VALIDATION REPORT

VistaPlex™ Mouse FF Cell Boundaries Assay Kit

For the CellScape™ Precise Spatial Proteomics platform

Product 531-125000001

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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 Mouse 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 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 Cell Boundaries Assay Kit. Data were obtained from murine FF spleen.

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

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

Tissue	Suitability
Spleen	Pass
Lymph Node	Pass
Colon	Pass

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

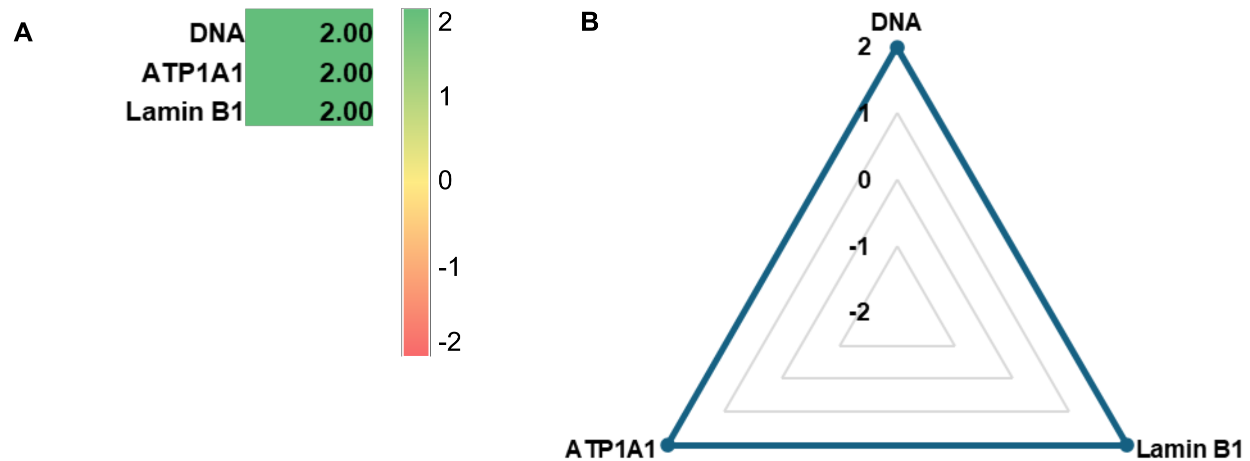


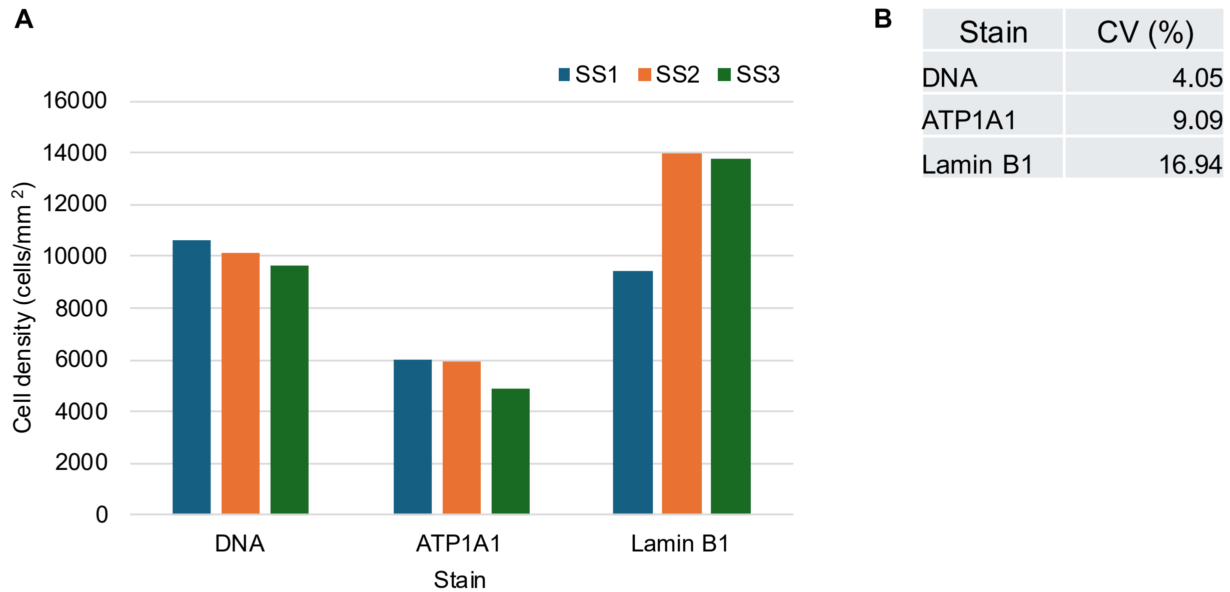
Figure 1. Scoring results of antibodies in the Mouse FF Cell Boundaries Assay Kit. Average scores from technical replicates of mouse FF spleen are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges.

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

Table 3. SNR values for stains in the Mouse FF Cell Boundaries Assay 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**
DNA	1201.38	354.89	3.39	2335.77	627.48	3.72
ATP1A1	202.49	62.19	3.26	988.90	53.96	18.33
Lamin B1	839.59	222.69	3.77	1504.92	267.62	5.62

Quantitative Reproducibility Assessment



Stain	CV (%)
DNA	4.05
ATP1A1	9.09
Lamin B1	16.94

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

Lymph Node

Qualitative Suitability and Specificity Assessment – Scoring

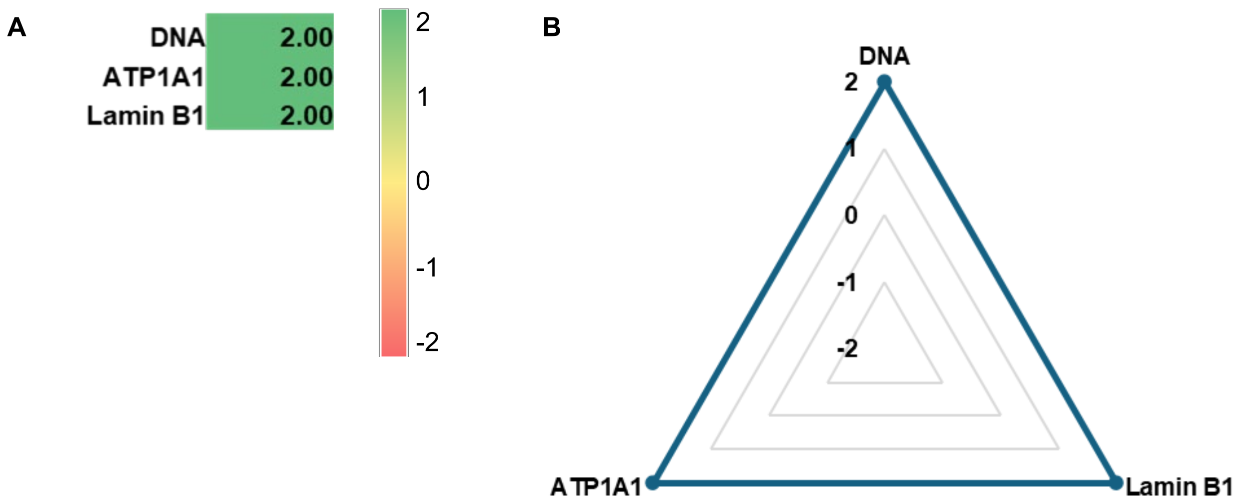


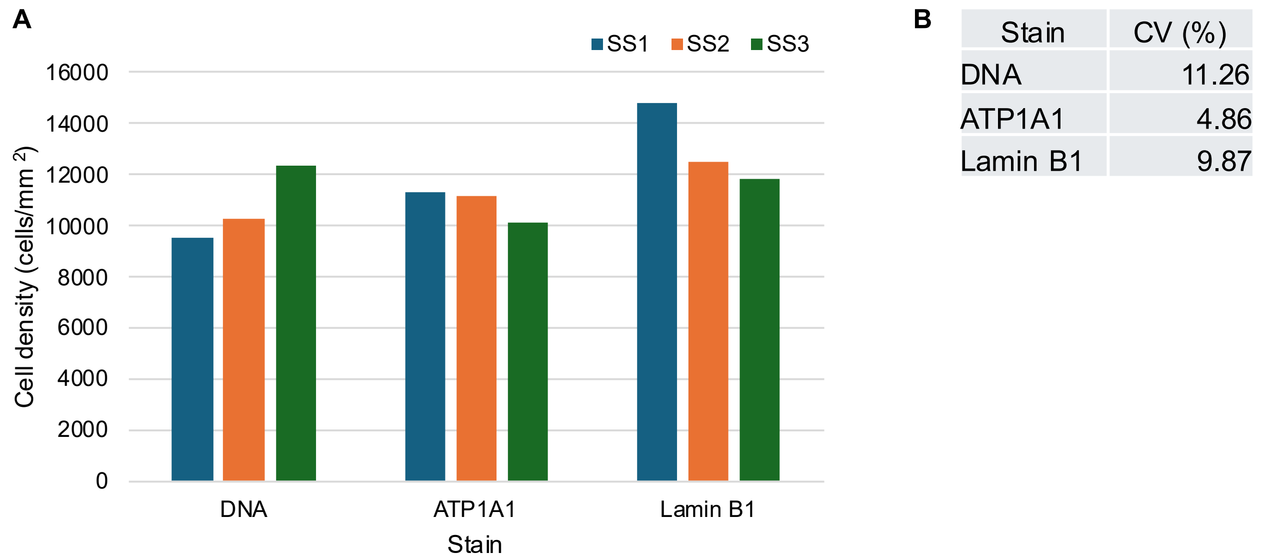
Figure 3. Scoring results of antibodies in the Mouse FF Cell Boundaries Assay Kit. Average scores from technical replicates of mouse FF lymph node are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges.

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

Table 4. SNR values for stains in the Mouse FF Cell Boundaries 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**
DNA	2087.80	994.19	2.10	4221.72	1093.58	3.86
ATP1A1	378.02	181.71	2.08	627.48	175.12	3.58
Lamin B1	638.01	326.84	1.95	1078.53	332.41	3.24

Quantitative Reproducibility Assessment



Stain	CV (%)
DNA	11.26
ATP1A1	4.86
Lamin B1	9.87

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

Colon

Qualitative Suitability and Specificity Assessment – Scoring

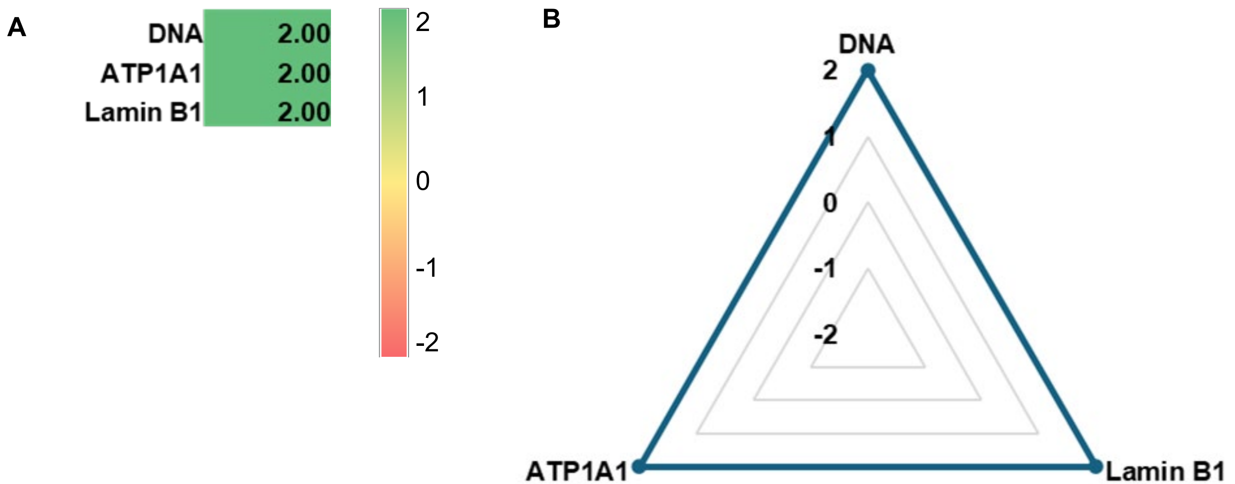


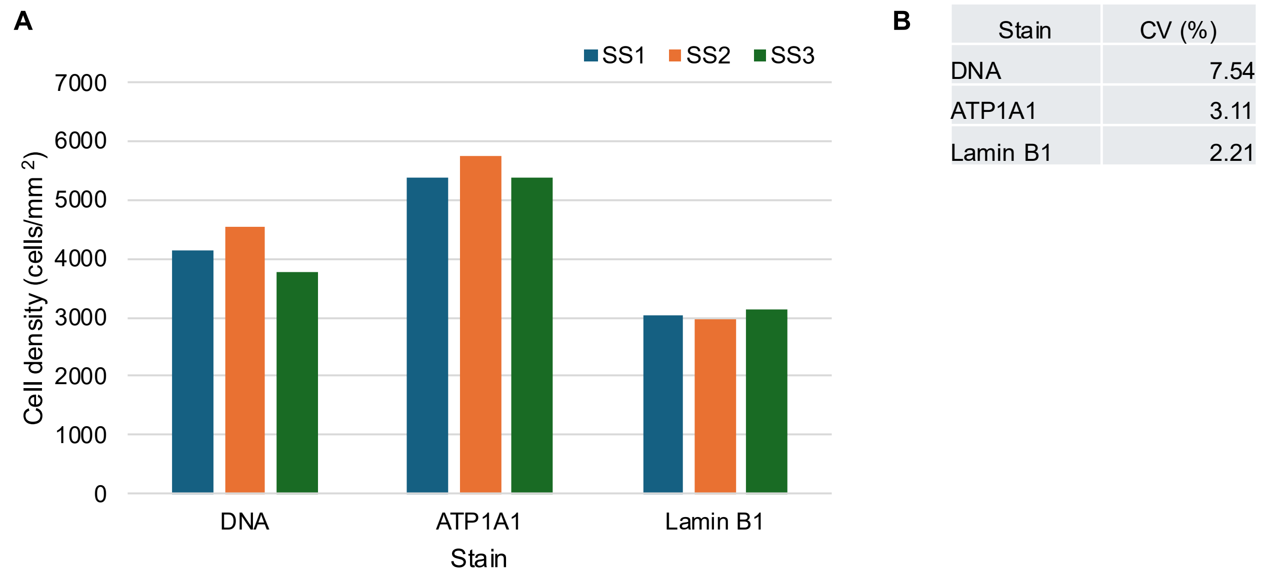
Figure 5. Scoring results of antibodies in the Mouse FF Cell Boundaries Assay Kit. Average scores from technical replicates of mouse FF colon are visualized in a heatmap (A) and a radar plot (B). n = 3 samples scored by three independent judges.

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

Table 5. SNR values for stains in the Mouse FF Cell Boundaries 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**
DNA	1849.06	250.01	7.40	3527.97	651.65	5.41
ATP1A1	1866.49	330.47	5.65	3638.78	75.85	47.97
Lamin B1	611.00	86.27	7.08	1457.30	149.75	9.73

Quantitative Reproducibility Assessment



Stain	CV (%)
DNA	7.54
ATP1A1	3.11
Lamin B1	2.21

Figure 6. Reproducibility of antibodies in the Mouse FF Cell Boundaries Assay Kit. Cell density measurements for each stain across technical replicates of mouse FF colon (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 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 Cell Boundaries Assay Kit in murine FF spleen.

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	-

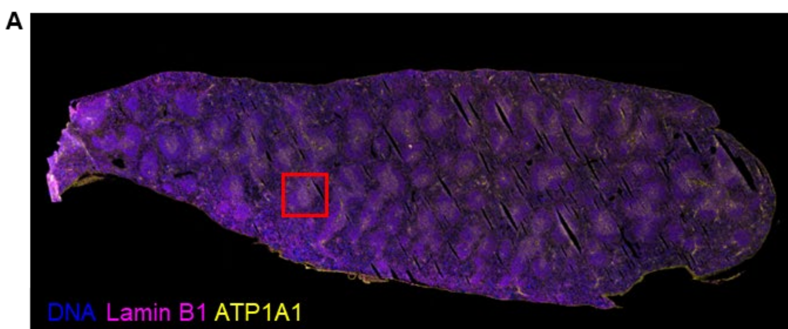
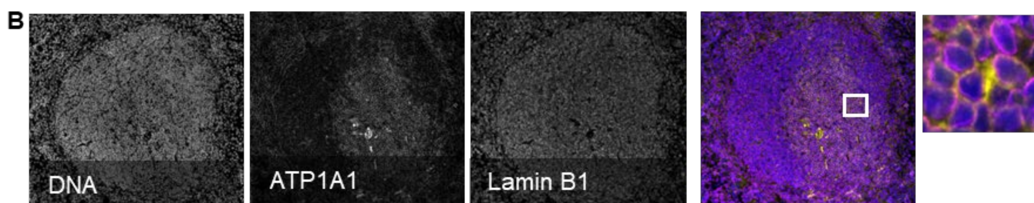


Figure 7. Example images for stains in the Mouse FF Cell Boundaries Assay Kit. Full overview of spleen (A) sample used in validation testing. The red box indicates region shown in enlarged images (B).



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 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.

<table>
<tr><th>Cycle</th><th>Target</th><th>Dilution</th><th>Stain Time (min)</th></tr>
<tr><td>1</td><td>DNA</td><td>1:8 million</td><td>5</td></tr>
<tr><td rowspan="2">2</td><td>ATP1A1</td><td>1:600</td><td rowspan="2">15</td></tr>
<tr><td>Lamin B1</td><td>1:1000</td></tr>
</table>

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.

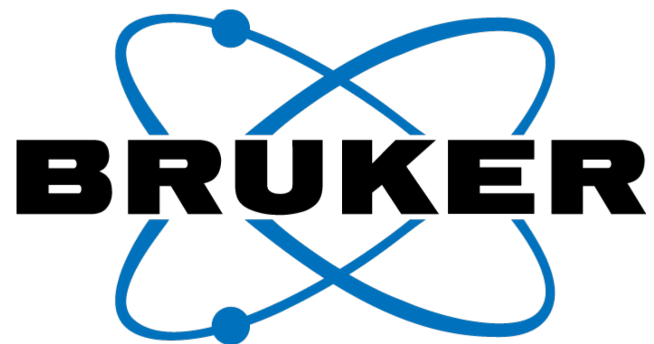
Score	Interpretation
2	Excellent, bright, specific stain
1	Acceptable but dim or high background
0	No staining
-1	Moderate, not abundant off target staining
-2	Strong and/or abundant unspecific staining

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/mm^2. The CV was calculated as the ratio of standard deviation to the mean expressed as a percent.



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