VALIDATION REPORT

VistaPlex™ Cell Boundaries Kit

For CellScape™ Precise Spatial Proteomics

Validation of the Cell Boundaries for Human FFPE multiplex antibody kit, product VISTAPLEX3101

PMR-11863-01

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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 Cell Boundaries 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 Cell Boundaries Kit. Data were obtained from human FFPE tonsil.

Antibody/Stain	Specificity	Sensitivity	Reproducibility
SYTOX® Orange (DNA)	Pass	Pass	Low Variability
ATP1A1	Pass	Pass	Low Variability
Lamin B1	Pass	Pass	Low Variability
B2M	Pass	Pass	Low Variability

Table 2. Results summary for suitability of the Cell Boundaries 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

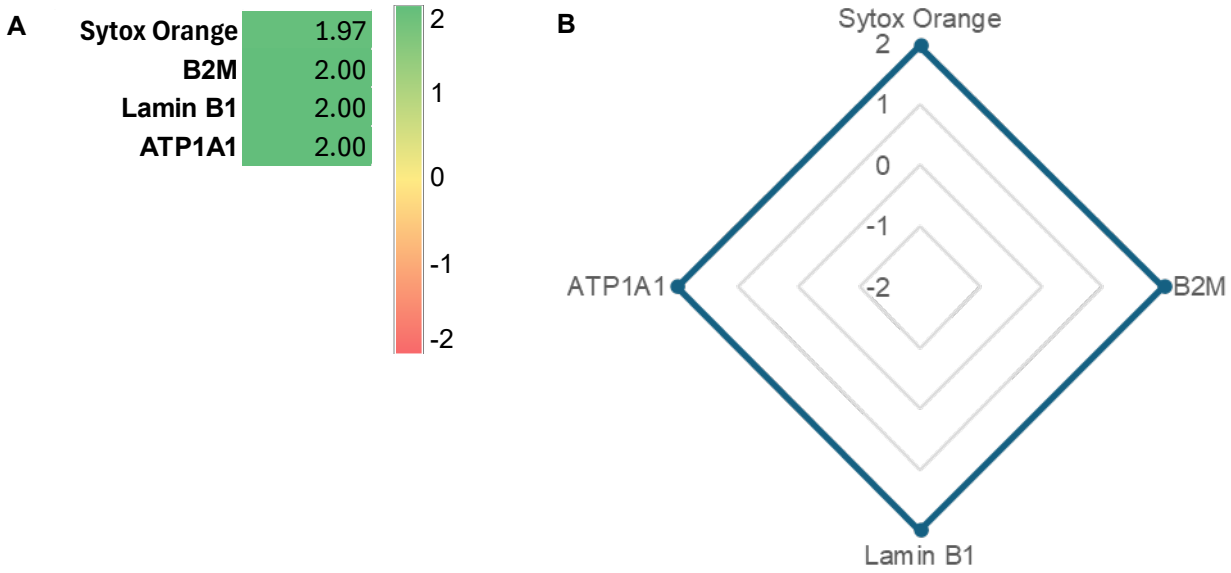


Figure 1. Scoring results of antibodies in the Cell Boundaries Kit. Average scores from technical replicates of human FFPE Tonsil are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 8 samples scored by four independent judges.

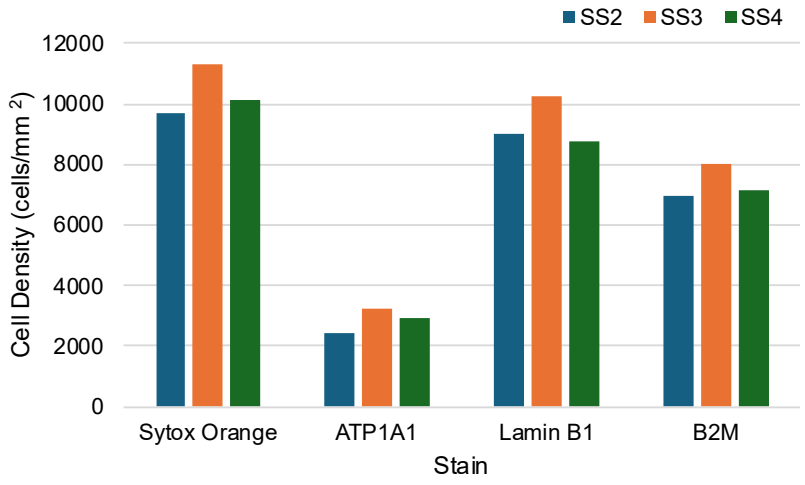
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 3. SNR values for stains in the Cell Boundaries 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**
DNA	4507.02	588.35	7.66	7912.57	2195.86	3.60
ATP1A1	1909.62	318.78	5.99	6340.49	361.02	17.56
Lamin B1	249.92	40.92	6.11	1404.19	217.17	6.47
B2M	499.16	179.32	2.78	1348.18	317.36	4.25

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
Sytox Orange	6.67
ATP1A1	11.77
LaminB1	6.92
B2M	6.31

Figure 2. Reproducibility of antibodies in the Cell Boundaries 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

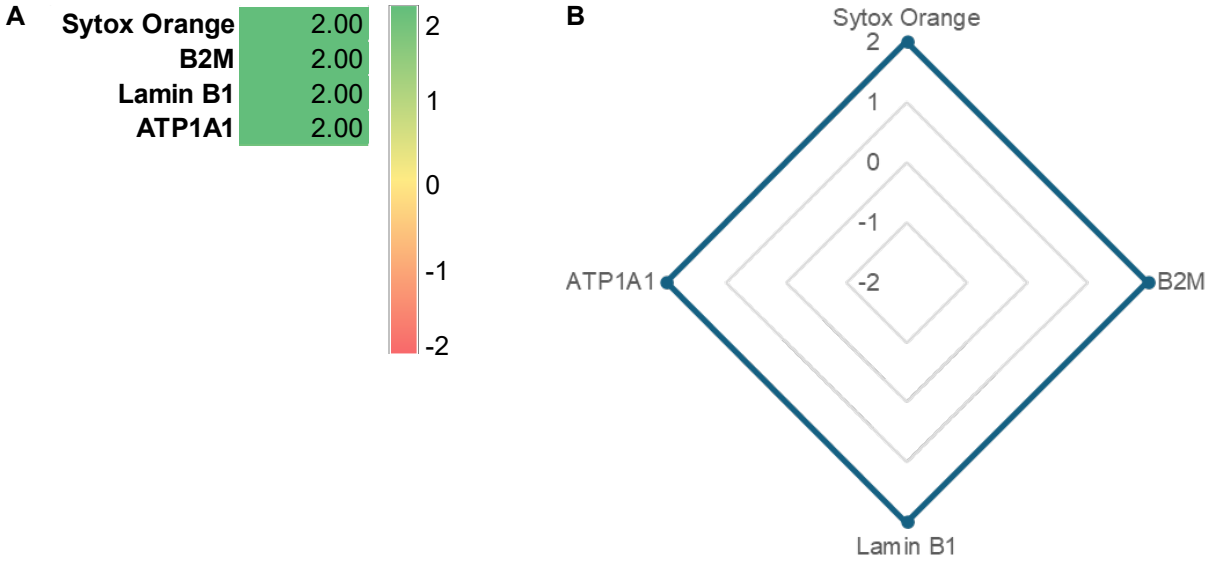


Figure 3. Scoring results of antibodies in the Cell Boundaries Kit. Average scores from technical replicates of human FFPE breast cancer are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 3 samples scored by four independent judges.

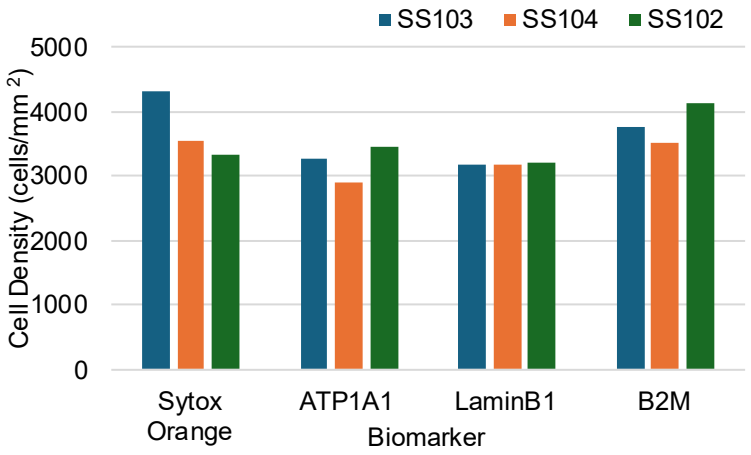
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 4. SNR values for stains in the Cell Boundaries 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**
DNA	5581.14	956.04	5.84	10209.55	1850.21	5.52
ATP1A1	541.65	224.88	2.41	4732.16	175.30	26.99
Lamin B1	244.84	47.92	5.11	773.42	51.98	14.88
B2M	2059.24	332.50	6.19	1729.77	220.83	7.83

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
Sytox Orange	11.35
ATP1A1	7.24
LaminB1	0.75
B2M	6.52

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

Melanoma

Qualitative Suitability and Specificity Assessment – Scoring

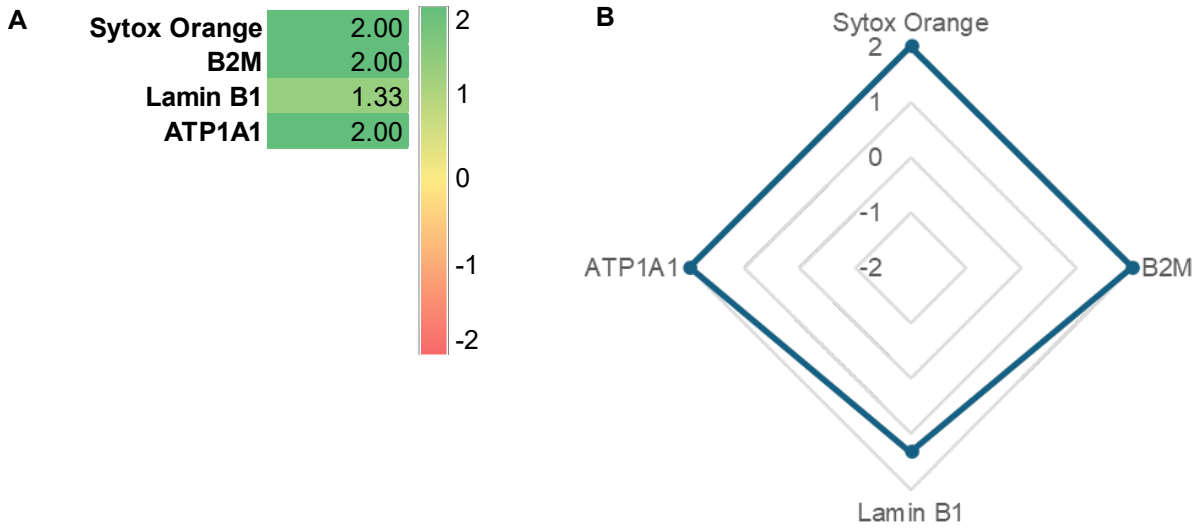


Figure 5. Scoring results of antibodies in the Cell Boundaries Kit. Average scores from technical replicates of human FFPE melanoma are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 3 samples scored by four independent judges.

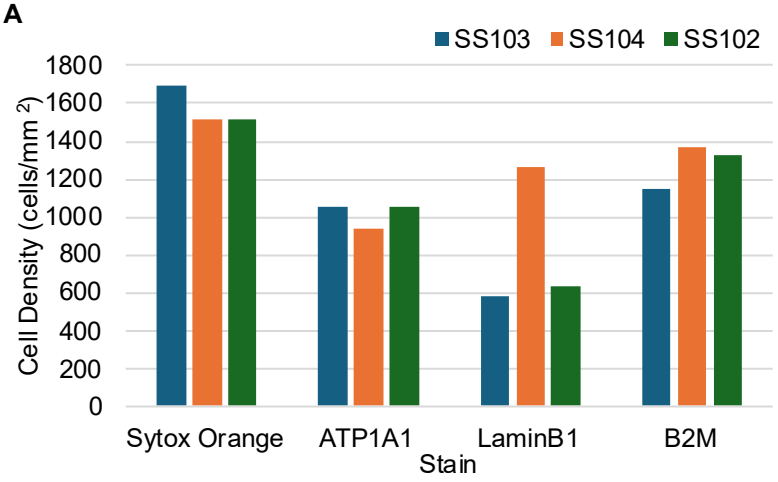
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 5. SNR values for stains in the Cell Boundaries 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**
DNA	2139.51	128.10	16.70	10209.55	779.94	13.09
ATP1A1	581.12	152.73	3.80	4732.16	75.94	62.31
Lamin B1	123.12	16.14	7.63	773.42	24.15	32.02
B2M	1132.46	97.91	11.57	1729.77	163.57	10.57

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
Sytox Orange	5.30
ATP1A1	5.09
LaminB1	37.15
B2M	7.60

Figure 6. Reproducibility of antibodies in the Cell Boundaries 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

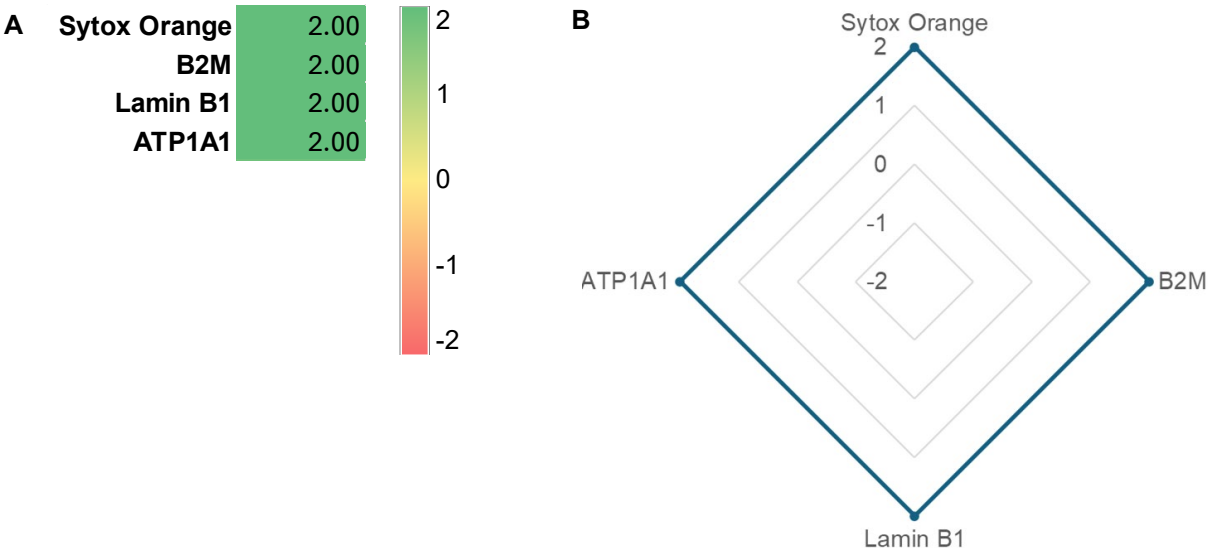


Figure 7. Scoring results of antibodies in the Cell Boundaries Kit. Average scores from technical replicates of human FFPE colon cancer are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 3 samples scored by four independent judges.

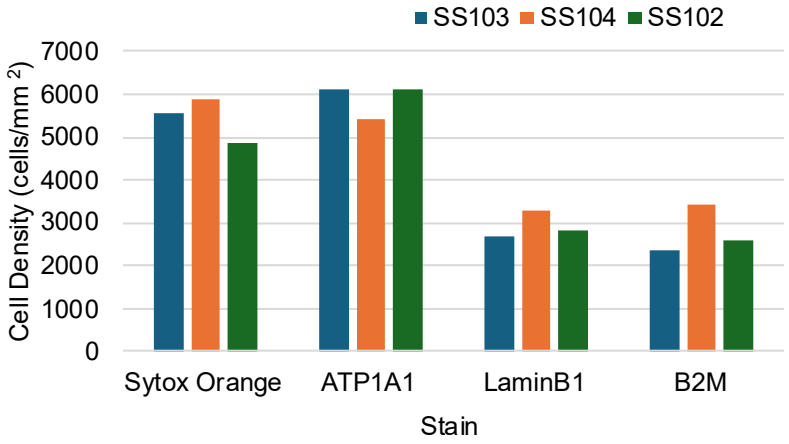
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 6. SNR values for stains in the Cell Boundaries 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**
DNA	4217.00	988.74	4.27	10209.55	2327.43	4.39
ATP1A1	369.24	174.50	2.12	4732.16	140.46	33.69
Lamin B1	298.17	87.66	3.40	773.42	81.48	9.49
B2M	2229.47	557.47	4.00	1729.77	159.77	10.83

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
Sytox Orange	7.89
ATP1A1	5.58
LaminB1	8.71
B2M	16.14

Figure 8. Reproducibility of antibodies in Cell Boundaries 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

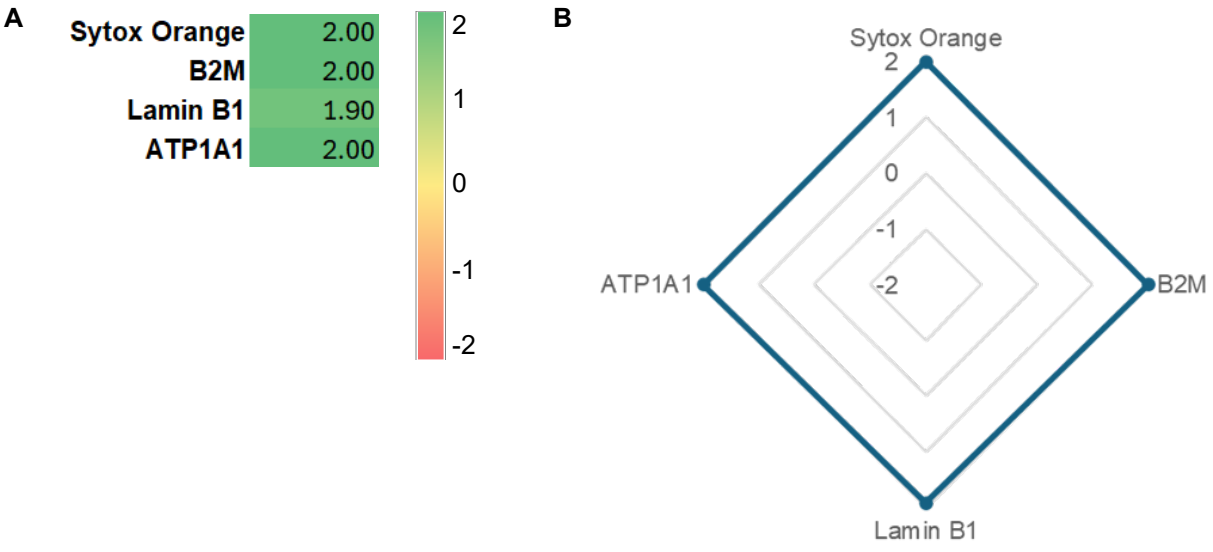


Figure 9. Scoring results of antibodies in the Cell Boundaries Kit. Average scores from three technical replicates of human FFPE head & neck cancer are visualized in a heatmap (A, green=pass, red=fail) and a radar plot (B). n = 3 samples scored by four independent judges.

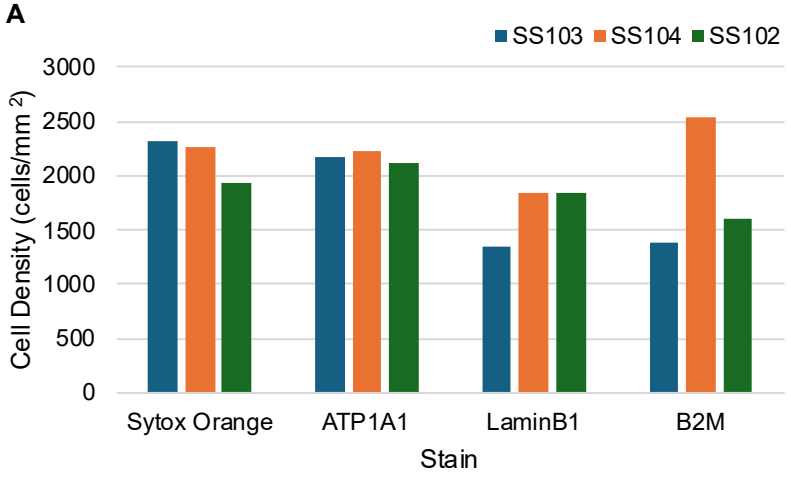
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 7. SNR values for stains in the Cell Boundaries 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**
DNA	4887.88	632.55	7.73	10209.55	1313.19	7.77
ATP1A1	366.87	154.17	2.38	4732.16	102.46	46.19
Lamin B1	206.58	34.95	5.91	773.42	28.98	26.69
B2M	1671.53	414.60	4.03	1729.77	125.79	13.75

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
Sytox Orange	7.80
ATP1A1	2.20
LaminB1	14.07
B2M	27.09

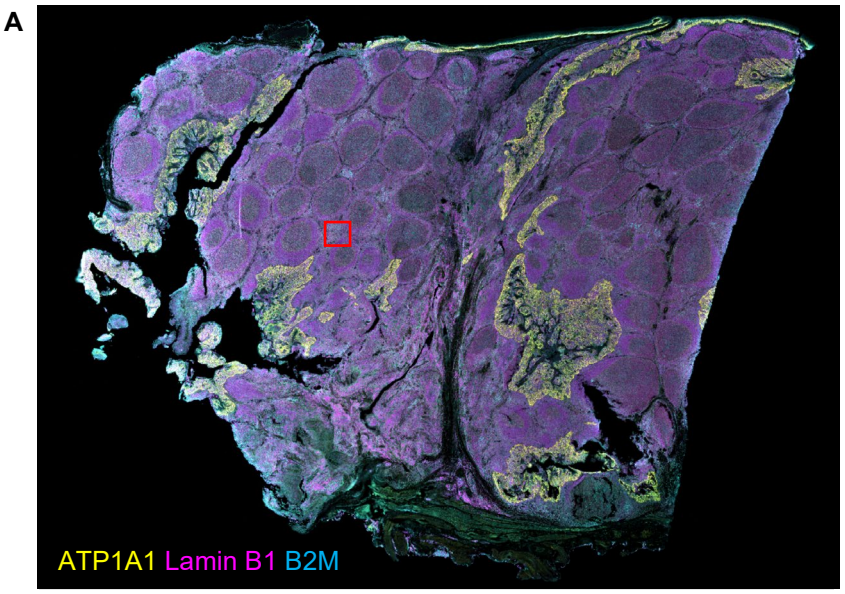
Figure 10. Reproducibility of antibodies in the Cell Boundaries Kit. Cell density measurements for each stain across three technical replicates of human FFPE head & neck cancer (A) and corresponding CV (B). n = 3 serial sections.

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 Cell Boundaries Kit in human FFPE tonsil.

Stain	Tissue Localization	Intracellular Localization	Positive counterstain	Negative counterstain
SYTOX Orange (DNA)	All regions	Nuclear	Lamin B1 or other DNA stain	n/a
ATP1A1	All regions, high expression in squamous epithelium	Surface membrane	PanCK	n/a
Lamin B1	All regions	Nuclear envelope	SYTOX Orange or other DNA stain	n/a
B2M	All regions	Surface membrane	CD45	n/a



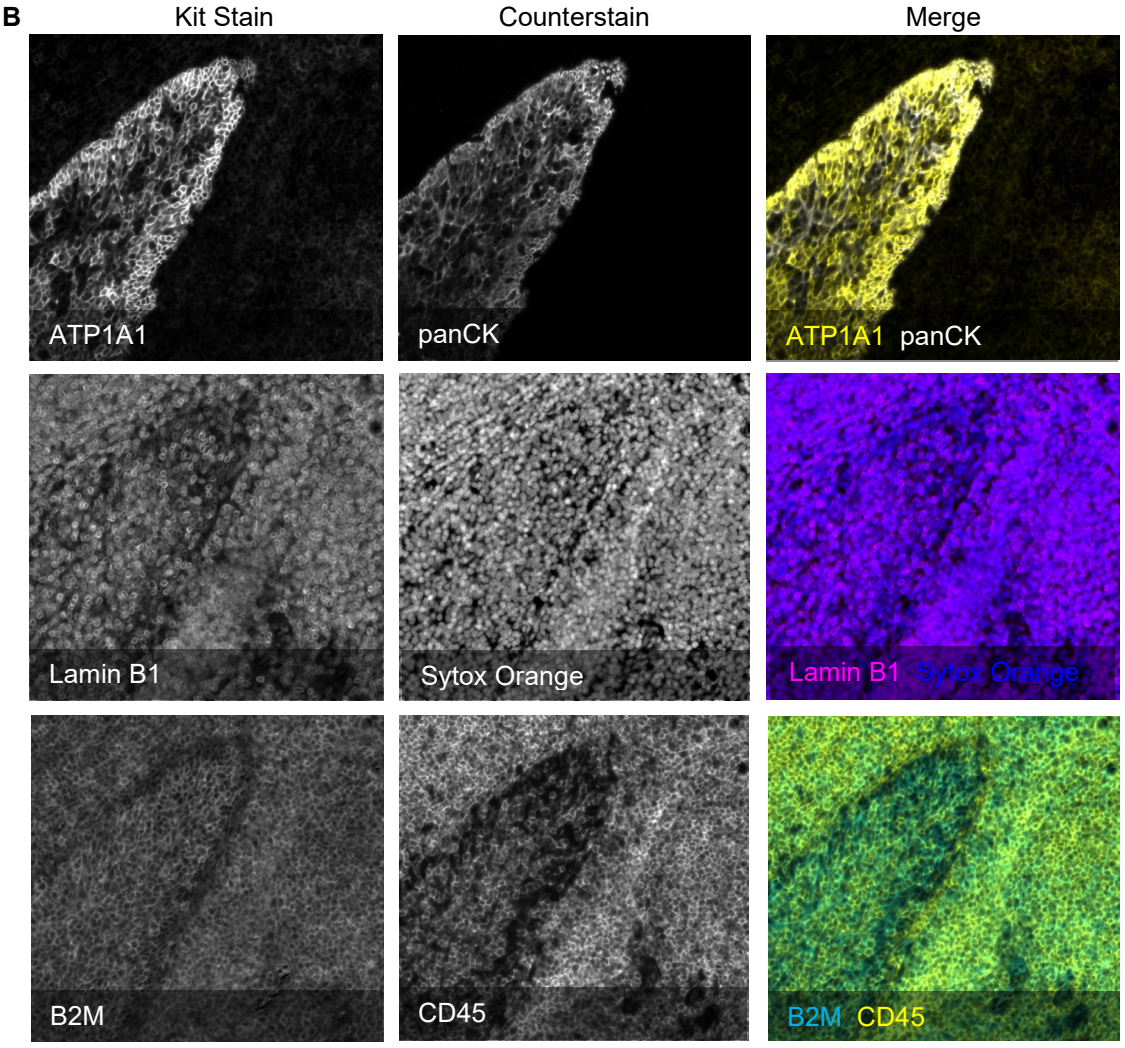


Figure 11. Example images for stains in the Cell Boundaries Kit.

A, Full overview of a tonsil sample used in validation testing. The red box indicates the region shown in enlarged images.

B, Enlarged images showing kit stains and appropriate 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 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	DNA	1:1 Million	5
2	B2M	1:250	60
	Lamin B1	1:250	
	ATP1A1	1:250	

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 –“).

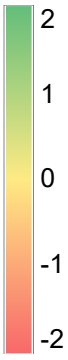
For reproducibility, cells were classified as positive or negative based on OTSU thresholding applied to average cell expression. The number of positive cells was quantified per unit area, expressed as cells/mm^2. The CV was calculated as the ratio of standard deviation to the mean expressed as a percent.

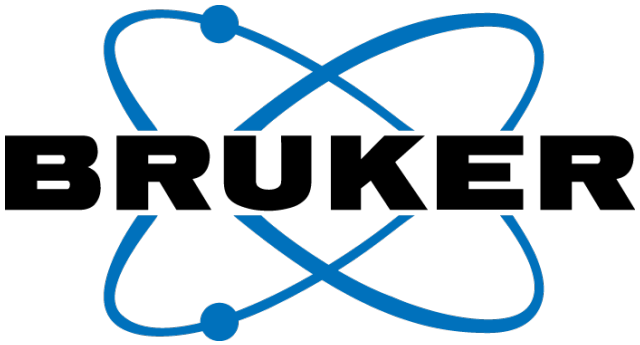
Supplemental Data

Table 12. Suitability scores from additional testing of the Cell Boundaries Kit. The Cell Boundaries 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
Sytox Orange	2.00	1.60	2.00	2.00	2.00	2.00	2.00	2.00	2.00
B2M	1.75	2.00	2.00	2.00	2.00	2.00	1.00	2.00	1.00
Lamin B1	1.88	2.00	2.00	2.00	2.00	2.00	2.00	2.00	1.00
ATP1A1	1.75	2.00	2.00	2.00	2.00	1.00	2.00	1.50	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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