VALIDATION REPORT

VistaPlex™ Human Fresh Frozen Cell Boundaries Assay Kit

For the CellScape™ Precise Spatial Proteomics platform

Product 531-125000002

Contents

Purpose

VistaPlex Assay Kits contain ready-to-use, reliable reagents and optimized protocols enabling researchers to obtain quick, robust data with the CellScape Precise Spatial Proteomics platform. The objective of this Validation Report is to quantitatively document the performance characteristics of the VistaPlex Human Fresh Frozen (FF) Cell Boundaries Assay Kit to demonstrate the specificity, sensitivity, and reproducibility of the kit. Kit validation is based on experiments performed on human FF tonsil 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 (tonsil) or 1.0 (other tissues)

Fail: Average score < 1.5 (tonsil) 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 (SS) 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 Human FF Cell Boundaries Assay Kit. Data were obtained from human FF tonsil.

Antibody/Stain	Specificity	Sensitivity	Reproducibility
ATP1A1	Pass	Pass	Low Variability
B2M	Pass	Pass	Low Variability
Nuclear Stain/DNA	Pass	Pass	Low Variability
Lamin B1	Pass	Pass	Low Variability

Table 2. Results summary for suitability of the Human FF Cell Boundaries Assay Kit.

Tissue	Suitability
Tonsil	Pass
Breast cancer	Pass
Lung cancer	Pass

Validation Data

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

- Tonsil
- Breast cancer
- Lung cancer

Tonsil

Qualitative Suitability and Specificity Assessment – Scoring

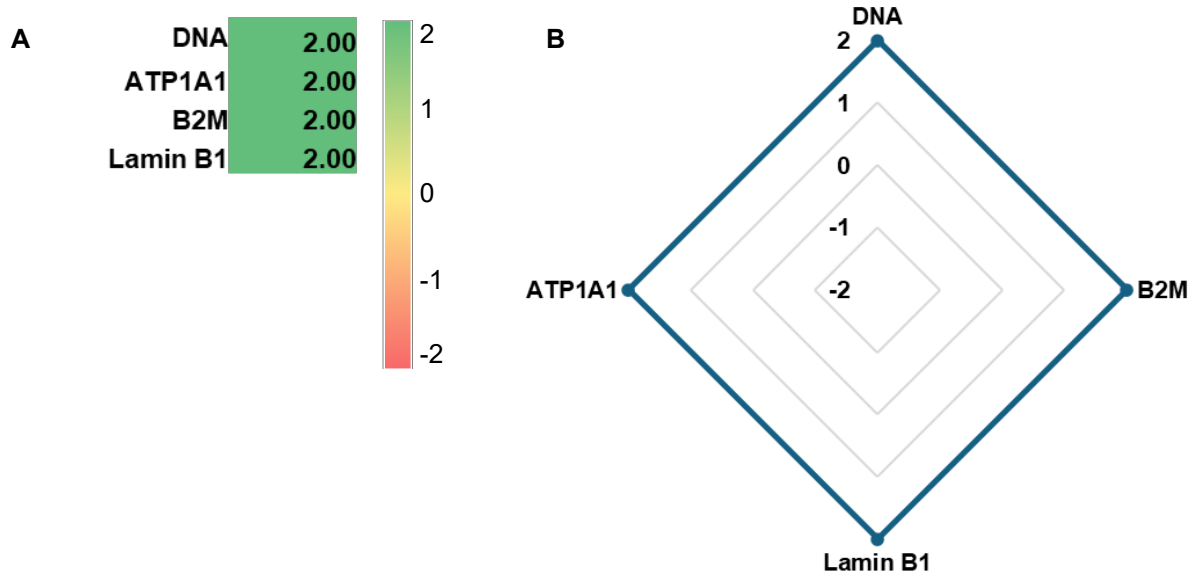


Figure 1. Scoring results of antibodies in the Human FF Cell Boundaries Assay Kit. Average scores from technical replicates of human FF tonsil are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges. PanCK data are excluded since this target is not expressed in tonsil.

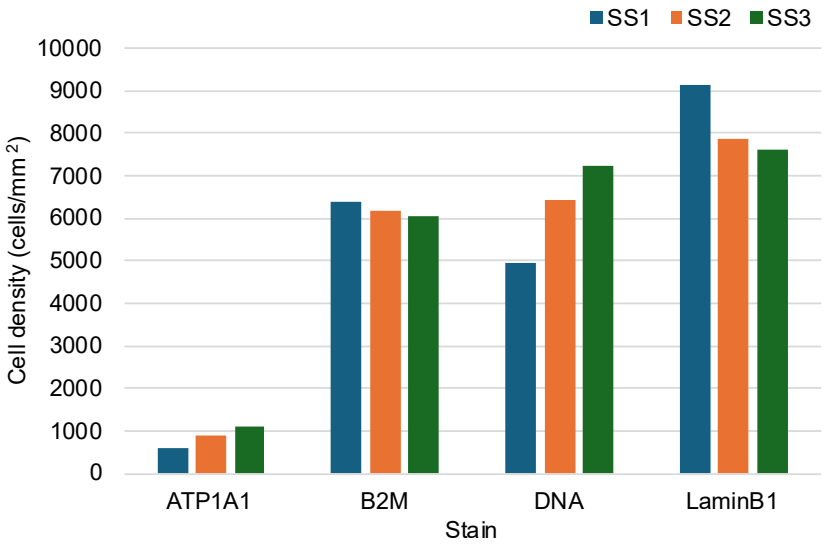
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 3. SNR values for stains in the Human FF Cell Boundaries Assay Kit. Average positive and negative signal intensities and SNR from three technical replicates of human FF tonsil.

	Method 1			Method 2		
	Mean +	**Mean -**	**SNR**	**Mean +**	**Mean -**	**SNR**
DNA	1988.14	383.34	5.19	3381.34	1095.48	3.09
ATP1A1	1876.67	263.84	7.11	3328.98	107.59	30.94
B2M	376.28	125.97	2.99	609.68	221.25	2.76
Lamin B1	1032.10	353.93	2.92	2013.48	417.16	4.83

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
ATP1A1	23.42
B2M	2.04
DNA	15.10
LaminB1	8.10

Figure 2. Reproducibility of antibodies in the Human FF Cell Boundaries Assay Kit. Cell density measurements for each stain across technical replicates of human FF tonsil (A) and corresponding CV (B). n = 3 serial sections.

Breast cancer

Qualitative Suitability and Specificity Assessment – Scoring

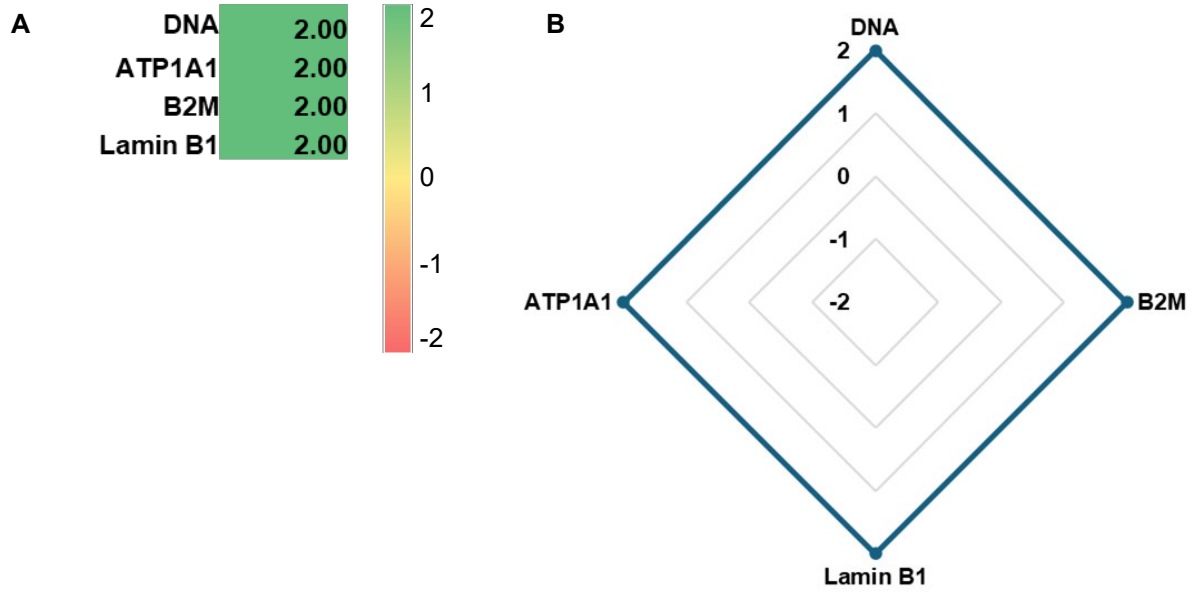


Figure 3. Scoring results of antibodies in the Human FF Cell Boundaries Assay Kit. Average scores from technical replicates of human FF breast cancer are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges.

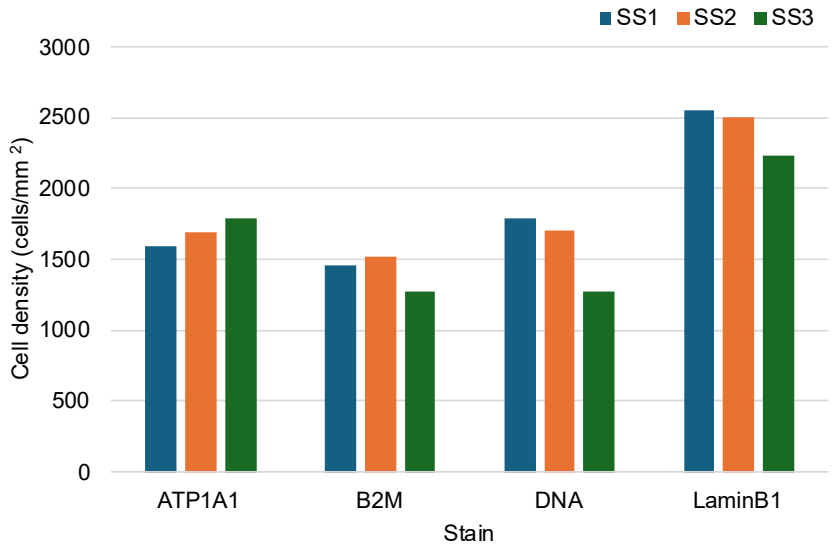
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 4. SNR values for stains in the Human FF Cell Boundaries Assay Kit. Average positive and negative signal intensities and SNR from three technical replicates of human FF breast cancer.

	Method 1			Method 2		
	Mean +	**Mean -**	**SNR**	**Mean +**	**Mean -**	**SNR**
DNA	3341.59	545.50	6.13	9046.07	1097.45	8.24
ATP1A1	362.44	85.58	4.24	760.08	84.95	8.95
B2M	294.16	113.18	2.60	546.17	102.96	5.30
Lamin B1	1273.09	131.52	9.68	2694.27	184.69	14.59

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
ATP1A1	4.63
B2M	7.27
DNA	14.06
LaminB1	5.68

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

Lung cancer

Qualitative Suitability and Specificity Assessment – Scoring

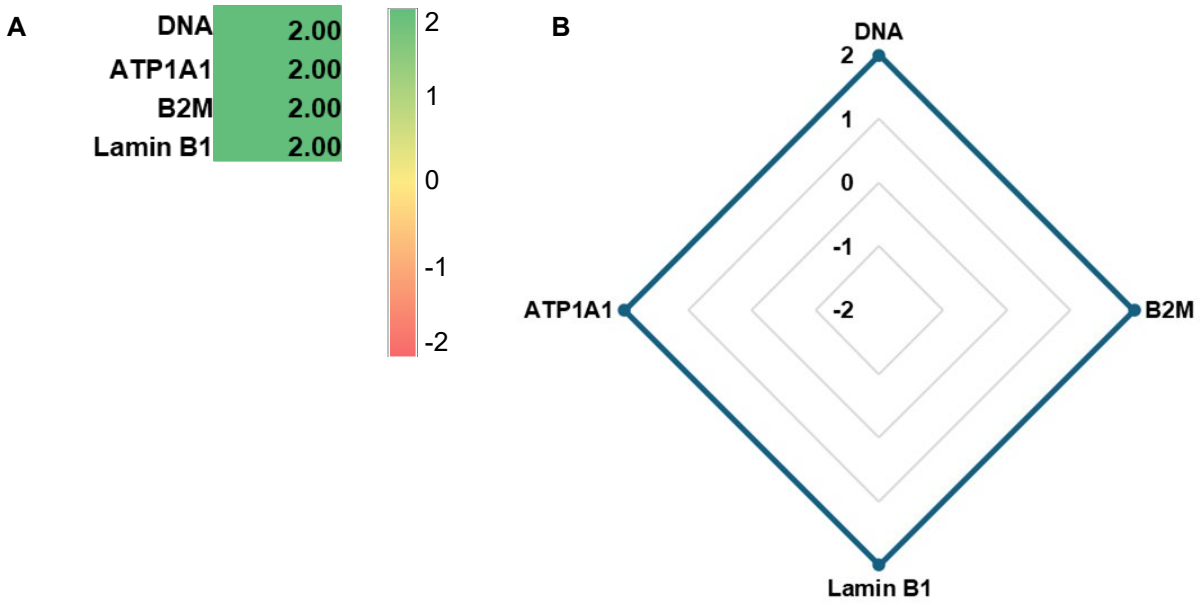


Figure 5. Scoring results of antibodies in the Human FF Cell Boundaries Assay Kit. Average scores from technical replicates of human FF lung cancer are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges.

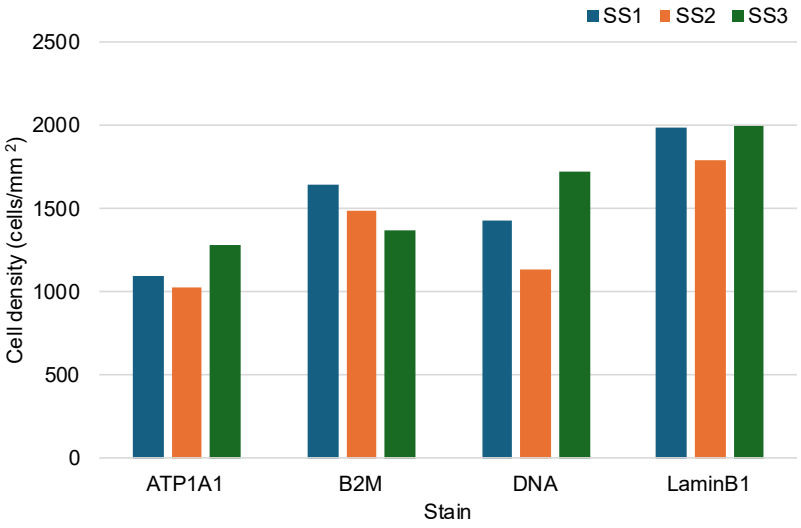
Quantitative Sensitivity Assessment – Signal-to-Noise Ratio (SNR)

Table 5. SNR values for stains in the Human FF Cell Boundaries Assay Kit. Average positive and negative signal intensities and SNR from three technical replicates of human FF lung cancer.

	Method 1			Method 2		
	Mean +	**Mean -**	**SNR**	**Mean +**	**Mean -**	**SNR**
DNA	3588.33	243.35	14.75	1465.97	0.09	17027.62
ATP1A1	628.19	77.83	8.07	1443.05	91.34	15.80
Lamin B1	1446.93	72.07	20.08	8396.16	1063.91	7.89
B2M	300.48	45.66	6.58	1172.23	1.05	1120.87

Quantitative Reproducibility Assessment

A



B

Stain	CV (%)
ATP1A1	9.57
B2M	7.49
DNA	16.71
LaminB1	5.01

Figure 6. Reproducibility of antibodies in the Human FF Cell Boundaries Assay Kit. Cell density measurements for each stain across technical replicates of Human FF lung cancer (A) and corresponding CV (B). n = 3 serial sections.

Stain Qualification and Specificity Criteria

Table 6 describes the areas of interest that were used for evaluating antibody performance in human FF tonsil. 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 Human FF Cell Boundaries Assay Kit in human FF tonsil.

Stain	Tissue Localization	Intracellular Localization	Positive Counterstain	Negative Counterstain
DNA	All regions	Nucleus	Lamin B1	-
Lamin B1	All regions	Nucleus (Nuclear lamina)	DNA	-
ATP1A1	All regions	Plasma membrane	panCK	-
B2M	All regions	Plasma membrane	CD45	-

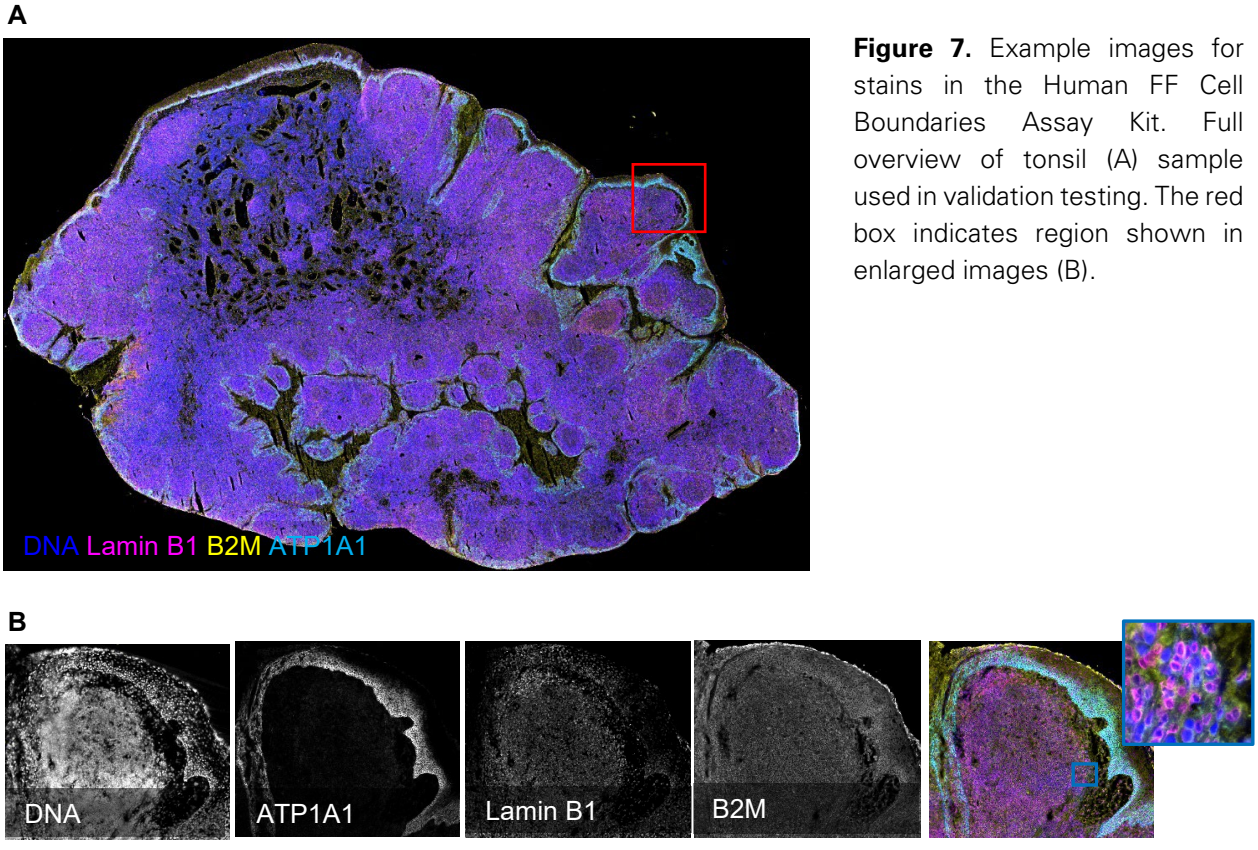


Figure 7. Example images for stains in the Human FF Cell Boundaries Assay Kit. Full overview of tonsil (A) sample used in validation testing. The red box indicates region shown in enlarged images (B).

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 human FF tonsil 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. Human tissues used for VistaPlex Kit validation.

Product Code	Description	Vendor
681074B2(3) / MZKL585620	Tissue – Tonsil	AmsBio
T1235086-DC	Tissue – Lung cancer	BioCat
T1235152-DC	Tissue – Breast cancer	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 20 seconds (DNA) or 10 seconds (antibodies) of enhanced photobleaching at 50% lamp power after each stain. 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	DNA	1:8 million	5
2	B2M	1:500	15
	Lamin B1	1:1000	
	ATP1A1	1:600	

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 (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 9. Score Definitions.

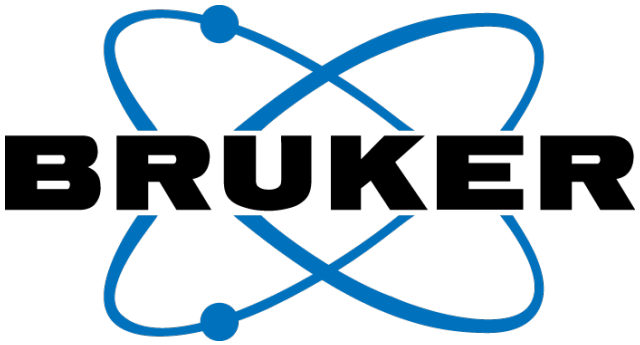
Score	Interpretation
2	Excellent, bright, specific stain
1	Acceptable but dim or high background
0	No staining
-1	Moderate, not abundant off target staining
-2	Strong and/or abundant unspecific staining

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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