



VALIDATION REPORT

VistaPlex™ Mouse FF Spatial Immune Profiling Assay Kit

For the CellScape™ Precise Spatial Proteomics platform

Product 531-125000005

Contents

Purpose	1
Validation Metrics and Pass/Fail Criteria	2
Validation Summary	3
Validation Data	3
Spleen	4
Lymph Node.....	6
Colon	8
Stain Qualification and Specificity Criteria.....	10
Methods.....	12

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 Fresh Frozen (FF) Spatial Immune Profiling Assay Kit to demonstrate specificity, sensitivity, and reproducibility of the kit. Kit validation is based on experiments performed on murine FF 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 FF Spatial Immune Profiling Assay Kit. Data were obtained from murine FF spleen.

Antibody/Stain	Specificity	Sensitivity	Reproducibility
B220	Pass	Pass	Low Variability
CD127	Pass	Pass	Low Variability
CD138	Pass	Pass	Low Variability
CD274/PD-L1	Pass	Pass	Low Variability
CD279/PD-1	Pass	Pass	Medium Variability
CD335	Pass	Pass	Low Variability
CD3e	Pass	Pass	Low Variability
CD4	Pass	Pass	Low Variability
CD44	Pass	Pass	Low Variability
CD45	Pass	Pass	Low Variability
CD62L	Pass	Pass	Low Variability
CD8a	Pass	Pass	Low Variability
F4/80	Pass	Pass	Low Variability
FoxP3	Pass	Pass	Low Variability
Ly6C	Pass	Pass	High Variability
panCK	Pass	Pass	Low Variability

Table 2. Results summary for suitability of the Mouse FF 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

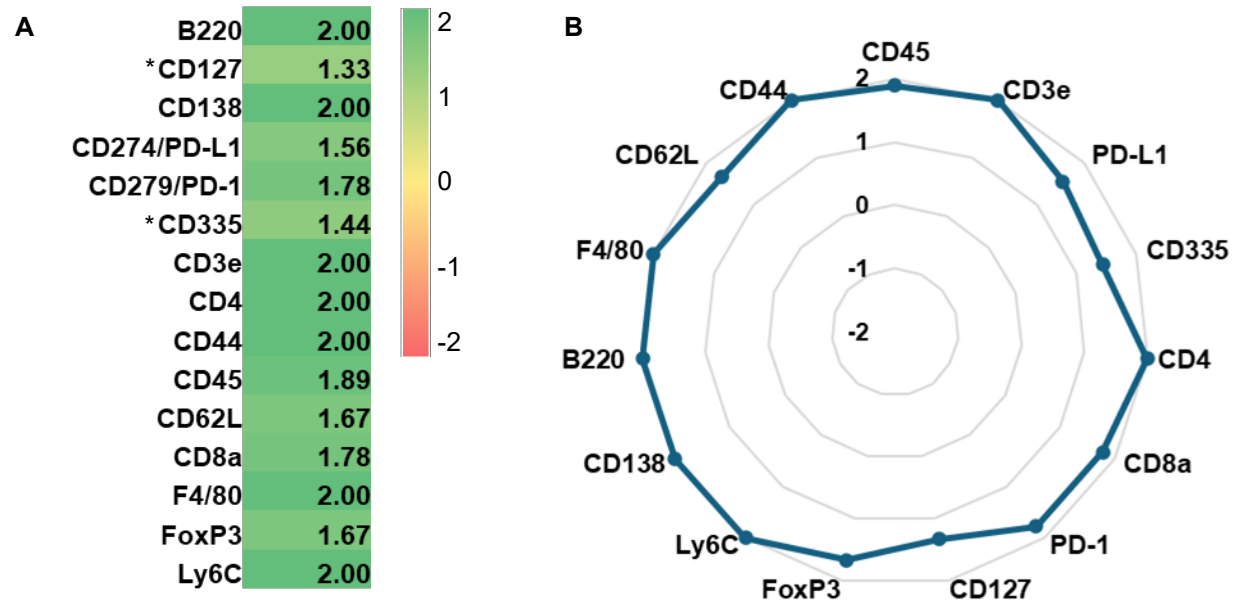


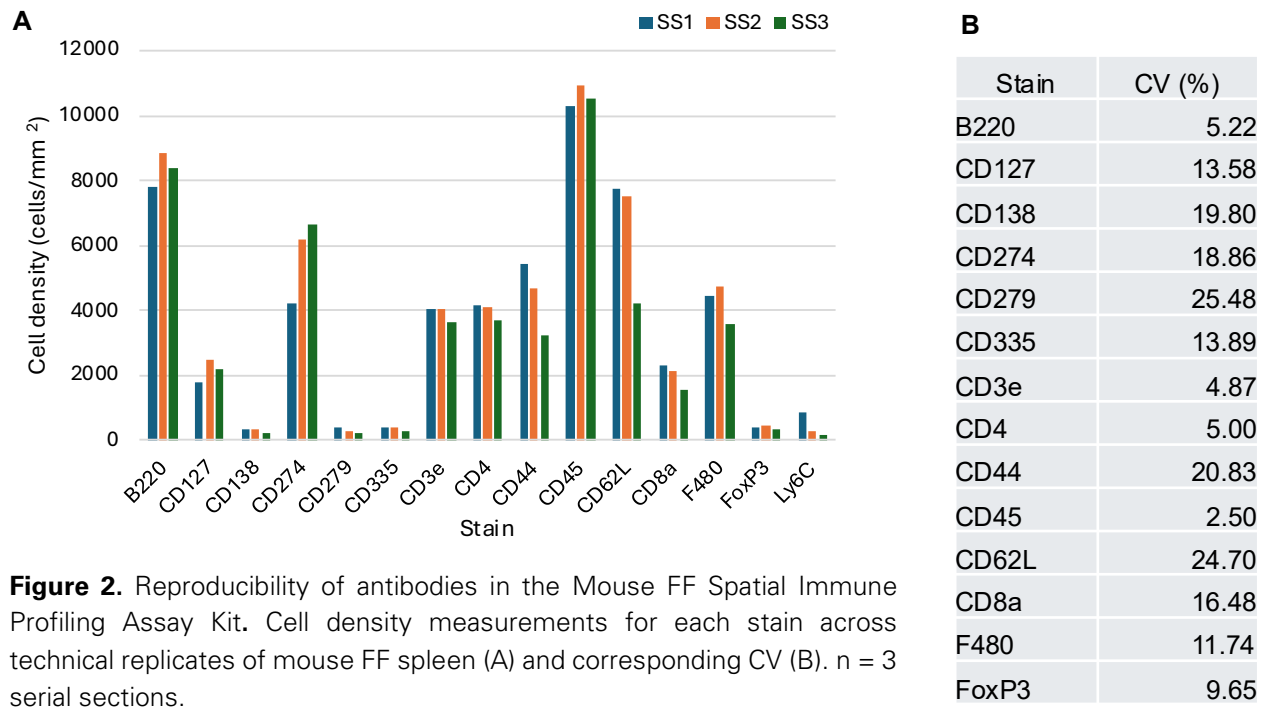
Figure 1. Scoring results of antibodies in the Mouse FF Spatial Immune Profiling Assay Kit. Average scores from technical replicates of mouse FF spleen are visualized in a heatmap (A) and a radar plot (B). $n = 3$ samples scored by three independent judges. PanCK data are excluded since this target is not expressed in spleen. *While the composite scores for CD127 and CD335 are < 1.5 , analysis of the score distributions revealed that all scores were passing (2: 30%; 1: 70% and 2: 40%; 1: 60% for CD127 and CD335, respectively).

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

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

	Method 1			Method 2		
	Mean +	Mean -	SNR	Mean +	Mean -	SNR
B220	207.96	31.28	6.65	406.26	2.75	147.51
CD127	33.93	2.44	13.90	618.08	0.90	688.44
CD138	180.20	2.88	62.60	461.13	0.95	487.18
CD274/PD-L1	42.15	7.69	5.48	312.77	3.96	78.99
CD279/PD-1	158.96	4.20	37.87	542.68	1.77	305.74
CD335	106.31	3.96	26.85	386.87	1.78	217.29
CD3e	419.52	25.73	16.30	1197.85	1.94	617.84
CD4	768.72	58.11	13.23	1721.27	5.53	311.32
CD44	422.27	71.82	5.88	967.88	34.52	28.04
CD45	127.76	2.94	43.41	249.07	0.87	286.18
CD62L	329.72	7.96	41.43	907.94	1.10	821.79
CD8a	203.22	7.69	26.43	796.92	3.96	201.26
F480	375.34	38.63	9.72	944.28	7.84	120.47
FoxP3	110.96	2.52	44.01	256.84	1.23	208.49
Ly6C	287.30	4.58	62.77	509.51	0.36	1404.73

Quantitative Reproducibility Assessment



Lymph Node

Qualitative Suitability and Specificity Assessment – Scoring

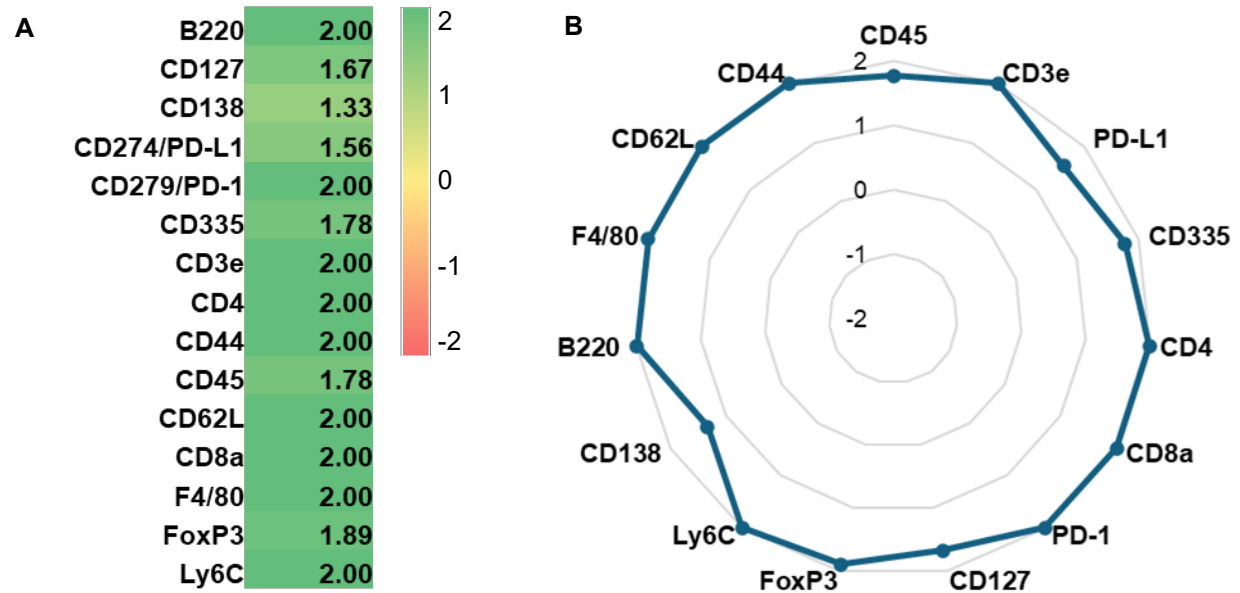


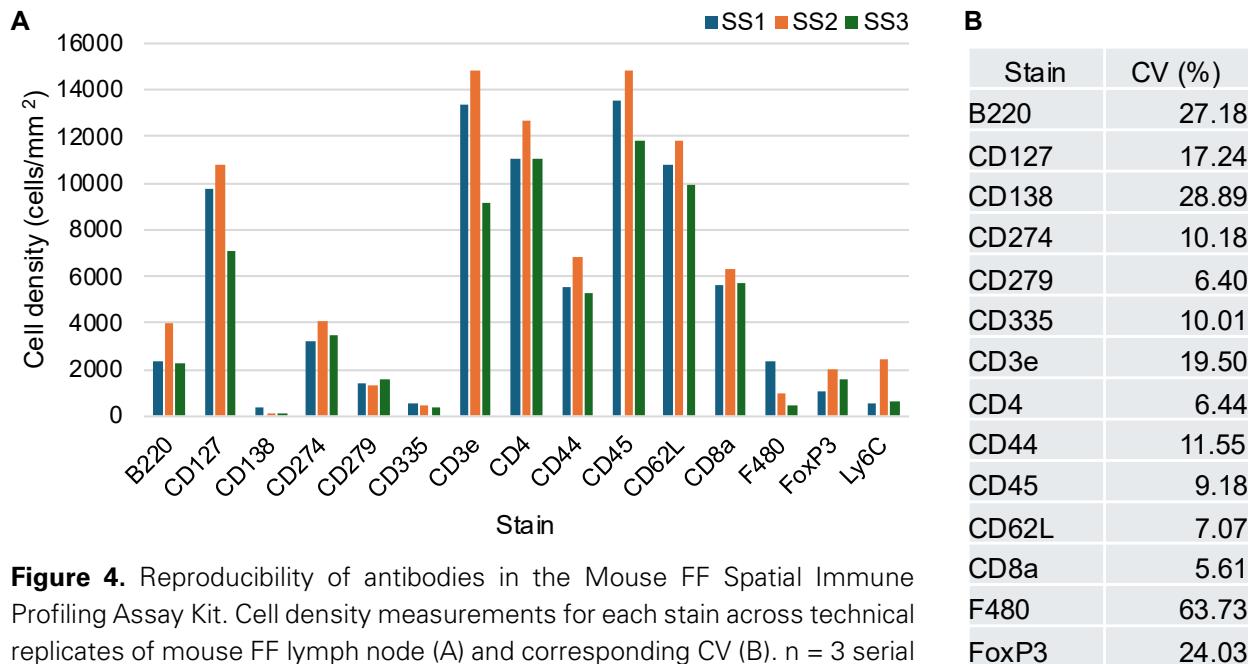
Figure 3. Scoring results of antibodies in the Mouse FF Spatial Immune Profiling Kit. Average scores from technical replicates of mouse FF lymph node are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges. PanCK data are excluded since this target is not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

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

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

	Method 1			Method 2		
	Mean +	Mean -	SNR	Mean +	Mean -	SNR
B220	252.65	18.52	13.64	487.30	1.39	349.59
CD127	92.46	27.43	3.37	180.98	16.63	10.88
CD138	303.47	0.37	825.39	191.64	0.00	90370.50
CD274/PD-L1	167.51	37.00	4.53	438.94	22.55	19.47
CD279/PD-1	441.54	33.25	13.28	993.64	10.16	97.81
CD335	291.97	14.02	20.83	496.61	7.26	68.39
CD3e	881.00	307.99	2.86	1662.70	176.25	9.43
CD4	1451.21	490.99	2.96	2365.17	311.62	7.59
CD44	704.60	191.99	3.67	1393.10	100.55	13.85
CD45	200.34	24.27	8.25	314.11	15.31	20.52
CD62L	542.38	64.31	8.43	907.66	21.08	43.05
CD8a	387.47	37.00	10.47	825.15	22.55	36.60
F480	263.78	48.47	5.44	694.74	31.07	22.36
FoxP3	227.83	12.55	18.15	368.35	3.83	96.15
Ly6C	248.26	8.29	29.94	385.36	1.18	327.61

Quantitative Reproducibility Assessment



Colon

Qualitative Suitability and Specificity Assessment – Scoring

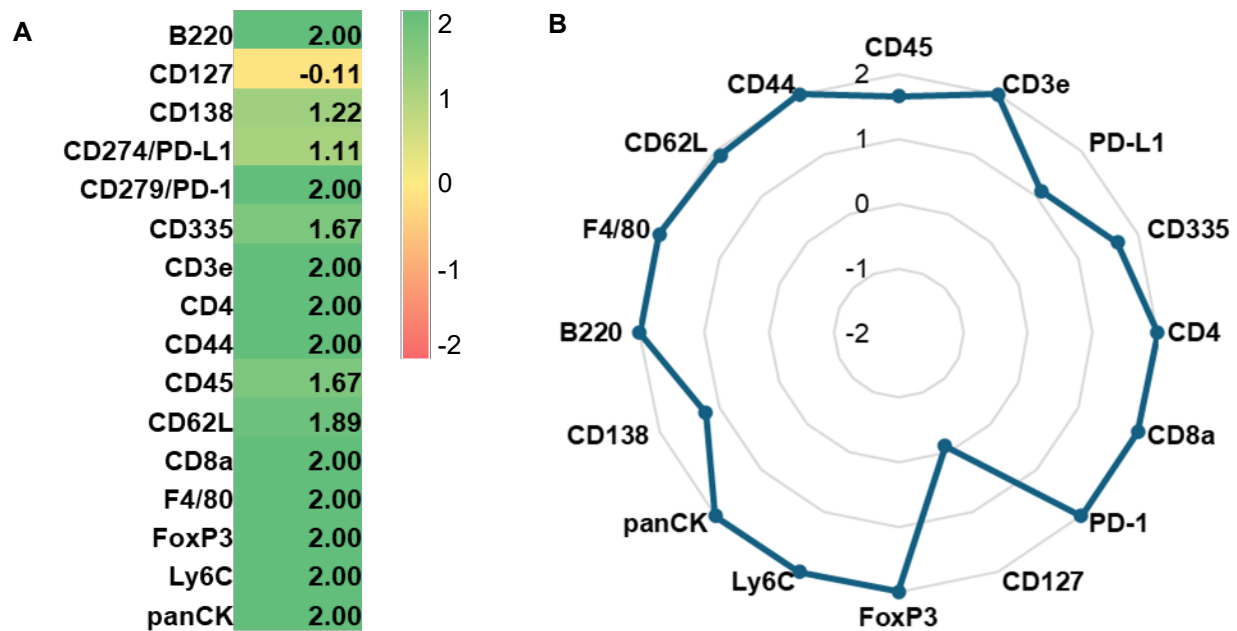


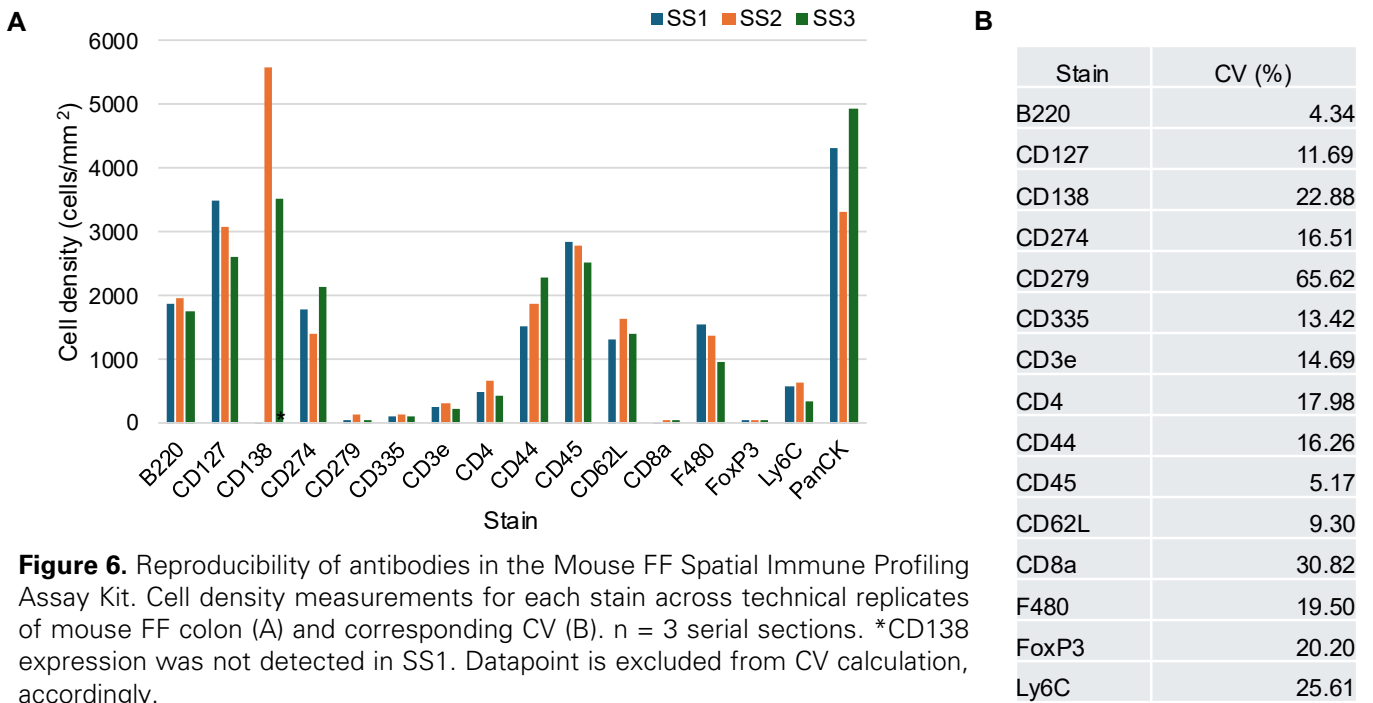
Figure 5. Scoring results of antibodies in the Mouse FF Spatial Immune Profiling Assay Kit. Average scores from technical replicates of mouse FF colon are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges.

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

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

	Method 1			Method 2		
	Mean +	Mean -	SNR	Mean +	Mean -	SNR
B220	233.01	6.04	38.59	426.34	0.79	540.10
CD127	49.76	10.35	4.81	117.98	11.06	10.67
CD138	83.05	10.97	7.57	207.59	1.02	204.13
CD274/PD-L1	72.32	8.74	8.27	156.89	3.67	42.79
CD279/PD-1	633.27	15.28	41.46	396.54	10.35	38.33
CD335	125.52	18.74	6.70	316.44	14.20	22.29
CD3e	439.72	4.00	109.88	567.56	0.16	3445.20
CD4	986.36	20.90	47.20	1439.71	0.38	3742.50
CD44	475.76	87.95	5.41	1508.64	50.86	29.66
CD45	160.73	16.12	9.97	269.74	1.75	154.35
CD62L	306.88	11.72	26.19	468.13	7.37	63.55
CD8a	517.61	8.74	59.19	209.45	3.67	57.12
F480	614.06	85.58	7.18	894.60	57.99	15.43
FoxP3	171.18	1.62	105.70	101.08	1.25	80.75
Ly6C	504.36	6.35	79.41	593.39	0.11	5196.22
panCK	182.59	23.34	7.82	243.20	0.68	356.32

Quantitative Reproducibility Assessment



Stain Qualification and Specificity Criteria

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

Stain	Tissue Localization	Intracellular Localization	Positive counterstain	Negative counterstain
B220	Germinal center	Plasma membrane	CD45	CD3
CD127	T cell zones	Plasma membrane	CD45	
CD138	All regions	Plasma membrane	CD45	CD3
CD274/PD-L1	Immune-responsive cells or activated areas	Plasma membrane	CD45	-
CD279/PD-1	T cell zone	Plasma membrane	CD3	F4/80
CD335	All regions (interfollicular areas, T cell zones)	Plasma membrane	CD45	B220, F4/80
CD3e	T cell zone (white pulp)	Plasma membrane	CD45	B220
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
CD8a	T cell zone (marginal zone/paracortex)	Plasma membrane	CD3	B220
F4/80	Red pulp, marginal zone, medullary regions	Plasma membrane	CD45	CD3, B220
FoxP3	T 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
panCK	Epithelial regions, tumor regions	Intracellular, plasma membrane	PD-L1	CD45

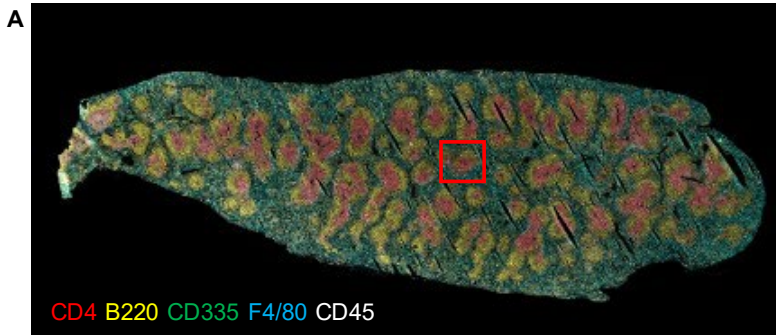
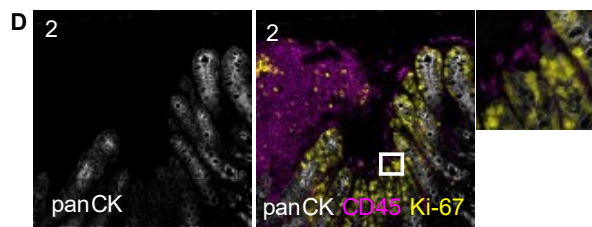
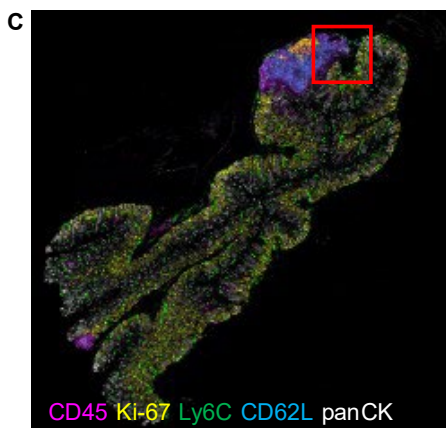
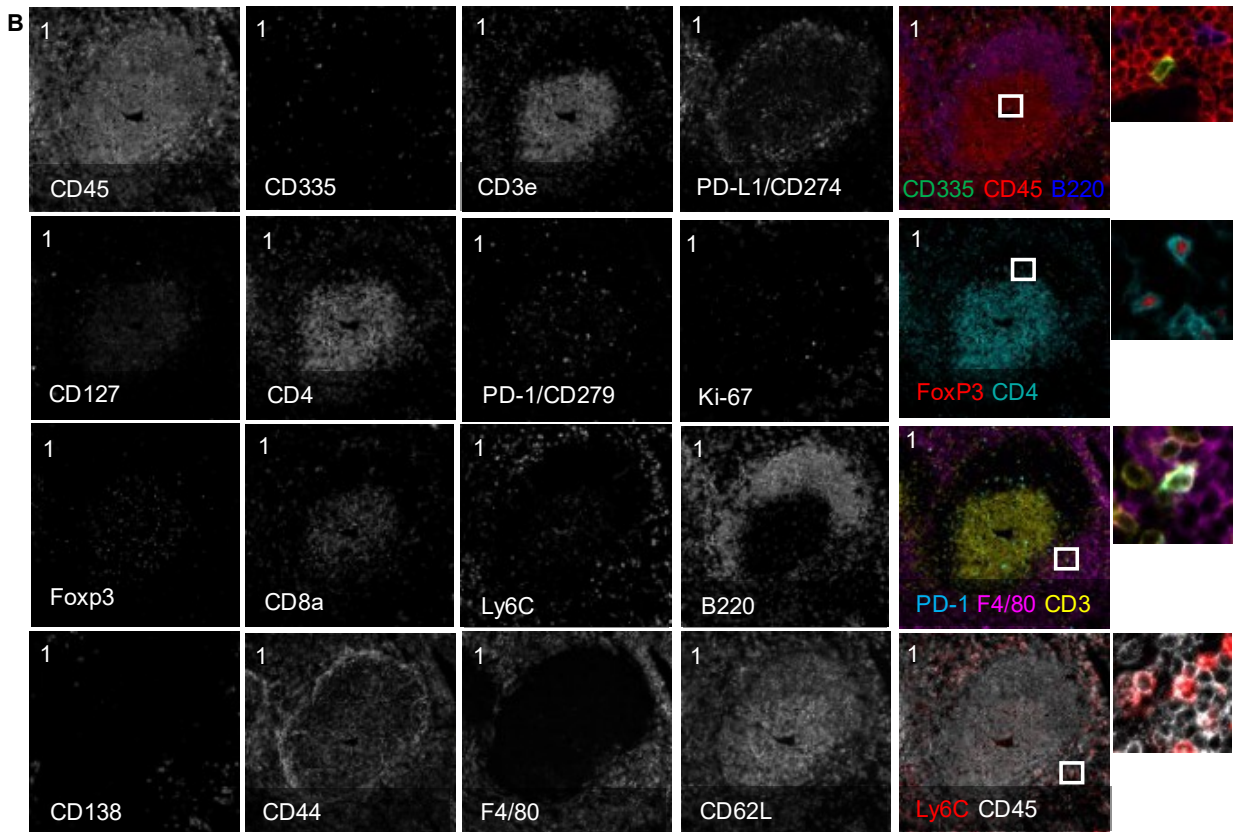


Figure 7. Example images for stains in the Mouse FF Spatial Immune Profiling Assay Kit. Full overview of spleen (A) and colon (C) samples used in validation testing. The red boxes indicate regions shown in enlarged images (B and D).



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 murine FF spleen 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. Murine tissues used for VistaPlex Kit validation.

Product Code	Description	Vendor
TFM0490-Y_1	Tissue – Spleen	BioCat
TFM0130-Y	Tissue – Colon	BioCat
TFM0320-Y	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 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	PD-L1	1:60	15
	CD3e	1:100	
	CD45	1:4000	
	CD335	1:60	
2	CD127	1:100	15
	CD4	1:500	
	PD-1	1:100	
3	Foxp3	1:80	15
	CD8a	1:1000	
	Ly6C	1:15000	
4	B220	1:1000	15
	panCK	1:3000	
	CD138	1:4000	
5	CD44	1:300	15
	F4/80	1:500	
	CD62L	1:200	

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 (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.

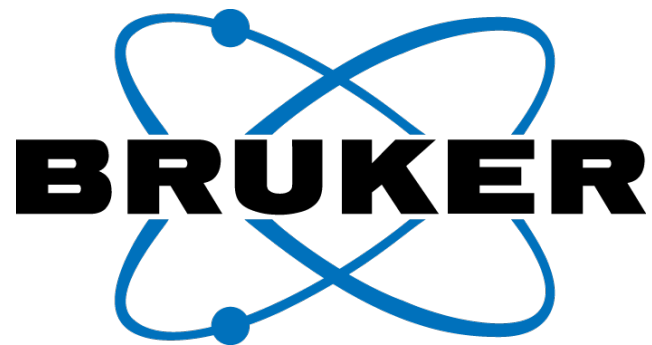
Score	Interpretation
2	Excellent, bright, specific stain
1	Acceptable but dim or high background
0	No staining
-1	Moderate, not abundant off target staining
-2	Strong and/or abundant unspecific staining

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/mm². The CV was calculated as the ratio of standard deviation to the mean expressed as a percent.



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