VALIDATION REPORT

VistaPlex™ Tissue Architecture Kit

For CellScape™ Precise Spatial Proteomics

Validation of the Tissue Architecture for Human FFPE multiplex antibody kit, product VISTAPLEX3103

PMR-11861-01

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 Tissue Architecture 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 Tissue Architecture Kit. Data were obtained from human FFPE tonsil, except for EpCAM, which was assessed in colon as a positive control tissue.

Antibody/Stain	Specificity	Sensitivity	Reproducibility
CD138	Pass	Pass	Low Variability
CD31	Pass	Pass	Low Variability
Col IV	Pass	Pass	Low Variability
SMA	Pass	Pass	High Variability*
B catenin	Pass	Pass	Low Variability
Podoplanin	Pass	Pass	Medium Variability
Vimentin	Pass	Pass	Low Variability
E-cadherin	Pass	Pass	Low Variability
CD34	Pass	Pass	Low Variability
MUC1	Pass	Pass	Low Variability
CD105	Pass	Pass	Low Variability
EpCAM	Pass	Pass	Low Variability

*Low or medium variability was observed in every other tissue type tested. High CV measurements may be due to structural variations rather than differences in antibody performance.

Table 2. Results summary for suitability of the Tissue Architecture 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 Tissue Architecture Kit, organized by tissue type:

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

Tonsil

Qualitative Suitability and Specificity Assessment – Scoring

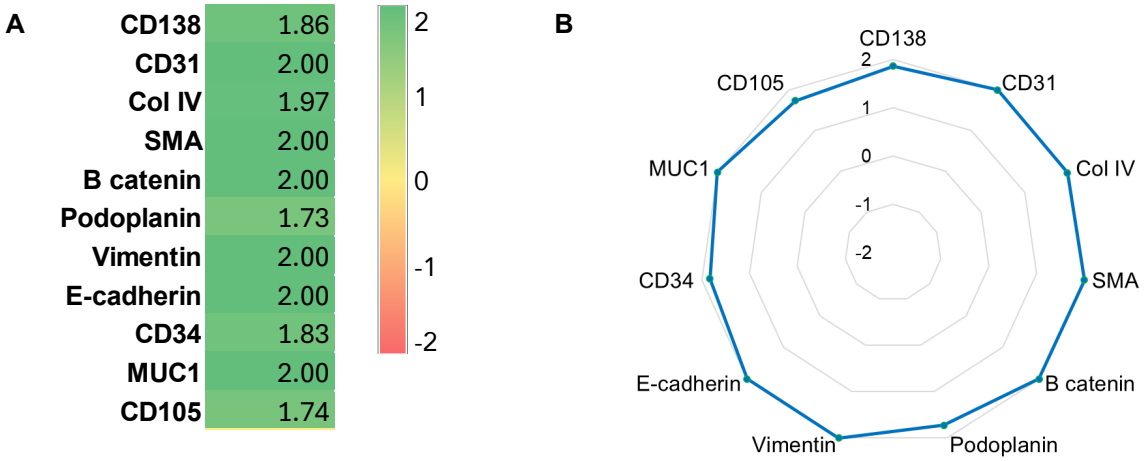


Figure 1. Scoring results of antibodies in the Tissue Architecture Kit. Average scores from technical replicates of human FFPE Tonsil are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 8 samples scored by four independent judges. Note that EpCAM is not included since it is not expressed in tonsil. See colon cancer tissue for positive control.

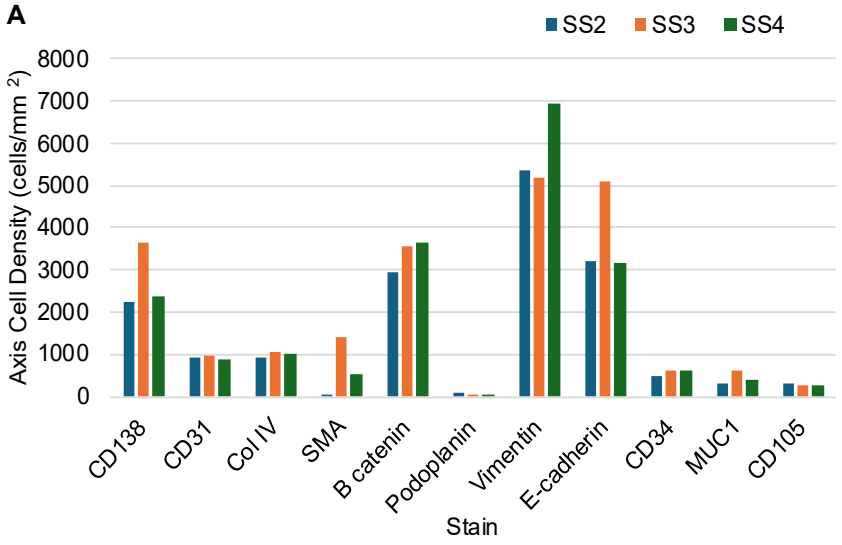
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 3. SNR values for stains in the Tissue Architecture 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**
CD138	521.88	73.41	7.11	930.95	6.29	147.95
CD31	1119.86	49.39	22.67	5926.48	45.23	131.02
Col IV	744.51	65.43	11.38	1778.52	52.52	33.86
SMA	7359.83	344.26	21.38	27255.05	89.90	303.18
B catenin	407.25	46.44	8.77	1066.13	20.24	52.68
Podoplanin	1602.80	31.58	50.76	1192.01	30.28	39.36
Vimentin	1819.74	199.42	9.13	3223.66	159.68	20.19
E-cadherin	1032.71	112.36	9.19	2372.55	15.07	157.43
CD34	204.97	38.48	5.33	1107.40	67.90	16.31
MUC1	7716.90	136.95	56.35	4064.59	36.44	111.53
CD105	322.13	17.24	18.69	773.29	21.49	35.98

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
CD138	22.95
CD31	3.84
Col IV	5.40
SMA	85.73
B catenin	9.30
Podoplanin	42.72
Vimentin	13.54
E-cadherin	23.61
CD34	8.93
MUC1	28.37
CD105	6.00

Figure 2. Reproducibility of antibodies in the Tissue Architecture Kit. Cell density measurements for each stain across three technical replicates of human FFPE tonsil (A) and corresponding CV (B). n = 3 serial sections. As EpCAM was not detected, it was not included in the quantitative reproducibility assessment.

Breast Cancer

Qualitative Suitability and Specificity Assessment – Scoring

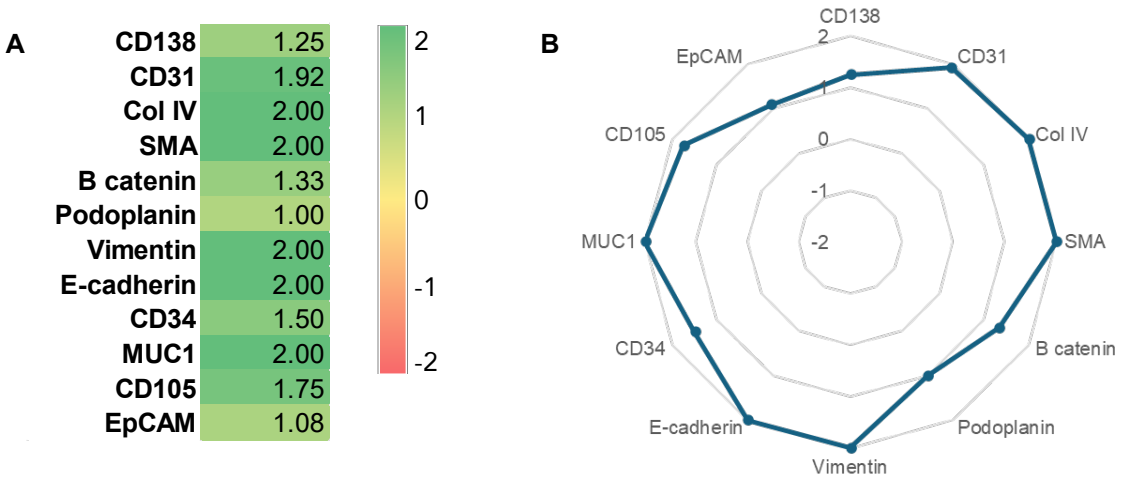


Figure 3. Scoring results of antibodies in the Tissue Architecture Kit. Average scores from technical replicates of human FFPE breast cancer are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 3 samples scored by four independent judges.

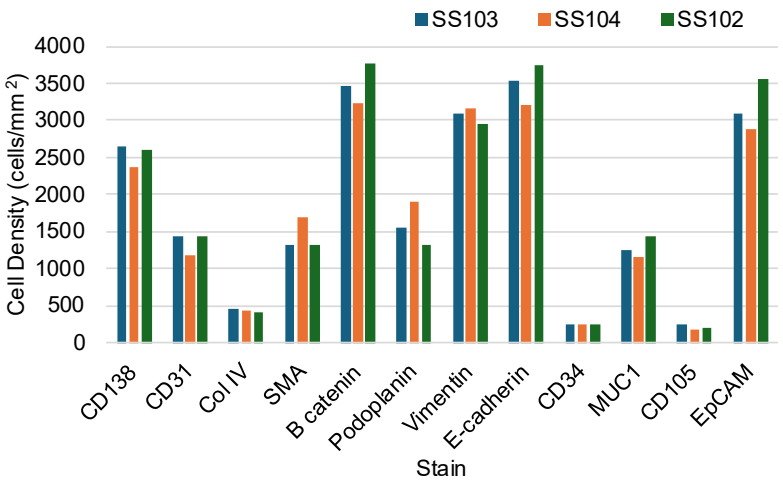
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 4. SNR values for stains in the Tissue Architecture 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**
CD138	270.13	58.27	4.64	2301.32	31.52	73.01
CD31	1128.29	81.21	13.89	2440.83	26.11	93.48
Col IV	535.42	65.45	8.18	2151.71	42.36	50.79
SMA	8069.89	615.54	13.11	33153.43	50.09	661.92
B catenin	154.56	24.70	6.26	1185.62	12.66	93.63
Podoplanin	37.77	18.79	2.01	1984.52	18.15	109.37
Vimentin	2066.38	286.82	7.20	5984.88	16.77	356.95
E-cadherin	1172.46	84.12	13.94	2792.07	22.35	124.90
CD34	211.88	43.70	4.85	551.03	35.83	15.38
MUC1	7905.54	343.12	23.04	9044.52	44.90	201.42
CD105	405.77	22.72	17.86	570.56	12.78	44.65
EpCAM	110.02	27.53	4.00	2142.19	19.90	107.63

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
CD138	4.58
CD31	9.25
Col IV	5.17
SMA	12.25
B catenin	6.14
Podoplanin	14.85
Vimentin	2.84
E-cadherin	6.15
CD34	0.63
MUC1	8.77
CD105	12.07
EpCAM	8.89

Figure 4. Reproducibility of antibodies in the Tissue Architecture 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

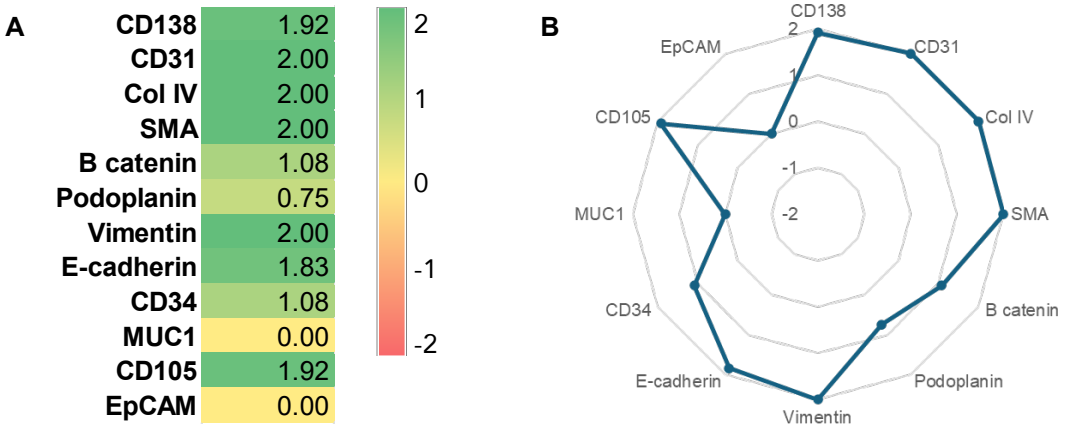


Figure 5. Scoring results of antibodies in the Tissue Architecture Kit. Average scores from technical replicates of human FFPE melanoma are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). EpCAM and MUC1 were not detected, which is not unexpected for a melanoma sample.

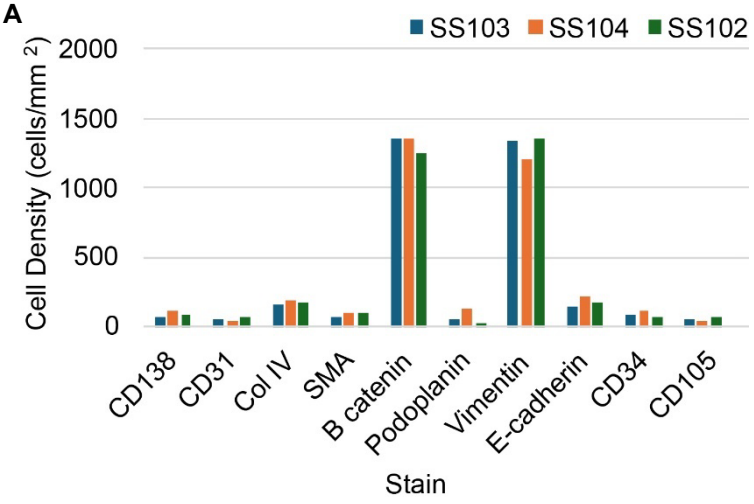
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 5. SNR values for stains in the Tissue Architecture 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**
CD138	236.58	12.86	18.40	2301.32	5.14	447.42
CD31	649.99	26.41	24.61	2440.83	15.74	155.06
Col IV	228.03	43.14	5.29	2151.71	39.10	55.03
SMA	4542.17	230.56	19.70	33153.43	36.00	920.94
B catenin	87.06	11.42	7.63	1185.62	9.34	126.97
Podoplanin	544.09	23.44	23.21	1984.52	16.08	123.38
Vimentin	1591.84	236.58	6.73	5984.88	290.49	20.60
E-cadherin	419.16	6.42	65.29	2792.07	6.86	406.78
CD34	133.90	25.10	5.34	551.03	29.18	18.89
CD105	25.03	4.98	5.03	570.56	7.75	73.65

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
CD138	21.00
CD31	13.82
Col IV	5.96
SMA	20.05
B catenin	3.57
Podoplanin	61.57
Vimentin	4.92
E-cadherin	18.34
CD34	20.40
CD105	22.31

Figure 6. Reproducibility of antibodies in the Tissue Architecture Kit. Cell density measurements for each stain across three technical replicates of human FFPE melanoma (A) and corresponding CV (B). n = 3 serial sections. As EpCAM and MUC1 were not detected, they were not included in the quantitative reproducibility assessment.

Colon Cancer

Qualitative Suitability and Specificity Assessment – Scoring

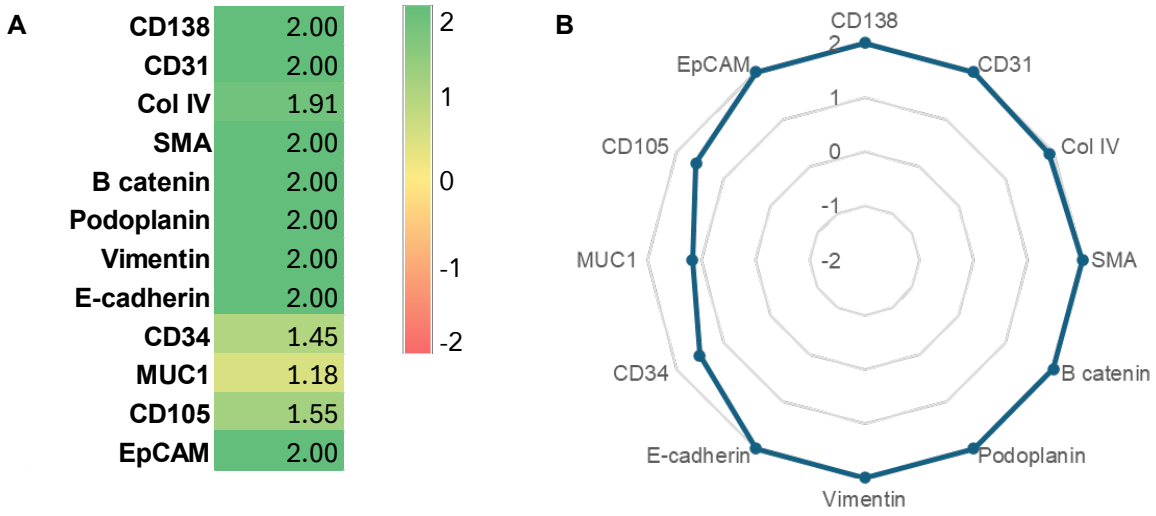


Figure 7. Scoring results of antibodies in the Tissue Architecture Kit. Average scores from technical replicates of human FFPE colon cancer are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 3 samples scored by four independent judges.

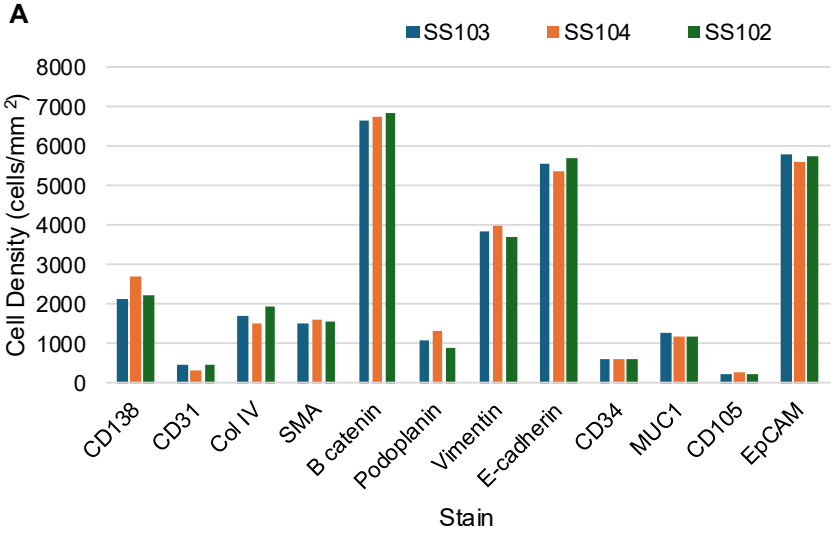
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 6. SNR values for stains in the Tissue Architecture 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**
CD138	709.07	116.81	6.07	2301.32	9.12	252.39
CD31	1029.91	45.53	22.62	2440.83	23.37	104.45
Col IV	848.33	92.12	9.21	2151.71	46.55	46.23
SMA	8159.80	435.44	18.74	33153.43	70.24	472.01
B catenin	450.27	108.05	4.17	1185.62	25.00	47.42
Podoplanin	1583.73	44.98	35.21	1984.52	24.27	81.76
Vimentin	1854.18	117.40	15.79	5984.88	10.73	557.54
E-cadherin	1196.45	283.81	4.22	2792.07	28.66	97.43
CD34	207.13	48.80	4.24	551.03	42.88	12.85
MUC1	915.82	107.39	8.53	9044.52	32.24	280.54
CD105	164.83	20.94	7.87	570.56	15.10	37.78
EpCAM	467.86	124.18	3.77	2142.19	27.41	78.16

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
CD138	9.94
CD31	16.00
Col IV	10.08
SMA	1.83
B catenin	0.97
Podoplanin	17.20
Vimentin	2.77
E-cadherin	2.38
CD34	1.85
MUC1	3.42
CD105	1.85
EpCAM	1.55

Figure 8. Reproducibility of antibodies in Tissue Architecture 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

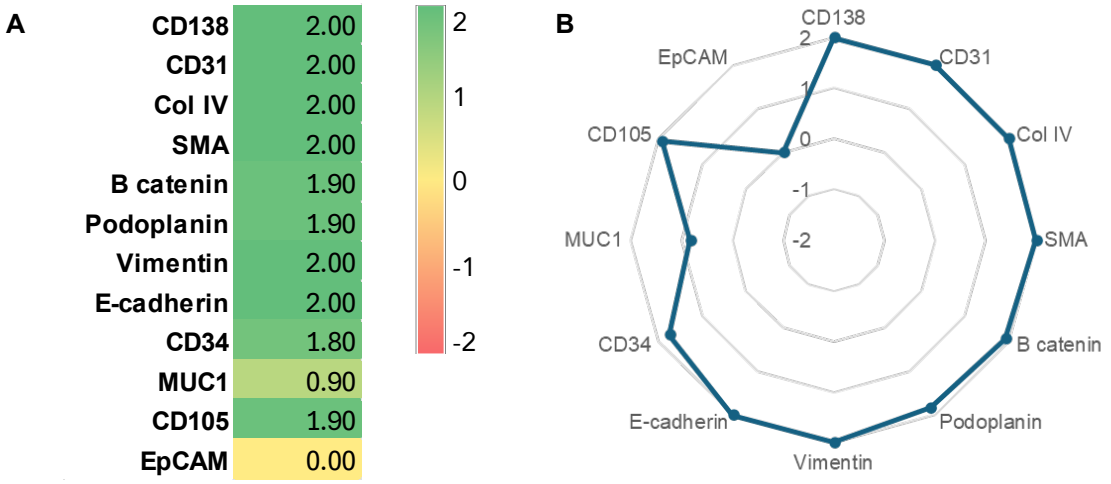


Figure 9. Scoring results of antibodies in the Tissue Architecture Kit. Average scores from technical replicates of human FFPE head & neck cancer are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). EpCAM was not detected, which is not unexpected for an individual head & neck cancer sample.

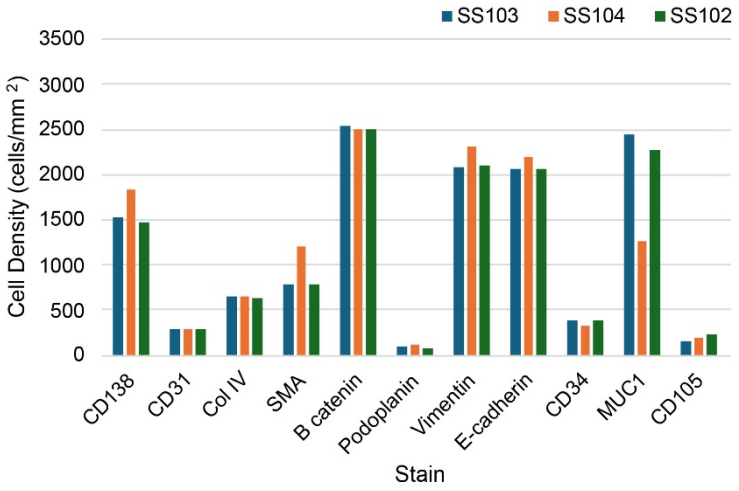
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 7. SNR values for stains in the Tissue Architecture 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**
CD138	435.50	116.15	3.75	2301.32	48.69	47.27
CD31	1406.86	44.66	31.50	2440.83	16.83	145.03
Col IV	832.46	55.90	14.89	2151.71	26.87	80.07
SMA	3865.48	93.27	41.44	33153.43	39.97	829.45
B catenin	229.10	53.16	4.31	1185.62	16.53	71.74
Podoplanin	449.42	24.18	18.58	1984.52	15.26	130.01
Vimentin	2154.46	184.77	11.66	5984.88	33.59	178.15
E-cadherin	1016.81	193.75	5.25	2792.07	28.82	96.89
CD34	326.05	33.87	9.63	551.03	25.33	21.76
MUC1	172.82	38.16	4.53	9044.52	16.56	546.05
CD105	357.14	14.63	24.41	570.56	8.16	69.95

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
CD138	10.04
CD31	2.09
Col IV	1.96
SMA	21.35
B catenin	0.71
Podoplanin	11.44
Vimentin	4.56
E-cadherin	3.35
CD34	7.81
MUC1	26.36
CD105	13.84

Figure 10. Reproducibility of antibodies in the Tissue Architecture 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. As EpCAM was not detected, it was not included in the quantitative reproducibility assessment.

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 Tissue Architecture Kit in human FFPE tonsil.

Stain	Tissue Localization	Intracellular Localization	Positive counterstain	Negative counterstain
CD138	Squamous and glandular epithelia, plasma cells	Plasma membrane	E-cadherin, CD38 (plasma cells)	SMA
CD31	Circulatory vascular endothelia	Plasma membrane	CD34	E-cadherin
Col IV	Basal lamina	Extracellular	CD31	E-cadherin
SMA	Smooth muscle surrounding vasculature	Cytoplasm	CD31	E-cadherin
B catenin	Squamous epithelia, vascular endothelia	Plasma membrane, cytoplasm	E-cadherin, CD31	Vimentin
Podoplanin	Lymphatic vasculature	Plasma membrane	Vimentin	CD31
Vimentin	Stromal fibroblasts	Cytoplasm	SMA, Podoplanin	E-cadherin
E-cadherin	Epithelia	Plasma membrane	B catenin	SMA
CD34	Circulatory vascular endothelia	Plasma membrane	CD31	E-cadherin
MUC1	Apical secretory epithelia	Apical plasma membrane	E-cadherin	SMA
CD105	Activated circulatory endothelia	Plasma membrane	CD31	E-cadherin
EpCAM*	Simple or tumor epithelia	Plasma membrane	E-cadherin	SMA

***Note:** Colon is the positive control tissue for EpCAM.

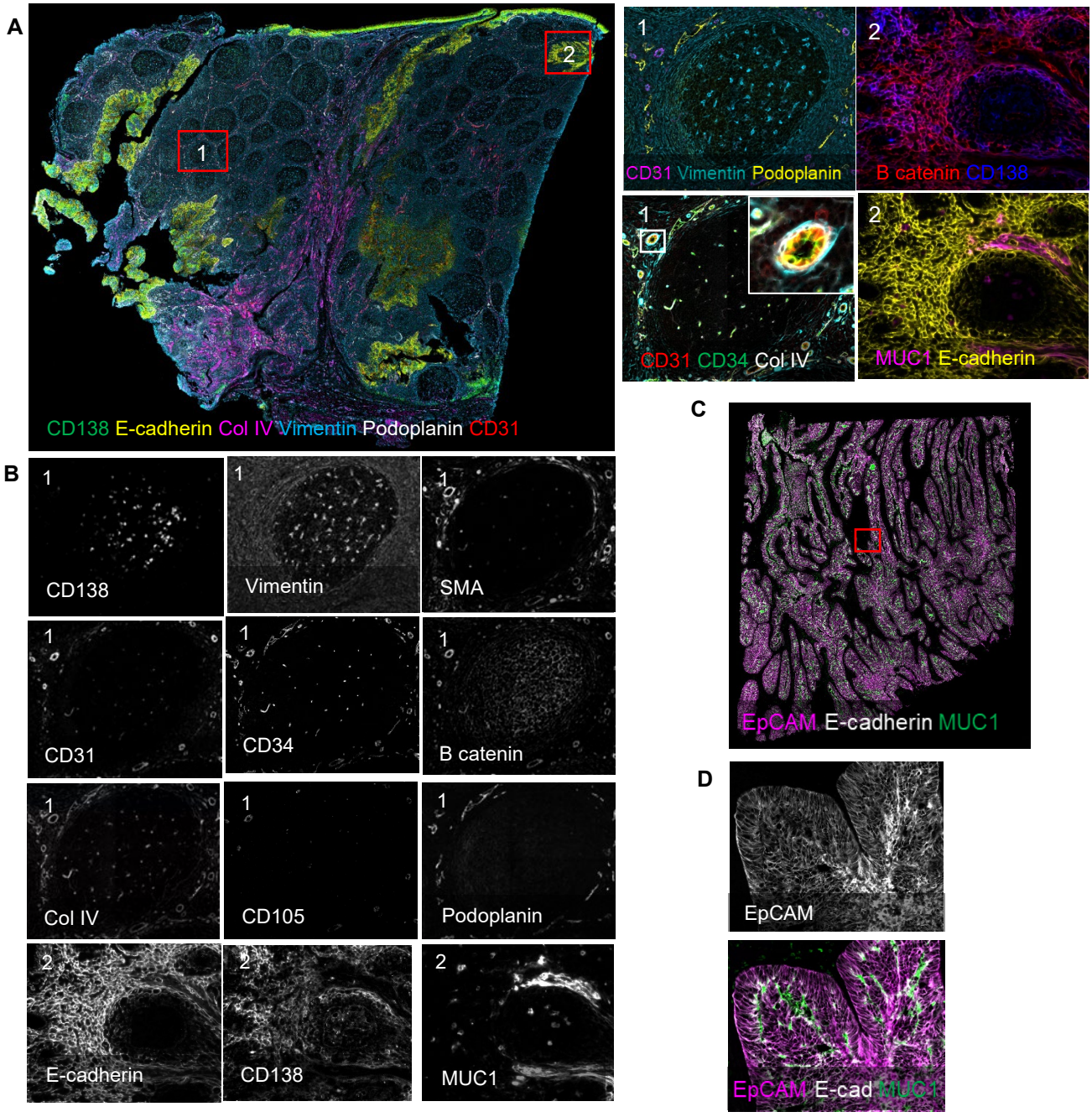


Figure 11. Example images for stains in the Tissue Architecture Kit. A. Full overview of a tonsil sample used in validation testing. The red boxes indicate the germinal center (1) and squamous epithelia (2) regions shown in enlarged images. B. Enlarged images showing individual antibody stains in grayscale and multicolor counterstain examples. C. Full overview of a colon cancer sample used in validation testing as positive control for EpCAM staining. The red box indicates epithelial region shown in enlarged images. D. Enlarged images showing positive EpCAM staining in grayscale and multicolor example counterstains.

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 5 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	CD138	1:50	60
	CD31	1:100	
	Col IV	1:50	
2	SMA	1:50	60
	B-catenin	1:100	
	Podoplanin	1:50	
3	Vimentin	1:500	60
	E-cadherin	1:250	
	CD34	1:50	
4	MUC1	1:100	60
	CD105	1:250	
	EpCAM	1:25	

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 –“).

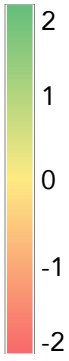
For reproducibility, cells were classified as positive or negative based on OTSU thresholding applied to average cell expression. The number of positive cells was quantified per unit area, expressed as cells/mm^2. The CV was calculated as the ratio of standard deviation to the mean expressed as a percent.

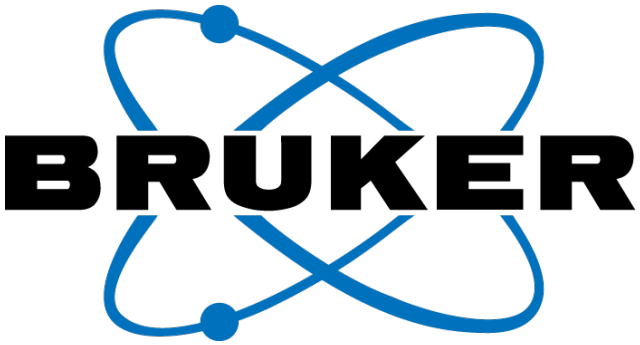
Additional Use Data

Table 12. Suitability scores from additional testing of the Tissue Architecture Assay Kit. The Tissue Architecture 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
CD138	1.50	2.00	1.50	2.00	2.00	1.00	0.00	1.50	1.00
CD31	1.80	2.00	2.00	2.00	2.00	2.00	0.00	2.00	2.00
Col IV	1.80	2.00	2.00	2.00	2.00	2.00	2.00	1.50	1.00
SMA	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
B catenin	1.70	2.00	1.50	2.00	2.00	2.00	1.00	1.50	1.00
Podoplanin	1.30	2.00	1.50	2.00	2.00	1.00	0.00	1.50	1.00
Vimentin	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
E-cadherin	1.70	2.00	2.00	2.00	2.00	2.00	1.00	1.50	1.00
CD34	0.80	2.00	1.50	1.00	1.00	1.00	1.00	1.50	2.00
MUC1	1.10	2.00	2.00	1.00	1.00	1.00	1.00	0.50	1.00
CD105	1.50	2.00	2.00	1.00	1.00	1.00	0.00	1.50	2.00
EpCAM	0.70	2.00	1.50	2.00	2.00	0.00	1.00	0.00	0.00

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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