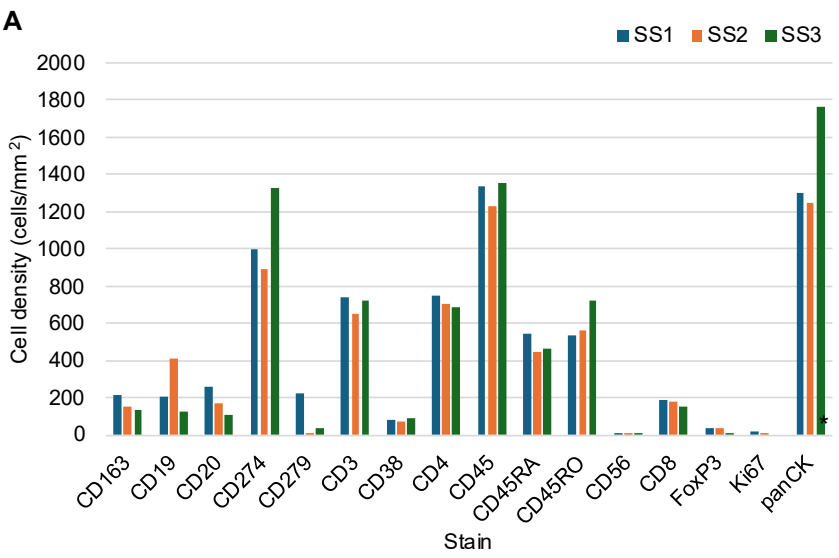
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 5. SNR values for stains in the Human FF Spatial Immune Profiling Assay Kit. Average positive and negative signal intensities and SNR from three technical replicates of human FF lung cancer.

	Method 1			Method 2		
	Mean +	**Mean -**	**SNR**	**Mean +**	**Mean -**	**SNR**
CD163	341.92	4.37	78.20	662.22	0.94	705.87
CD19	36.74	2.52	14.59	1084.68	2.04	532.00
CD20	23.92	0.52	46.03	565.67	0.28	2054.55
CD274	740.96	48.81	15.18	1563.93	169.64	9.22
CD279	68.73	0.20	339.84	2048.70	104.43	19.62
CD3	345.55	4.49	76.90	294.53	23.02	12.79
CD38	986.28	5.70	173.13	475.48	0.26	1813.30
CD4	237.42	11.64	20.40	704.20	1.83	384.32
CD45	638.88	21.64	29.52	456.60	1.47	311.19
CD45RA	243.50	1.70	142.84	637.66	55.97	11.39
CD45RO	217.44	3.34	65.12	880.04	52.77	16.68
CD56	145.74	0.33	439.00	1005.80	55.97	17.97
CD8	686.16	2.06	332.60	841.03	0.45	1860.82
FoxP3	34.03	0.53	64.54	1485.60	3.85	385.59
Ki67	174.93	0.13	1387.64	449.74	0.49	922.57
panCK	295.74	12.26	24.12	1600.89	26.39	60.66

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
CD163	22.97
CD19	47.44
CD20	34.09
CD274	17.37
CD279	69.42
CD3	5.32
CD38	8.91
CD4	3.45
CD45	4.47
CD45RA	8.28
CD45RO	13.45
CD56	68.63
CD8	8.38
FoxP3	52.20
Ki67	36.30
panCK	14.98

Figure 6. Reproducibility of antibodies in the Human FF Spatial Immune Profiling Assay Kit. Cell density measurements for each stain across technical replicates of human FF lung cancer (A) and corresponding CV (B). n = 3 serial sections. *Ki-67 was not stained in SS3. Datapoint is excluded from CV calculation, accordingly.

Stain Qualification and Specificity Criteria

Table 6 describes the areas of interest that were used for evaluating antibody performance in human FF tonsil. Specificity assessment was informed by counterstains that provide context on overall tissue organization. Example images of each stain and example counterstains are shown in Figure 7.

Table 6. Localization and specificity assessment criteria used for stains in the Human FF Spatial Immune Profiling Assay Kit in human FF tonsil.

Stain	Tissue Localization	Intracellular Localization	Positive counterstain	Negative counterstain
CD20	Germinal centers and interfollicular regions	Plasma membrane	CD45	CD3
CD45	Germinal centers and interfollicular regions	Plasma membrane	CD20	panCK
CD19	Germinal centers and interfollicular regions	Plasma membrane	CD20	CD3
CD38	Germinal centers and interfollicular regions	Plasma membrane	CD19	panCK
CD56	Interfollicular regions	Plasma membrane	CD45	CD3 (some proportion will be positive)
FoxP3	Interfollicular regions	Nucleus	CD4	CD8
CD45RA	Germinal centers and interfollicular regions	Plasma membrane	CD45	CD45RO
CD45RO	Germinal centers and interfollicular regions	Plasma membrane	CD45	CD45RA
CD8	Interfollicular regions, germinal centers	Plasma membrane	CD3	CD4
CD3	Germinal centers and interfollicular regions	Plasma membrane	CD45	CD20
CD4	Interfollicular regions, germinal centers	Plasma membrane	CD3	CD8
CD279 (PD-1)	Germinal centers	Plasma membrane	CD4	CD8
CD274 (PD-L1)	Squamous epithelia and germinal centers	Plasma membrane	panCK (epithelia), CD68 (germinal centers)	CD3
Ki-67	Germinal centers and interfollicular regions	Nucleus	CD20	n/a
CD163	Interfollicular regions	Plasma membrane	CD63 (subpopulation of CD63+)	CD3
panCK	Squamous epithelia	Intracellular, plasma membrane	PD-L1	CD45

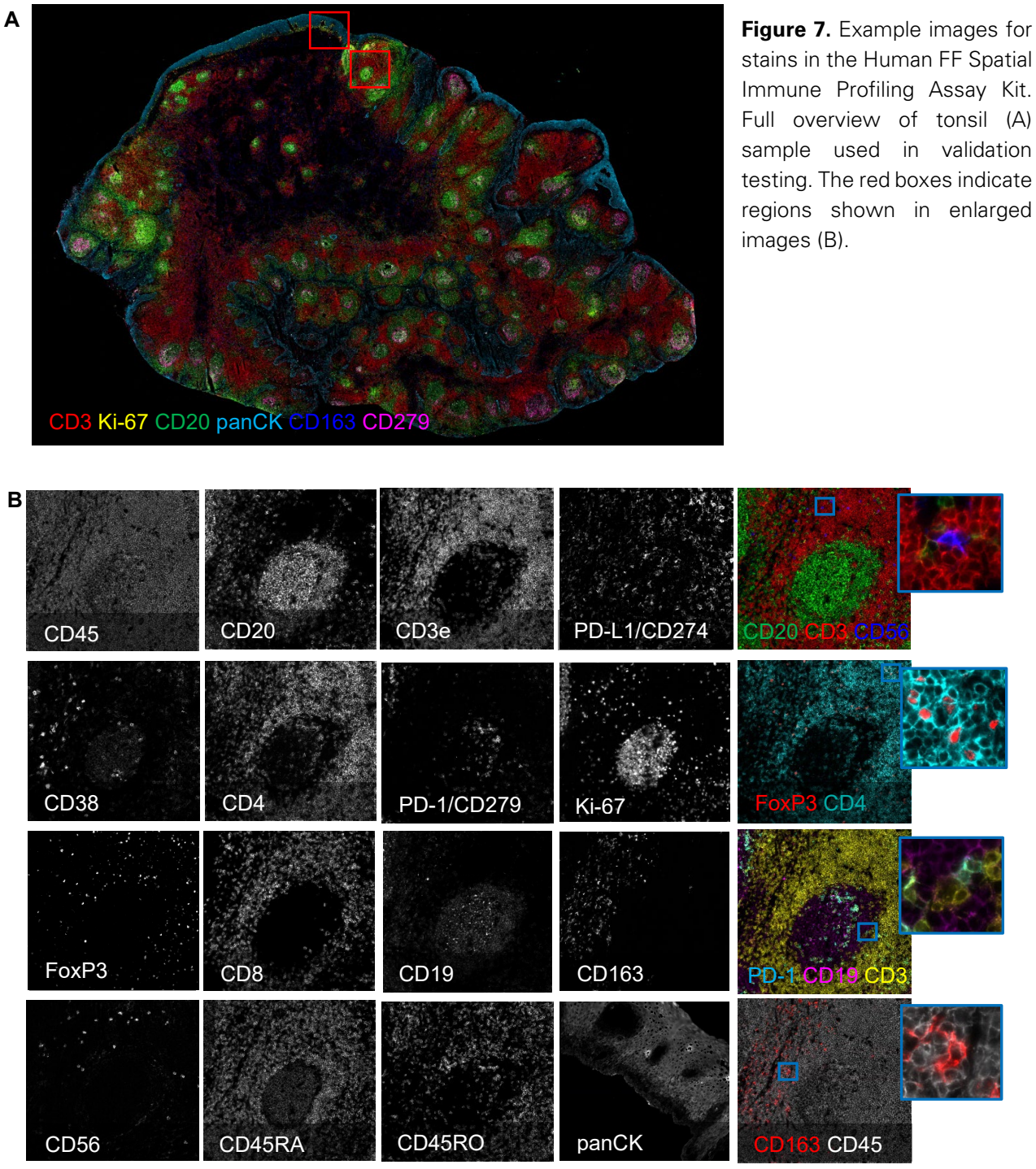


Figure 7. Example images for stains in the Human FF Spatial Immune Profiling Assay Kit. Full overview of tonsil (A) sample used in validation testing. The red boxes indicate regions shown in enlarged images (B).

Methods

Reagent Preparation

Tissue samples (Table 7) were prepared in Leipzig, Germany, and shipped to additional testing sites in Hannover, Germany and Saint Louis, MO. Serial sections of human FF tonsil were cut and mounted on Superfrost Plus Gold Slides (Fisher Scientific, 22-037-246) and stored at -80°C before shipping on dry ice. Acetone/Ethanol fixation was performed independently at each testing site following the CellScape Sample Preparation and Instrument Operation Manual (MAN-10200).

Table 7. Human tissues used for VistaPlex Kit validation.

Product Code	Description	Vendor
681074B2(3) / MZKL585620	Tissue – Tonsil	AmsBio
T1235086-DC	Tissue – Lung cancer	BioCat
T1235152-DC	Tissue – Breast cancer	BioCat

Antibodies were diluted in Storage Buffer (Bruker Spatial Biology, PRSM-BUF-STR-50mL) to create working solutions, which were then filtered through a 0.22 µm low protein-binding syringe filter (Millipore-Sigma, SLGV004SL) before use.

Image Acquisition

The cyclic multiplex immunofluorescence assay was executed on the CellScape platform powered by CellScape Navigator software, following the stain plan (Table 8) with 10 seconds of enhanced photobleaching at 50% lamp power before each cycle. Signal removal between cycles was facilitated by EpicIF™ Solution (Bruker Spatial Biology, PRSM-BUF-EPIC-500mL).

Table 8. Staining plan.

Cycle	Target	Dilution	Stain Time (min)
1	CD20	1:500	15
	CD45	1:60	
	CD19	1:100	
	CD38	1:500	
2	CD56	1:100	15
	FoxP3	1:100	
	CD45RA	1:2000	
	CD45RO	1:500	
3	CD8	1:900	15
	CD3	1:50	
	CD4	1:900	
4	CD279/PD-1	1:100	15
	CD274/PD-L1	1:100	
	Ki-67	1:2000	
5	CD163	1:100	15
	panCK	1:4000	

Image Scoring

Exported OME-TIFF files were viewed in QuPath to assess stain quality, suitability and specificity. Four independent judges scored all images according to the scoring definitions in Table 9. All scores were averaged for each marker and sample type. An acceptable average score for the positive control tissue (tonsil) was defined as ≥ 1.5. We based this cutoff on the requirement that all stains must be acceptable (scored ≥ 1) in the positive control tissue. Given two scores, the average of the greatest passing score (2) and the greatest failing score (0) is 1 while the average of the greatest passing score and the lowest passing score (1) is 1.5. Therefore, 1.5 is an acceptable cutoff demonstrating a passing score from all judges.

Table 9. Score Definitions.

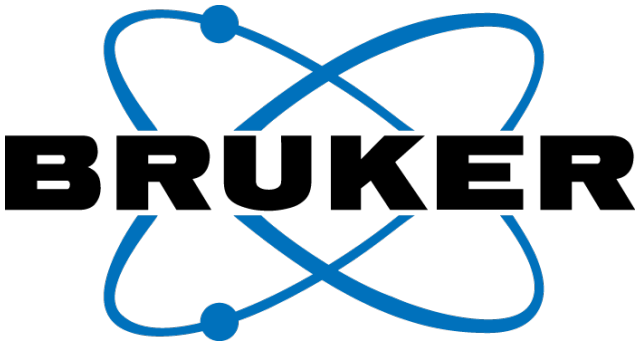
Score	Interpretation
2	Excellent, bright, specific stain
1	Acceptable but dim or high background
0	No staining
-1	Moderate, not abundant off target staining
-2	Strong and/or abundant unspecific staining

Computational Image Analysis, Thresholding, and Signal-to-Noise Ratios

Serial sections were used for quantitative reproducibility analysis. Briefly, 32-bit OME-TIFF images were used to create a single QuPath project, and matching regions were selected with the annotation tool. The selected regions were exported and analyzed. For each region, cells were segmented using DeepCell, a publicly available pre-trained model, including nuclear and cytoplasm compartments. Nuclear segmentation was based on DNA (SYTOX™ Orange), while membrane segmentation used the max-projection of ATP1A1. Marker expression levels were extracted for each cell, enabling downstream quantification of regions and slides.

Signal-to-noise ratios were calculated using two different methods. Method 1 (referenced here) applied Otsu thresholding to raw, non-segmented pixel data to classify pixels as positive or negative. The SNR is then computed as the ratio of the mean positive intensity to the mean negative intensity. Method 2 (referenced here) defined signal intensity using per-cell quantifications. The signal was determined by the average intensity of the top 20 brightest cells ("mean +"), while noise was defined as the 10th percentile of cell intensities ("mean –").

For reproducibility, cells were classified as positive or negative based on Otsu thresholding applied to average cell expression. The number of positive cells was quantified per unit area, expressed as cells/mm^2. The CV was calculated as the ratio of standard deviation to the mean expressed as a percent.



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