VALIDATION REPORT

VistaPlex™ Mouse FFPE Spatial Immune Profiling Assay Kit

For the CellScape™ Precise Spatial Proteomics platform

Product 531-125000004

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 Mouse Formaldehyde Fixed, Paraffin-Embedded (FFPE) Spatial Immune Profiling Kit to demonstrate the specificity, sensitivity, and reproducibility of the kit. Kit validation is based on experiments performed on mouse FFPE spleen 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 (spleen) or 1.0 (other tissues)

Fail: Average score < 1.5 (spleen) 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 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 Mouse FFPE Spatial Immune Profiling Assay Kit. Data were obtained from mouse FFPE spleen.

Antibody/Stain	Specificity	Sensitivity	Reproducibility
B220	Pass	Pass	Low Variability
CD138	Pass	Pass	Low Variability
CD279/PD-1	Pass	Pass	Medium Variability
CD3	Pass	Pass	Low Variability
CD4	Pass	Pass	Low Variability
CD44	Pass	Pass	Low Variability
CD45	Pass	Pass	Low Variability
CD62L	Pass	Pass	Low Variability
F4/80	Pass	Pass	Medium Variability
FoxP3	Pass	Pass	Low Variability
Ki67	Pass	Pass	Low Variability
Ly6C	Pass	Pass	Low Variability
NK1.1	Pass	Pass	Low Variability
panCK	Pass	Pass	Low Variability

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

Tissue	Suitability
Spleen	Pass
Lymph Node	Pass
Colon	Variable

Validation Data

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

- Spleen
- Lymph Node
- Colon

Spleen

Qualitative Suitability and Specificity Assessment – Scoring

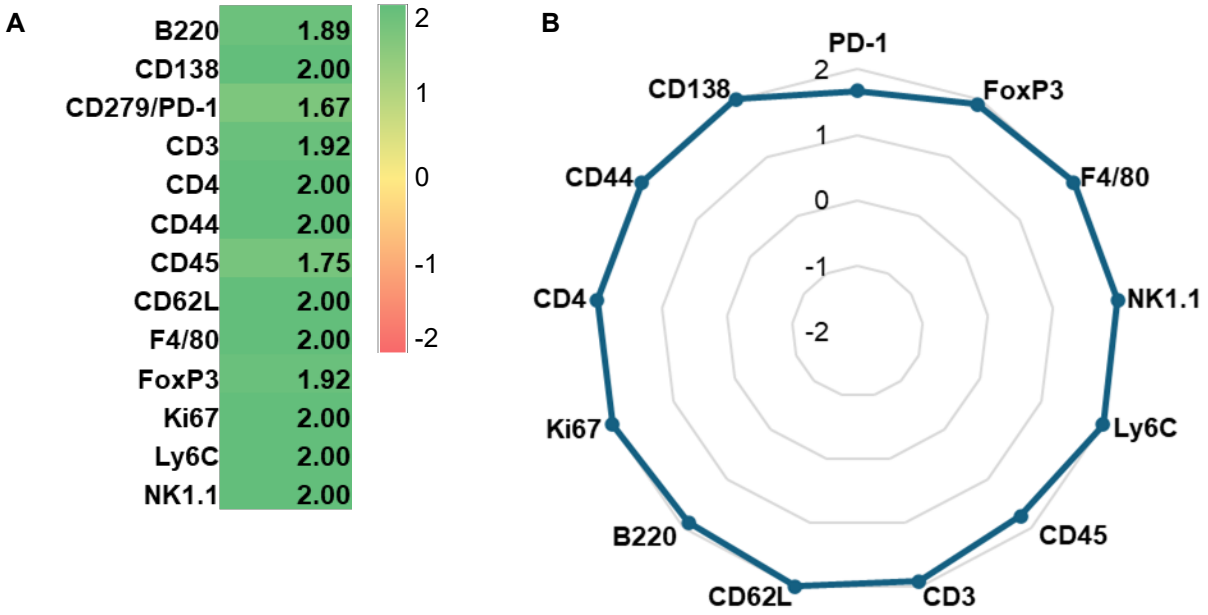


Figure 1. Scoring results of antibodies in the Mouse FFPE Spatial Immune Profiling Assay Kit. Average scores from technical replicates of mouse FFPE spleen are visualized in a heatmap (A) and a radar plot (B). n = 4 samples scored by three independent judges. PanCK data are excluded since this target is not expressed in spleen.

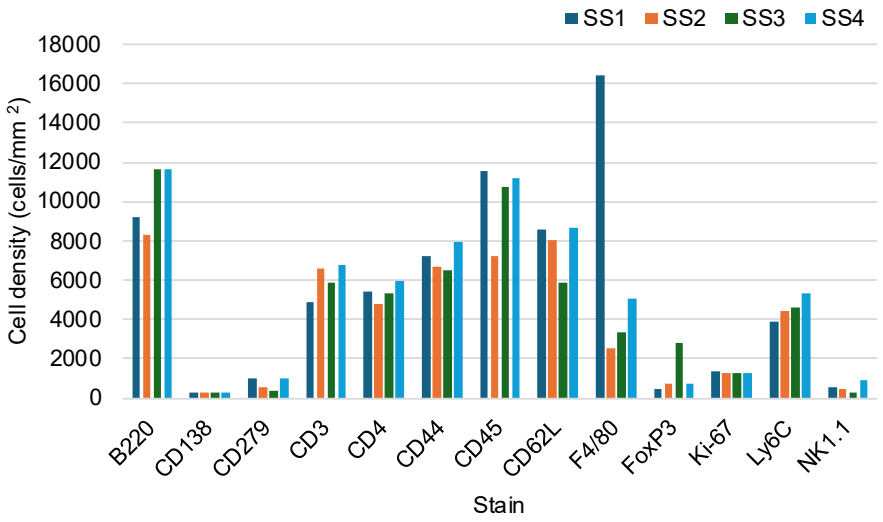
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

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

	Method 1			Method 2		
	Mean +	**Mean -**	**SNR**	**Mean +**	**Mean -**	**SNR**
B220	372.69	87.65	4.25	611.92	16.87	36.27
CD138	2091.06	27.34	76.50	2064.30	0.20	10392.47
CD279/PD-1	2411.21	27.75	86.89	421.42	3.38	124.51
CD3	629.22	102.09	6.16	1147.26	2.70	424.86
CD4	752.78	98.53	7.64	1347.07	1.54	873.26
CD44	3287.74	1181.04	2.78	5037.80	359.78	14.00
CD45	250.56	85.00	2.95	348.78	18.05	19.32
CD62L	522.79	212.29	2.46	873.32	26.75	32.65
F4/80	1115.61	355.60	3.14	1482.74	124.84	11.88
FoxP3	386.03	10.56	36.56	444.56	0.45	978.43
Ki-67	3371.01	180.21	18.71	5366.01	3.65	1469.91
Ly6C	617.54	121.36	5.09	1167.50	9.27	125.91
NK1.1	1162.14	49.94	23.27	1240.67	6.27	197.83

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
B220	14.61
CD138	10.08
CD279/PD-1	34.88
CD3	12.1
CD4	8.46
CD44	8.24
CD45	17.56
CD62L	11.7
F4/80	25.45
FoxP3	17.45
Ki-67	5.2
Ly6C	13.33
NK1.1	10.02

Figure 2. Reproducibility of antibodies in the Mouse FFPE Spatial Immune Profiling Assay Kit. Cell density measurements for each stain across technical replicates of mouse FFPE spleen (A) and corresponding CV (B). n = 4 serial sections.

Lymph Node

Qualitative Suitability and Specificity Assessment – Scoring

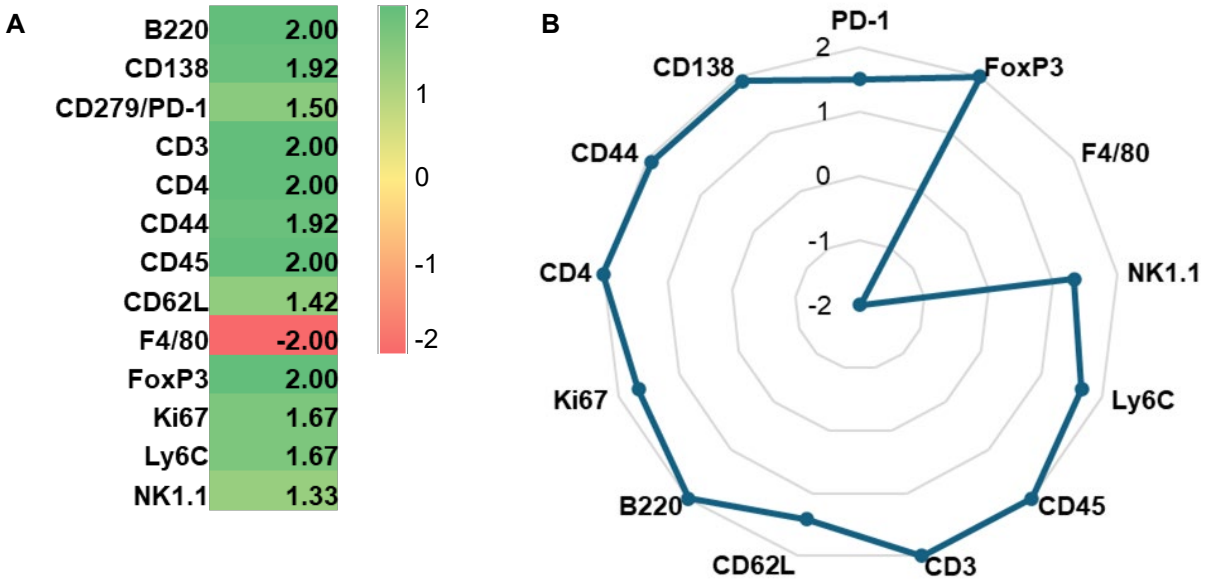


Figure 3. Scoring results of antibodies in the Mouse FFPE Spatial Immune Profiling Assay Kit. Average scores from technical replicates of mouse FFPE lymph node are visualized in a heatmap (A) and a radar plot (B). n = 4 samples scored by three independent judges. PanCK data are excluded since this target is not expressed in lymph node.

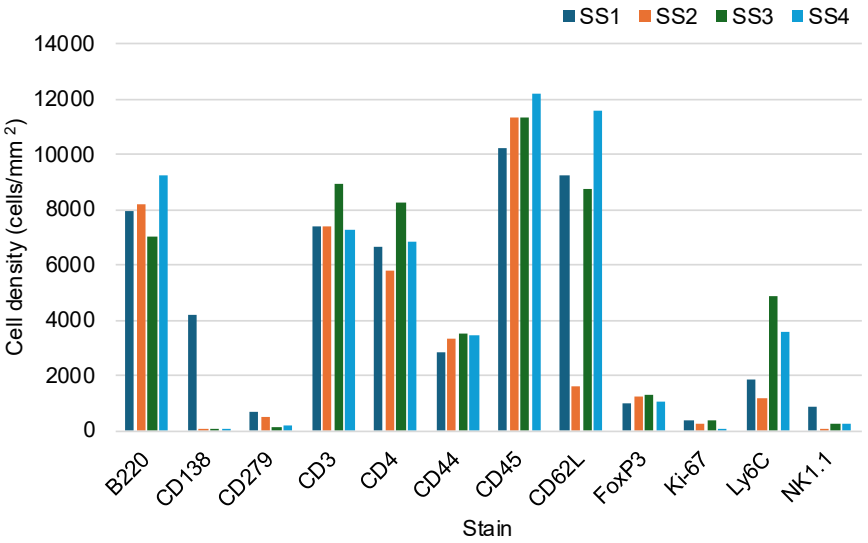
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 4. SNR values for stains in the Mouse FFPE Spatial Immune Profiling Assay Kit. Average positive and negative signal intensities and SNR from three technical replicates of mouse FFPE lymph node.

	Method 1			Method 2		
	Mean +	**Mean -**	**SNR**	**Mean +**	**Mean -**	**SNR**
B220	696.79	177.73	3.92	1110.11	52.94	20.97
CD138	318.96	2.71	117.50	192.70	0.05	3618.71
CD279/PD-1	517.50	5.68	91.14	193.31	0.32	607.04
CD3	382.62	40.68	9.40	895.85	1.48	606.82
CD4	472.49	56.80	8.32	1077.05	2.53	426.37
CD44	1886.27	192.32	9.81	3604.47	1.79	2011.47
CD45	220.06	86.60	2.54	346.32	15.04	23.03
CD62L	168.03	59.33	2.83	265.49	7.47	35.52
F4/80	81.00	19.07	4.25	133.24	3.09	43.08
FoxP3	180.08	7.03	25.61	258.27	0.63	407.22
Ki-67	5889.32	37.14	158.58	3447.77	0.03	131423.07
Ly6C	254.63	17.02	14.96	502.69	0.51	993.95
NK1.1	161.03	1.85	87.14	59.67	0.37	161.38

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
B220	9.88
CD138	167.25
CD279	62.77
CD3	8.67
CD4	12.91
CD44	8.62
CD45	6.21
CD62L	47.69
FoxP3	10.13
Ki-67	55.24
Ly6C	50.05
NK1.1	92.03

Figure 4. Reproducibility of antibodies in the Mouse FFPE Spatial Immune Profiling Assay Kit. Cell density measurements for each stain across technical replicates of mouse FFPE lymph node (A) and corresponding CV (B). n = 4 serial sections.

Colon

Qualitative Suitability and Specificity Assessment – Scoring

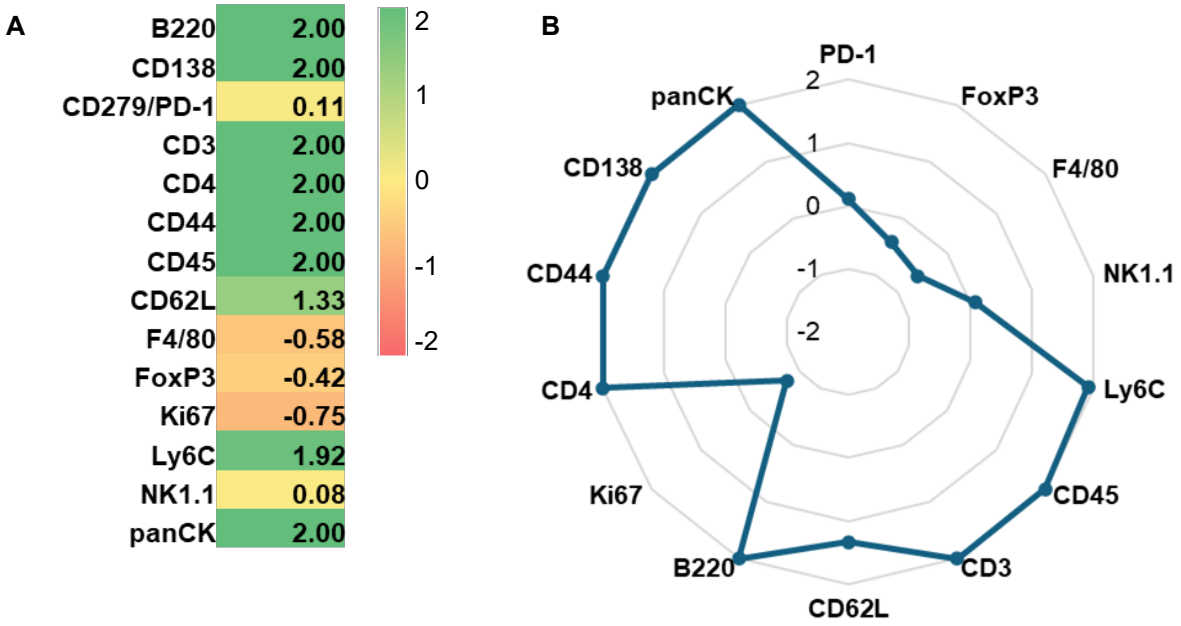


Figure 5. Scoring results of antibodies in the Mouse FFPE Spatial Immune Profiling Assay Kit. Average scores from technical replicates of mouse FFPE colon are visualized in a heatmap (A) and a radar plot (B). n = 4 samples scored by three independent judges.

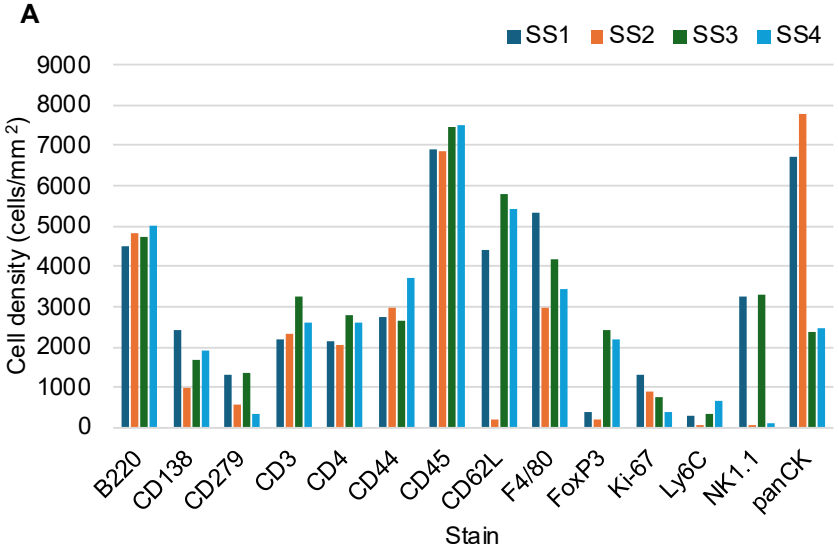
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 5. SNR values for stains in the Mouse FFPE Spatial Immune Profiling Assay Kit. Average positive and negative signal intensities and SNR from three technical replicates of mouse FFPE colon.

	Method 1			Method 2		
	Mean +	**Mean -**	**SNR**	**Mean +**	**Mean -**	**SNR**
B220	553.17	32.28	17.14	1173.99	1.47	801.21
CD138	536.08	31.48	17.03	1045.80	0.13	8011.66
CD279/PD-1	79.30	4.65	17.04	84.36	0.91	92.52
CD3	591.35	35.33	16.74	928.43	0.83	1122.15
CD4	528.66	30.17	17.52	780.23	0.79	987.52
CD44	2073.90	315.79	6.57	6662.76	7.11	937.46
CD45	302.44	24.92	12.14	491.17	0.86	568.29
CD62L	174.71	9.30	18.79	246.60	0.45	542.02
F4/80	146.54	36.99	3.96	269.81	4.63	58.33
FoxP3	67.90	4.82	14.08	104.86	0.99	105.74
Ki-67	556.11	32.46	17.13	1622.60	0.09	19052.73
Ly6C	910.73	12.40	73.46	642.84	0.08	7592.21
NK1.1	179.37	1.94	92.68	33.22	0.42	78.56
panCK	316.92	29.98	10.57	556.19	1.25	446.05

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
B220	3.71
CD138	30.12
CD279	51.00
CD3	15.59
CD4	13.11
CD44	13.67
CD45	4.19
CD62L	56.53
F4/80	22.29
FoxP3	79.08
Ki-67	39.28
Ly6C	64.81
NK1.1	97.12
panCK	50.76

Figure 6. Reproducibility of antibodies in the Mouse FFPE Spatial Immune Profiling Assay Kit. Cell density measurements for each stain across technical replicates of mouse FFPE colon (A) and corresponding CV (B). n = 4 serial sections.

Stain Qualification and Specificity Criteria

The following Table describes the areas of interest that were used for evaluating antibody performance in mouse FFPE spleen. 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 Mouse FFPE Spatial Immune Profiling Kit in mouse FFPE spleen.

Stain	Tissue Localization	Intracellular Localization	Positive Counterstain	Negative Counterstain
B220	Germinal center	Plasma membrane	CD45	CD3
CD138	All regions (plasma cells in medullary cords, marginal zones, red pulp)	Plasma membrane	CD45	CD3
CD279/ PD-1	T cell zone (germinal centers, chronically activated T cells)	T cell zone (germinal centers, chronically activated T cells)	T cell zone (germinal centers, chronically activated T cells)	T cell zone (germinal centers, chronically activated T cells)
CD3	T cell zone (white pulp)	Plasma membrane	CD3	F4/80
CD4	T cell zone(white pulp/paracortex)	Plasma membrane	CD3	B220
CD44	All regions	Plasma membrane	CD45	panCK
CD45	All hematopoietic regions	Plasma membrane	All immune cell markers	panCK
CD62L	All regions (T cell zones, blood vessels)	Plasma membrane	CD45	F4/80
F4/80	Red pulp, marginal zone, medullary regions	Plasma membrane	CD45	CD3, B220
FoxP3	cell zone (white pulp)	Nucleus	CD4	F4/80
Ki-67	All regions, proliferating areas	Nucleus	CD45	n/a
Ly6C	All regions	Plasma membrane	CD45	B220, F4/80
NK1.1	All regions (interfollicular areas, T cell zones)	Plasma membrane	CD45	B220, F4/80
panCK	Epithelial regions, tumor regions	Intracellular, plasma membrane	PD-L1	CD45

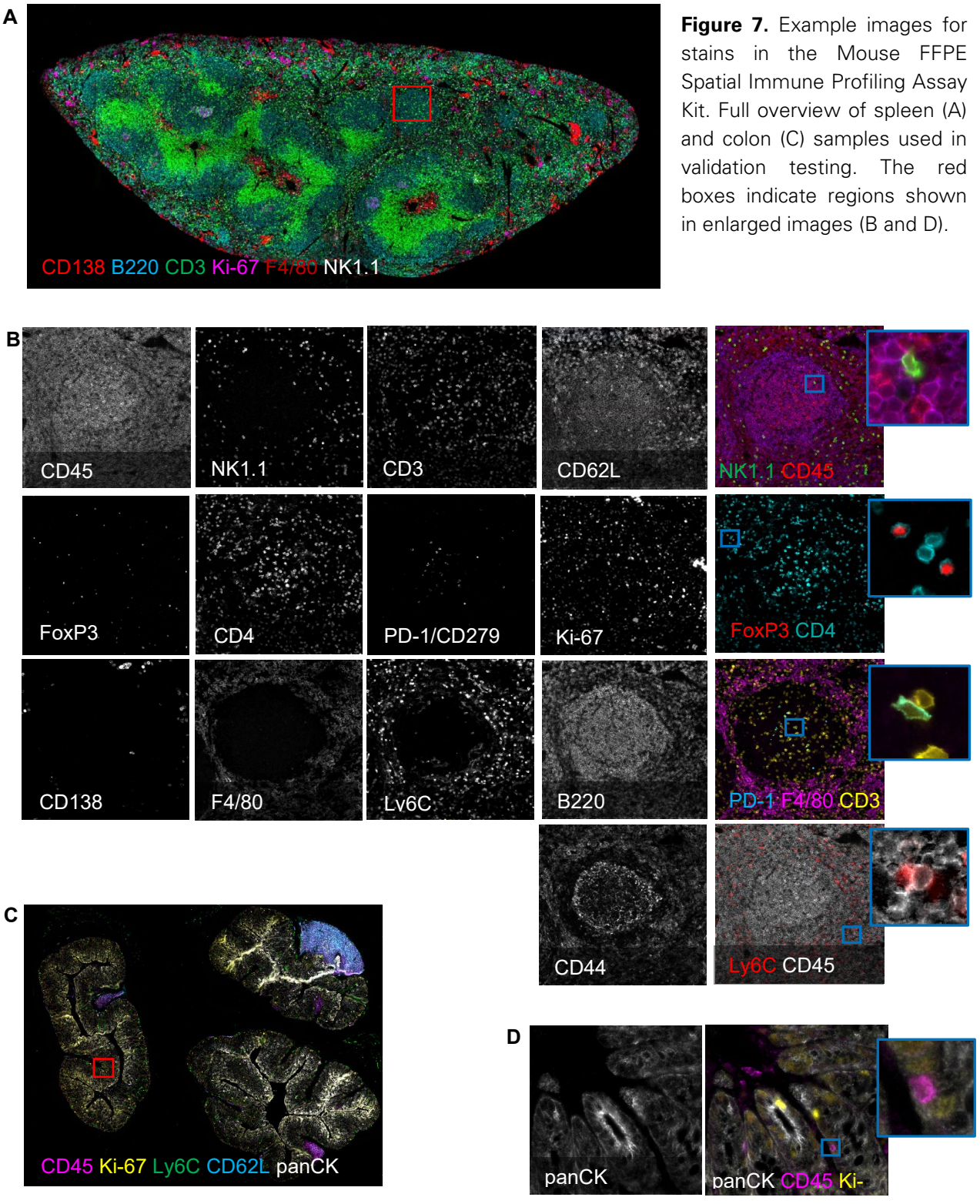


Figure 7. Example images for stains in the Mouse FFPE Spatial Immune Profiling Assay Kit. Full overview of spleen (A) and colon (C) samples used in validation testing. The red boxes indicate regions shown in enlarged images (B and D).

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 mouse FFPE spleen 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 Sample Preparation and Instrument Operation Manual (MAN-10200).

Table 7. Mouse tissues used for VistaPlex Kit validation.

Product Code	Description	Vendor
MZKL594802	Tissue – Spleen	Internal
Custom	Tissue – Colon	BioCat
Custom	Tissue – Lymph Node	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 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	CD279/PD-1	1:60	60
	FoxP3	1:1000	
	F4/80	1:500	
	NK1.1	1:60	
2	CD3	1:1000	60
	CD45	1:100	
	Ly6C	1:500	
3	B220	1:1000	60
	panCK	1:300	
	CD62L	1:500	
4	Ki-67	1:600	60
	CD4	1:1000	
	CD44	1:100	
5	CD138	1:1000	60

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 (spleen) 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.

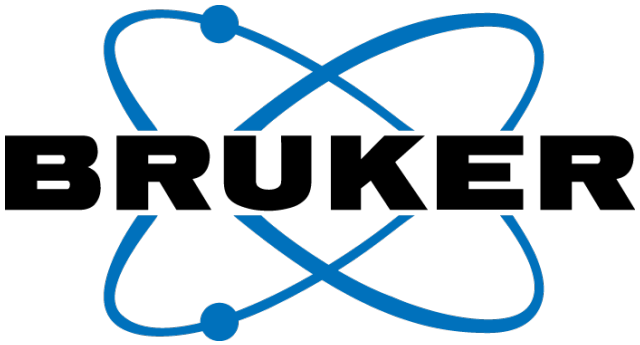
Score	Interpretation
2	Excellent, bright, specific stain
1	Acceptable but dim or high background
0	No staining
-1	Moderate, not abundant off target staining
-2	Strong and/or abundant unspecific staining

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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