



VALIDATION REPORT

VistaPlex™ Spatial Immune Profiling Kit

For CellScape™ Precise Spatial Proteomics

Validation of the Spatial Immune Profiling for Human FFPE multiplex antibody kit, product VISTAPLEX3102

PMR-11862-01

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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 Spatial Immune Profiling Kit antibody panel to demonstrate the repeatability, reproducibility, and specificity of the kit. Kit validation is based on experiments performed on human FFPE tonsil samples. Validation metrics for tumor 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.

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 (tumor)

Fail: Average score < 1.5 (tonsil) or 1.0 (tumor)

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 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 Spatial Immune Profiling Kit. Data were obtained from human FFPE tonsil.

Antibody/Stain	Specificity	Sensitivity	Reproducibility
CD20	Pass	Pass	Low Variability
CD3	Pass	Pass	Low Variability
CD279	Pass	Pass	Low Variability
CD274	Pass	Pass	Low Variability
FoxP3	Pass	Pass	Low Variability
CD4	Pass	Pass	Low Variability
CD45	Pass	Pass	Low Variability
CD19	Pass	Pass	Low Variability
GrnB	Pass	Pass	Medium Variability
CD8	Pass	Pass	Low Variability
CD45RO	Pass	Pass	Low Variability
CD38	Pass	Pass	Low Variability
Ki-67	Pass	Pass	Low Variability
CD68	Pass	Pass	Medium Variability
CD45RA	Pass	Pass	Low Variability
PanCK	Pass	Pass	Low Variability
CD163	Pass	Pass	Low Variability

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

Tissue	Suitability
Breast Cancer	Pass
Melanoma	Pass
Colon Cancer	Pass
Head and Neck Cancer	Pass

Validation Data

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

- Tonsil
- Breast Cancer
- Melanoma
- Colon Cancer
- Head & Neck Cancer

Tonsil

Qualitative Suitability and Specificity Assessment – Scoring

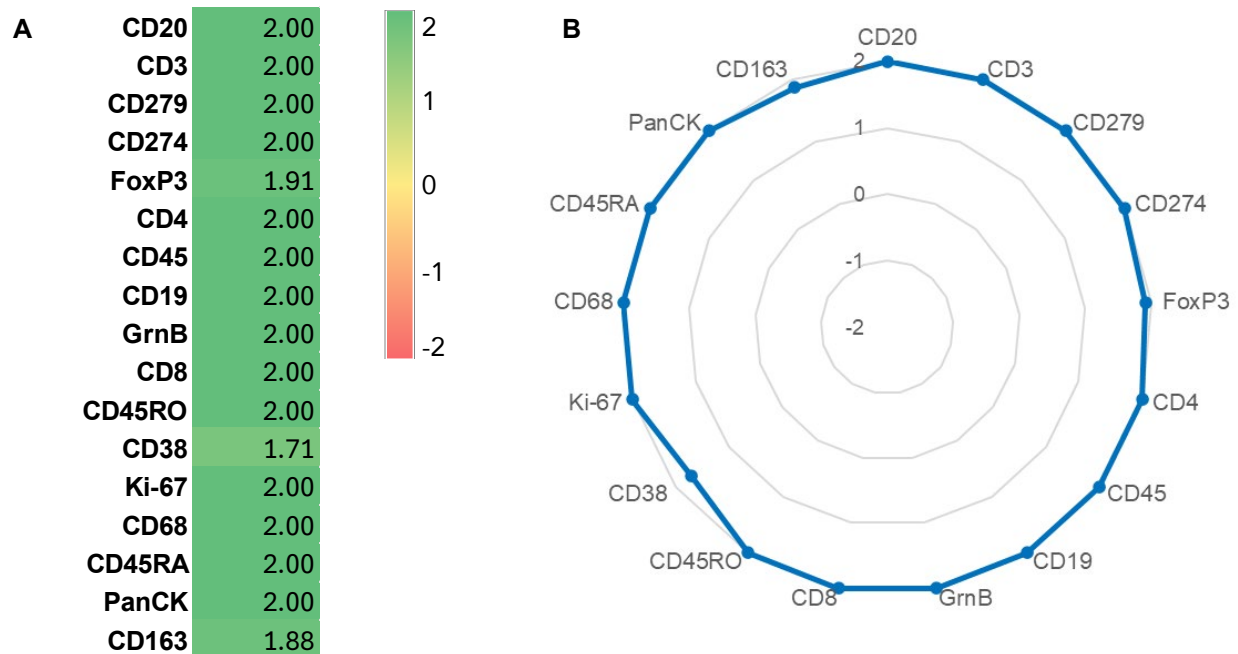


Figure 1. Scoring results of antibodies in the Spatial Immune Profiling Kit. Average scores from technical replicates of human FFPE Tonsil are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 8 samples scored by four independent judges.

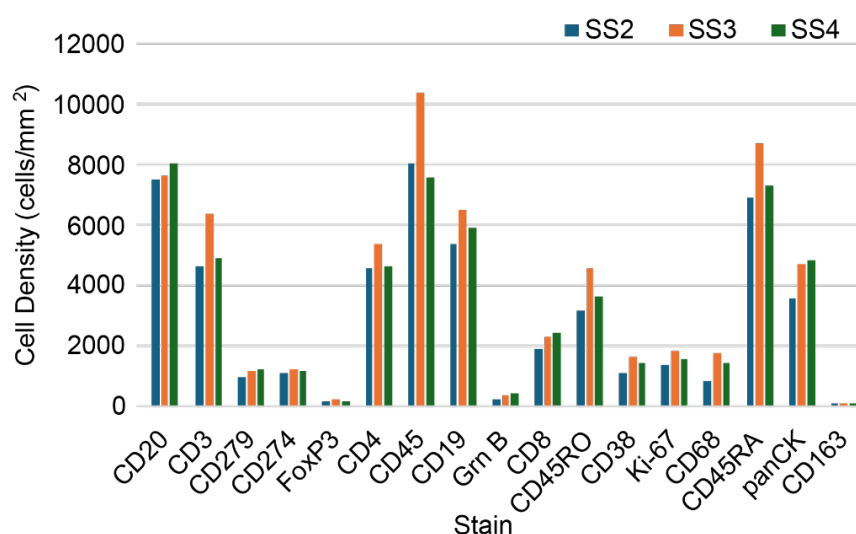
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

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

	Method 1			Method 2		
	Mean +	Mean -	SNR	Mean +	Mean 1-	SNR
CD20	646.60	36.57	17.68	5129.43	303.87	16.88
CD3	602.81	55.58	10.85	3257.34	92.09	35.37
CD279	15.84	2.95	5.37	894.63	13.09	68.33
CD274	2775.36	15.83	175.35	2192.52	31.70	69.15
FoxP3	587.21	25.80	22.76	1048.60	31.17	33.64
CD4	280.42	50.65	5.54	4050.50	158.69	25.52
CD45	1119.01	98.09	11.41	13820.49	965.47	14.31
CD19	48.67	15.26	3.19	1465.69	85.19	17.20
GrnB	787.63	8.57	91.95	2279.90	16.47	138.39
CD8	296.65	9.47	31.33	1607.47	20.70	77.66
CD45RO	361.97	33.59	10.78	1262.17	52.62	23.99
CD38	486.60	47.34	10.28	1769.83	78.35	22.59
Ki-67	2180.12	88.95	24.51	9483.40	34.07	278.34
CD68	1115.49	89.68	12.44	2982.80	102.71	29.04
CD45RA	342.74	13.97	24.53	615.75	60.22	10.22
panCK	946.32	94.59	10.00	2733.15	12.45	219.61
CD163	577.11	45.39	12.72	710.93	37.27	19.08

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
CD20	2.82
CD3	14.28
CD279	8.32
CD274	5.68
FoxP3	11.89
CD4	7.40
CD45	14.21
CD19	7.76
GrnB	25.00
CD8	10.14
CD45RO	15.05
CD38	15.86
Ki-67	11.18
CD68	28.22
CD45RA	9.85
panCK	13.07
CD163	6.36

Figure 2. Reproducibility of antibodies in the Spatial Immune Profiling Kit. Cell density measurements for each stain across three technical replicates of human FFPE tonsil (A) and corresponding CV (B). n = 3 serial sections.

Breast Cancer

Qualitative Suitability and Specificity Assessment – Scoring

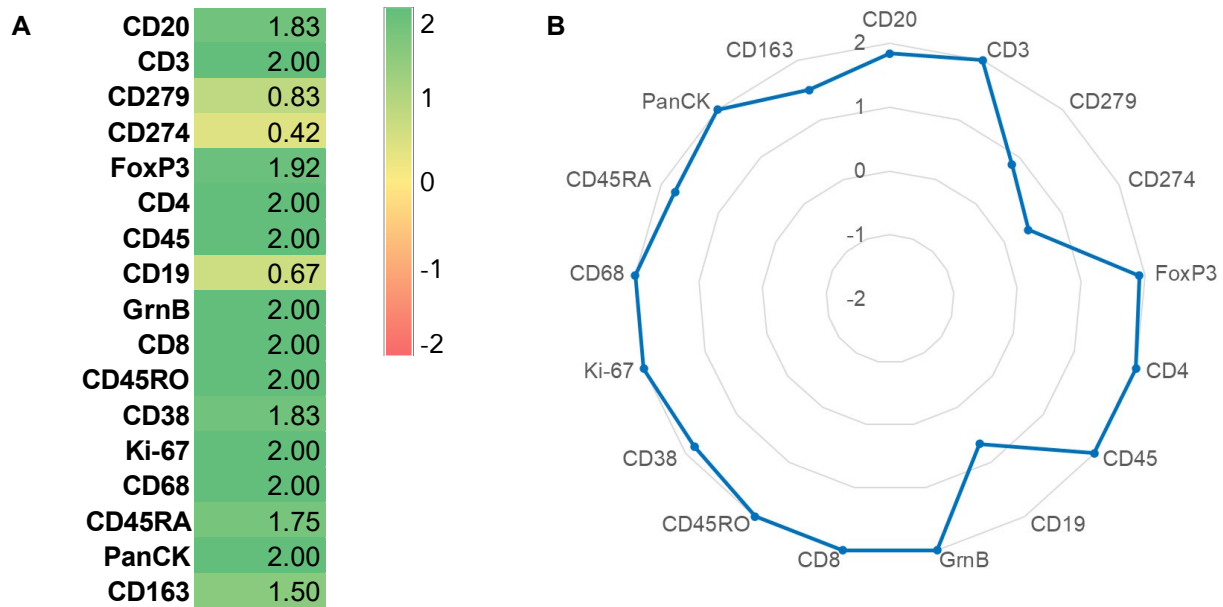


Figure 3. Scoring results of antibodies in the Spatial Immune Profiling Kit. Average scores from technical replicates of human FFPE breast cancer are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 3 samples scored by four independent judges.

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

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

	Method 1			Method 2		
	Mean +	Mean -	SNR	Mean +	Mean -	SNR
CD20	29.53	11.87	2.49	17467.75	34.21	510.66
CD3	580.77	75.55	7.69	1402.61	48.60	28.86
CD279	15.70	3.21	4.89	148.07	4.90	30.22
CD274	70.22	23.36	3.01	369.73	18.61	19.87
FoxP3	343.95	32.10	10.71	1171.98	24.40	48.03
CD4	321.17	58.75	5.47	842.52	32.23	26.14
CD45	1254.08	214.28	5.85	4771.24	31.99	149.15
CD19	69.81	25.15	2.78	485.17	19.75	24.57
GrnB	798.12	9.27	86.11	1570.19	5.14	305.48
CD8	318.64	12.68	25.13	585.39	7.88	74.29
CD45RO	382.04	48.46	7.88	1332.16	24.90	53.50
CD38	525.41	86.42	6.08	991.66	40.48	24.50
Ki-67	2145.35	38.21	56.14	6616.17	13.93	474.96
CD68	999.97	109.99	9.09	3183.34	52.31	60.86
CD45RA	347.28	34.45	10.08	1312.89	8.92	147.18
panCK	1471.29	72.40	20.32	3412.15	15.91	214.47
CD163	262.36	40.43	6.49	1243.50	26.10	47.64

Quantitative Reproducibility Assessment

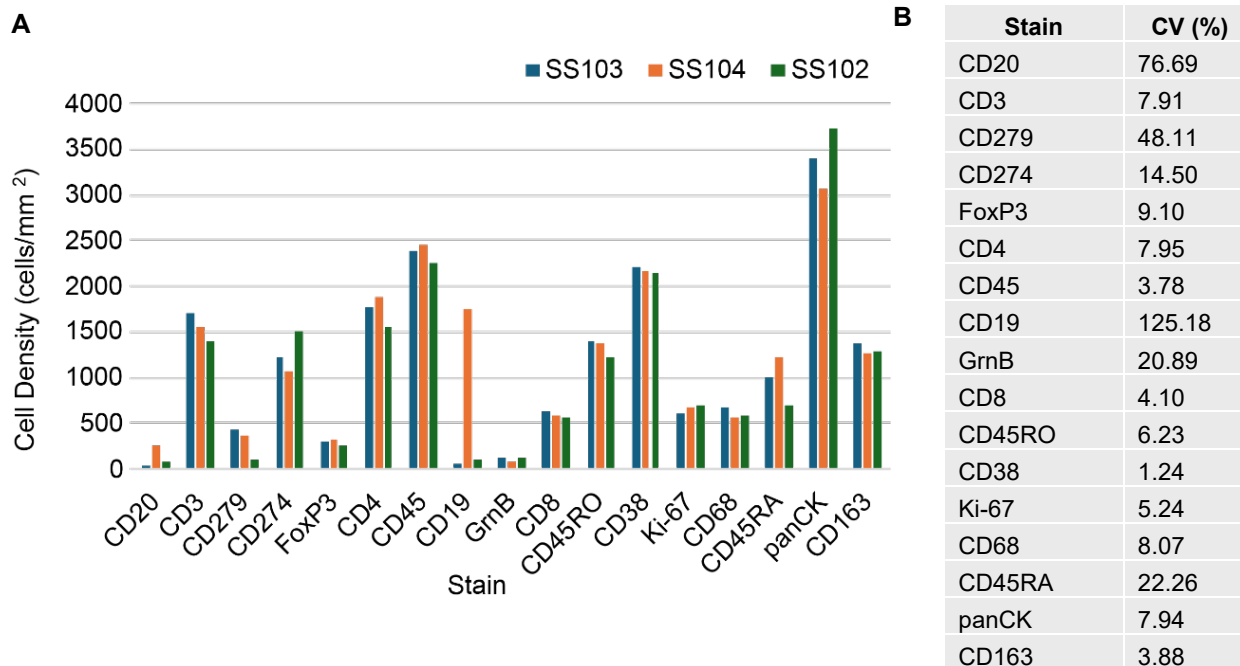


Figure 4. Reproducibility of antibodies in the Spatial Immune Profiling Kit. Cell density measurements for each stain across three technical replicates of human FFPE breast cancer (A) and corresponding CV (B). n = 3 serial sections.

Melanoma

Qualitative Suitability and Specificity Assessment – Scoring

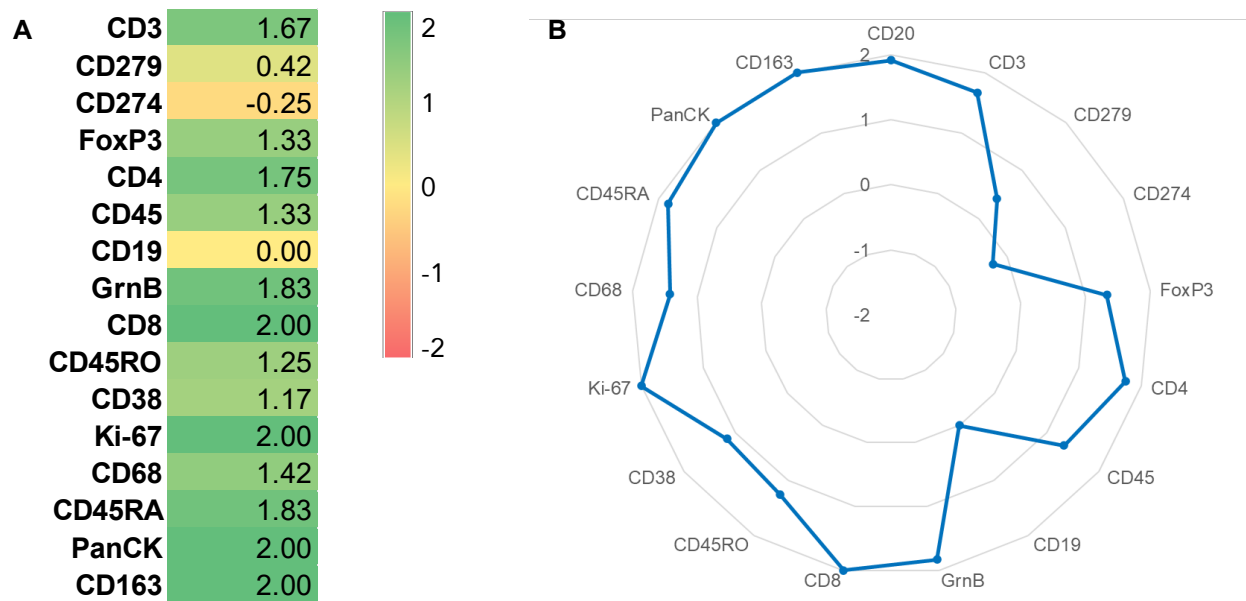


Figure 5. Scoring results of antibodies in the Spatial Immune Profiling Kit. Average scores from technical replicates of human FFPE melanoma are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 3 samples scored by four independent judges.

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

Table 5. SNR values for stains in the Spatial Immune Profiling Kit. Average positive and negative intensities and SNR from three technical replicates of human FFPE melanoma.

	Method 1			Method 2		
	Mean +	Mean -	SNR	Mean +	Mean -	SNR
CD20	29.53	11.87	2.49	17467.75	34.21	510.66
CD3	580.77	75.55	7.69	1402.61	48.60	28.86
CD279	15.70	3.21	4.89	148.07	4.90	30.22
CD274	70.22	23.36	3.01	369.73	18.61	19.87
FoxP3	343.95	32.10	10.71	1171.98	24.40	48.03
CD4	321.17	58.75	5.47	842.52	32.23	26.14
CD45	1254.08	214.28	5.85	4771.24	31.99	149.15
CD19	69.81	25.15	2.78	485.17	19.75	24.57
GrnB	798.12	9.27	86.11	1570.19	5.14	305.48
CD8	318.64	12.68	25.13	585.39	7.88	74.29
CD45RO	382.04	48.46	7.88	1332.16	24.90	53.50
CD38	525.41	86.42	6.08	991.66	40.48	24.50
Ki-67	2145.35	38.21	56.14	6616.17	13.93	474.96
CD68	999.97	109.99	9.09	3183.34	52.31	60.86
CD45RA	347.28	34.45	10.08	1312.89	8.92	147.18
panCK	1471.29	72.40	20.32	3412.15	15.91	214.47
CD163	262.36	40.43	6.49	1243.50	26.10	47.64

Quantitative Reproducibility Assessment

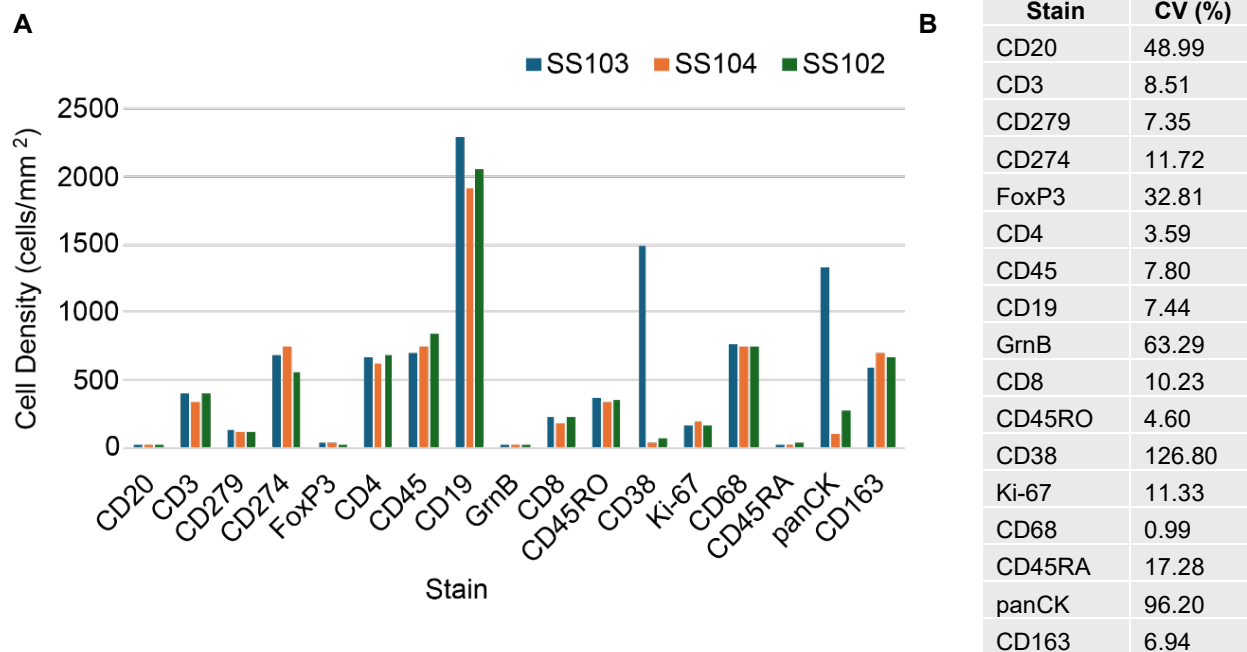


Figure 6. Reproducibility of antibodies in the Spatial Immune Profiling Kit. Cell density measurements for each stain across three technical replicates of human FFPE melanoma (A) and corresponding CV (B). n = 3 serial sections.

Colon Cancer

Qualitative Suitability and Specificity Assessment – Scoring

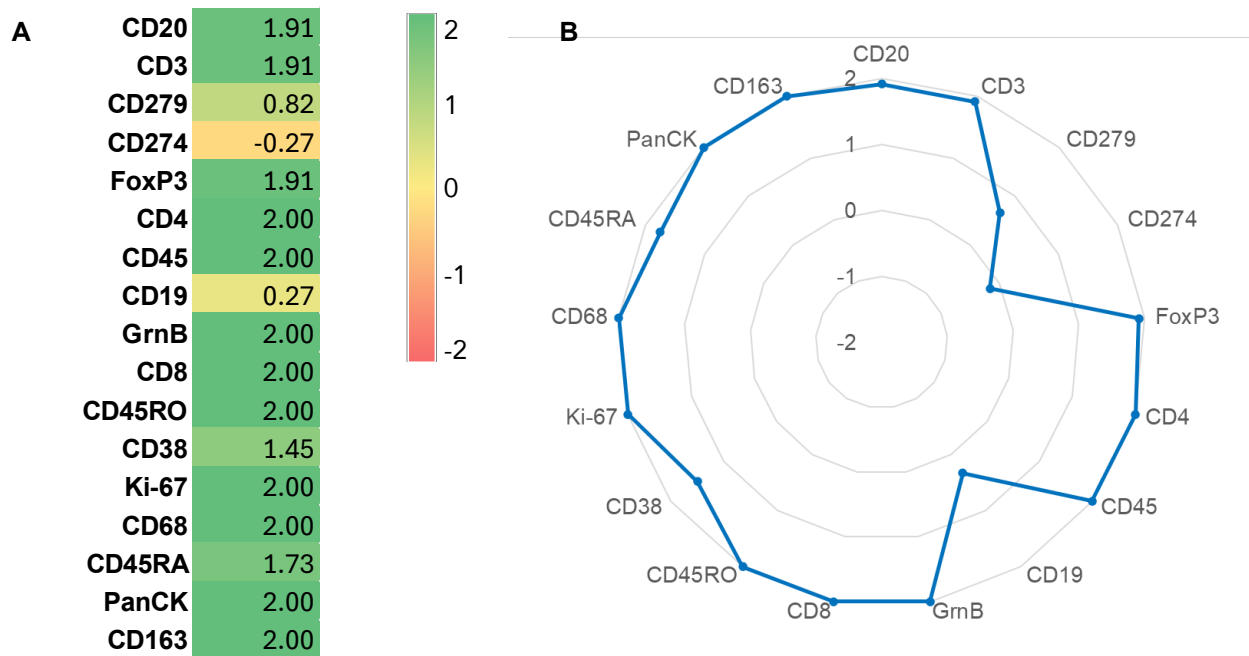


Figure 7. Scoring results of antibodies in the Spatial Immune Profiling Kit. Average scores from technical replicates of human FFPE colon cancer are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 3 samples scored by four independent judges.

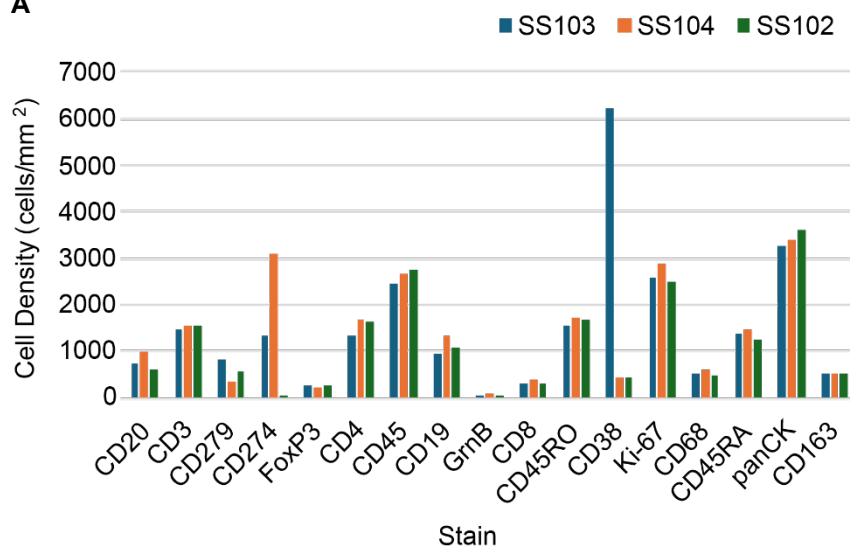
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 6. SNR values for stains in the Spatial Immune Profiling Kit. Average positive and negative intensities and SNR from three technical replicates of human FFPE colon cancer.

	Method 1			Method 2		
	Mean +	Mean -	SNR	Mean +	Mean -	SNR
CD20	743.84	66.82	11.13	17467.75	37.89	461.05
CD3	754.28	75.65	9.97	1402.61	57.59	24.36
CD279	15.09	3.20	4.71	148.07	3.96	37.37
CD274	40.81	12.12	3.37	369.73	12.77	28.96
FoxP3	676.03	33.55	20.15	1171.98	27.60	42.46
CD4	369.02	59.68	6.18	842.52	37.54	22.44
CD45	1399.76	70.43	19.87	4771.24	24.18	197.29
CD19	62.46	22.04	2.83	485.17	24.63	19.70
GrnB	389.15	10.14	38.39	1570.19	5.77	271.93
CD8	316.33	12.50	25.31	585.39	9.82	59.60
CD45RO	413.86	38.72	10.69	1332.16	26.37	50.52
CD38	185.07	37.66	4.91	991.66	24.45	40.56
Ki-67	2283.32	270.61	8.44	6616.17	22.97	288.01
CD68	1080.59	72.85	14.83	3183.34	52.81	60.28
CD45RA	425.79	9.87	43.16	1312.89	5.74	228.86
panCK	552.36	109.07	5.06	3412.15	15.88	214.81
CD163	1054.37	42.82	24.62	1243.50	30.29	41.06

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
CD20	21.60
CD3	3.52
CD279	31.30
CD274	86.27
FoxP3	9.50
CD4	9.38
CD45	4.95
CD19	14.48
GrnB	109.49
CD8	10.38
CD45RO	4.52
CD38	115.38
Ki-67	6.21
CD68	9.40
CD45RA	6.74
panCK	4.46
CD163	1.78

Figure 8. Reproducibility of antibodies in Spatial Immune Profiling Kit. Cell density measurements for each stain across three technical replicates of human FFPE colon cancer (A) and corresponding CV (B). n = 3 serial sections.

Head & Neck Cancer

Qualitative Suitability and Specificity Assessment – Scoring

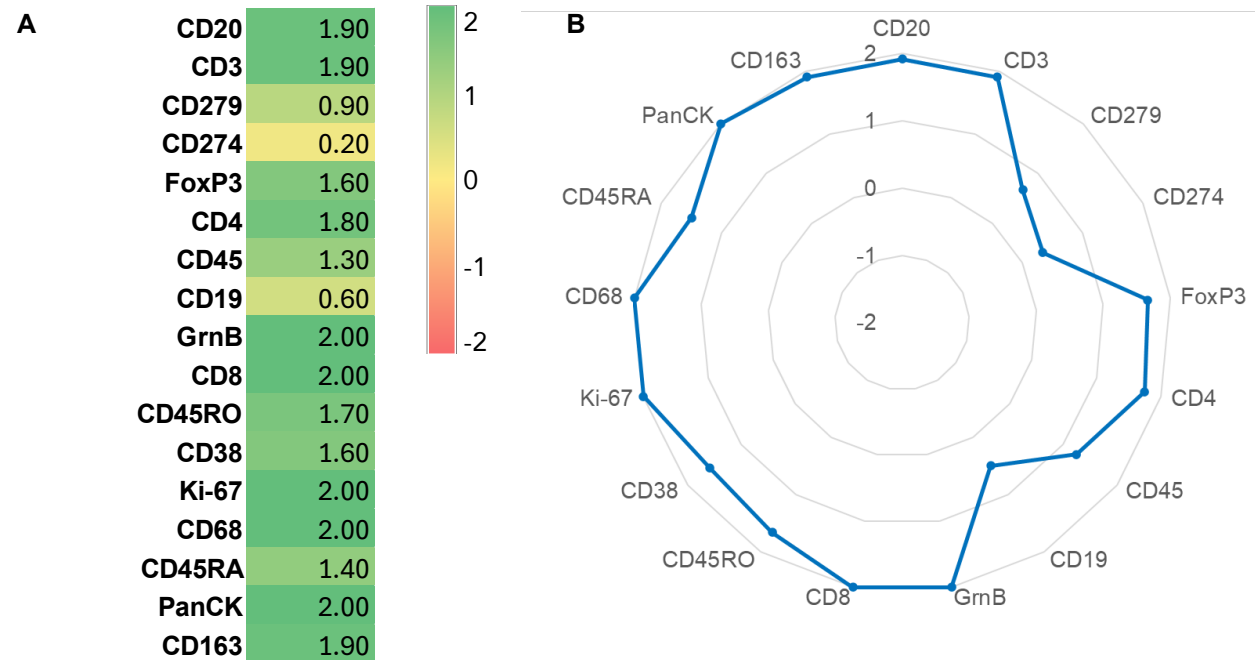


Figure 9. Scoring results of antibodies in the Spatial Immune Profiling Kit. Average scores from technical replicates of human FFPE head & neck cancer are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 3 samples scored by four independent judges.

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

Table 7. SNR values for stains in the Spatial Immune Profiling Kit. Average positive and negative intensities and SNR from three technical replicates of human FFPE head & neck cancer.

	Method 1			Method 2		
	Mean +	Mean -	SNR	Mean +	Mean -	SNR
CD20	121.56	31.83	3.82	17467.75	20.33	859.12
CD3	453.43	43.59	10.40	1402.61	23.40	59.94
CD279	15.91	4.14	3.84	148.07	4.80	30.87
CD274	2300.45	18.29	125.78	369.73	10.33	35.78
FoxP3	746.83	20.22	36.94	1171.98	13.74	85.32
CD4	179.58	44.98	3.99	842.52	22.76	37.02
CD45	344.74	41.91	8.23	4771.24	9.98	478.21
CD19	18.95	6.26	3.03	485.17	6.31	76.86
GrnB	427.61	7.14	59.92	1570.19	5.49	286.21
CD8	310.27	6.28	49.44	585.39	4.25	137.82
CD45RO	205.95	22.99	8.96	1332.16	13.17	101.17
CD38	79.93	24.85	3.22	991.66	17.20	57.67
Ki-67	2534.47	81.44	31.12	6616.17	8.94	739.70
CD68	1354.06	127.31	10.64	3183.34	38.48	82.73
CD45RA	187.18	5.73	32.66	1312.89	2.78	472.13
panCK	758.25	194.19	3.90	3412.15	13.32	256.10
CD163	601.63	44.18	13.62	1243.50	14.46	86.01

Quantitative Reproducibility Assessment

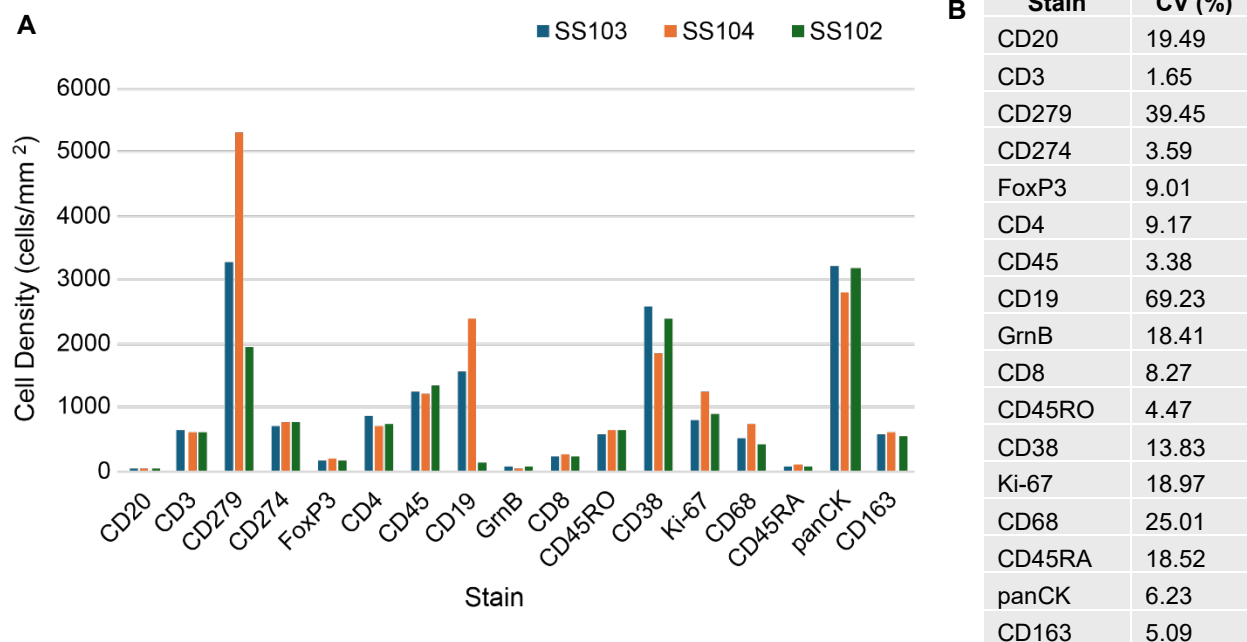


Figure 10. Reproducibility of antibodies in the Spatial Immune Profiling Kit. Cell density measurements for each stain across three technical replicates of human FFPE head & neck cancer (A) and corresponding CV (B). n = 3 serial sections.

Stain Qualification and Specificity Criteria

The following Table describes the areas of interest that were used for evaluating antibody performance in human FFPE tonsil. The [Human Protein Atlas](#) was referenced to determine tissue structure, organization and biomarker expression as needed. 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 11.

Table 8. Localization and specificity assessment criteria used for stains in the Spatial Immune Profiling Kit in human FFPE tonsil.

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

A

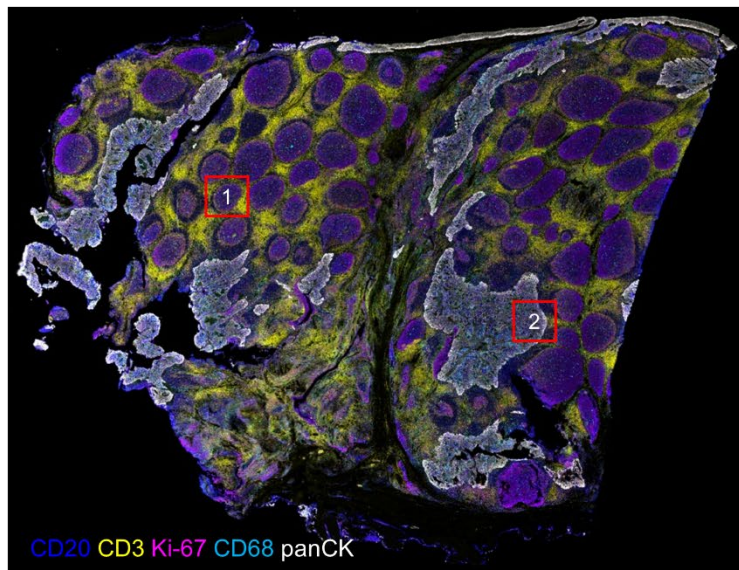
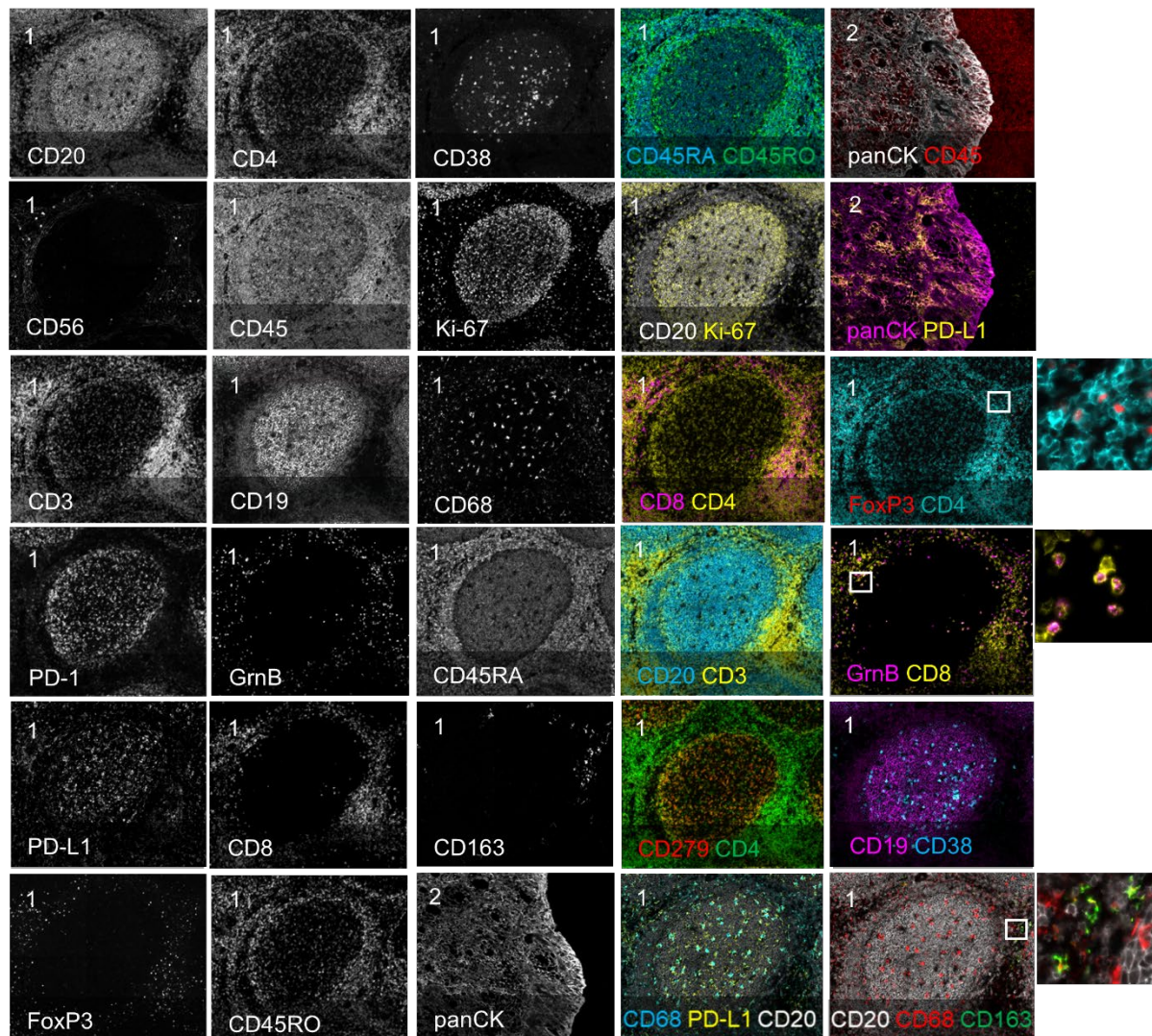


Figure 11. Example images for stains in the Spatial Immune Profiling Kit. A, Full overview of a tonsil sample used in validation testing. The red boxes indicate the germinal center (1) and squamous epithelia (2) regions shown in enlarged images. B, Enlarged images showing individual antibody stains in grayscale and multicolor counterstain examples.

B



Methods

Reagent Preparation

Tissue samples (Table 9) were prepared in Saint Louis, MO, and shipped to additional testing sites in Hannover and Leipzig, Germany. Serial sections of human FFPE tonsil were cut and mounted on Superfrost Plus Gold Slides (Fisher Scientific, 22-037-246) and dried overnight before shipping. Overnight baking, deparaffinization, and antigen retrieval was performed independently at each testing site following the [CellScape User Manual \(MAN-10200-02\)](#).

Table 9. Human tissues used for VistaPlex Kit validation.

Product Code	Description	Vendor
CS-FFPE Tissue Service	Tissue panel – 8 tissue	BioChain
AMS6022	Normal tonsil	AMS Bio

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 10) with 10 seconds of enhanced photobleaching before each cycle. Signal removal between cycles was facilitated by EpicIF™ Buffer (Bruker Spatial Biology, PRSM-BUF-EPIC-500mL).

Table 10. Staining plan.

Cycle	Target	Dilution	Stain Time (min)
1	CD20	1:500	60
	CD3	1:100	
2	CD279	1:50	60
	CD274	1:100	
	FoxP3	1:250	
3	CD4	1:100	60
	CD45	1:50	
	CD19	1:50	
4	GrnB	1:100	60
	CD8	1:100	
	CD45RO	1:250	
5	CD38	1:100	60
	Ki-67	1:100	
	CD68	1:500	
6	CD45RA	1:100	60
	PanCK	1:500	
	CD163	1:100	

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 11. 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 11. 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. In tonsil, three regions were selected comprising one of the primary organ structures: germinal center, interfollicular region and squamous epithelia. In tumor tissues, one representative region per tissue was selected based on the inclusion of all markers present on the sample. 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 B2M and 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 -").

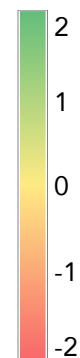
For reproducibility, cells were classified as positive or negative based on OTSU thresholding applied to average cell expression. The number of positive cells was quantified per unit area, expressed as cells/mm². The CV was calculated as the ratio of standard deviation to the mean expressed as a percent.

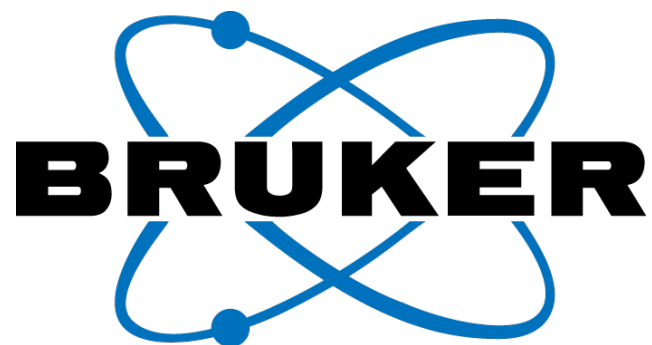
Supplemental Data

Table 12. Suitability scores from additional testing of the Spatial Immune Profiling Kit. The Spatial Immune Profiling Kit was utilized for other projects outside of the validation process. Suitability scores were obtained from the individual(s) overseeing each project. Scoring of cancer tissues (Tumor) and non-cancer tissues (Other) were grouped and averaged. Average scores of some common tumor types are also shown independently.

	Tumor	Other	Breast	Colon	Head&Neck	Prostate	Lung	Skin	Pancreas
CD20	1.90	2.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00
CD3	1.90	2.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00
CD279	0.90	1.50	1.00	1.00	1.00	1.00	1.00	0.50	1.00
CD274	0.78	0.50	2.00	1.00	1.00	0.00	0.00	0.50	0.00
FoxP3	1.90	1.83	2.00	2.00	2.00	1.00	2.00	2.00	2.00
CD4	1.90	1.33	2.00	2.00	2.00	2.00	1.00	2.00	2.00
CD45	1.60	1.33	2.00	2.00	2.00	2.00	1.00	1.50	1.00
CD19	0.90	0.50	1.50	1.00	1.00	1.00	1.00	0.00	0.00
GrnB	1.40	-0.33	2.00	2.00	2.00	2.00	-2.00	1.50	1.00
CD8	1.50	2.00	2.00	2.00	2.00	1.00	0.00	1.50	1.00
CD45RO	1.80	2.00	2.00	2.00	2.00	2.00	1.00	1.50	2.00
CD38	1.30	2.00	2.00	2.00	2.00	1.00	1.00	1.00	0.00
Ki-67	1.70	0.67	2.00	2.00	2.00	2.00	0.00	2.00	1.00
CD68	1.90	2.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00
CD45RA	1.70	2.00	2.00	2.00	2.00	2.00	1.00	1.50	1.00
PanCK	1.80	2.00	2.00	2.00	2.00	2.00	2.00	1.00	2.00
CD163	1.80	2.00	2.00	2.00	2.00	1.00	2.00	2.00	2.00

Sample type	Number of samples
Tumors	18
Other	7
Breast	10
Colon	1
Head & Neck	1
Prostate	1
Lung	1
Skin	2
Pancreas	1
CNS DLBCL	1





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