VistaPlex™ Assay Kit Design and Validation Process

Introduction

Spatial biology research supports human understanding of the progression and treatment of disease. Ideally, this knowledge comes from distilling complex cellular phenotypes and cell-cell interactions into actionable spatial signatures. VistaPlex validated assay panels for the CellScape™ Precise Spatial Proteomics platform are designed to support the streamlined and efficient generation of high-quality data to support reliable and actionable analysis and conclusions.

The CellScape platform is a high dynamic range (HDR), high resolution imaging system for spatially resolved quantitative phenotyping. Bringing unmatched versatility to spatial biology, the CellScape platform utilizes enhanced photobleaching for cyclic immunofluorescence (EpicIF™) technology and is compatible with most commercially available fluorescent primary and secondary antibodies. To save time and effort on creating multiplex proteomic assays from scratch, VistaPlex assay kits contain rigorously validated antibody panels and optimized protocols to support rapid and robust data generation. The panels are modular and can be used in combination with each other or with additional antibodies to create comprehensive and bespoke spatial assays for individual research needs.

Accurate interpretation of imaging results is dependent on thorough validation of the antibodies used[1], but despite their common utilization, there are no universally accepted guidelines for antibody validation. We have developed robust processes to select high-performing primary antibodies and validate multiplex antibody panels for use in spatial biology and immuno-oncology applications that leverage the CellScape platform. VistaPlex antibody panels are validated in both single-marker and combination assays for key multiplex assay performance metrics[2], including specificity, sensitivity, and reproducibility. Our unique reproducibility method is completed across three different instruments and two different laboratories.

Panel Design

Antibody Selection

To identify candidate antibodies for VistaPlex assay panels, we look at commercially available clones that are validated for immunofluorescence (IF) or immunohistochemistry (IHC). Clones are also selected based on the available conjugated fluorophores, as the EpicIF workflow is compatible with most organic dyes.

To design efficient assays, complementary fluorophores covering the available filter range are thoughtfully chosen to achieve high multiplexing in as few cycles as possible.

Localization

Each candidate clone is evaluated for staining of intended tissue structures (e.g., epithelia, stroma, lymphoid follicles) and sub-cellular localization (e.g. nucleus, cell membrane, cytoplasm) using control tissues with known abundant expression of the targeted biomarkers. Selected clones must exhibit a strong and specific signal with minimal off-target staining **(FIGURE 1)**.

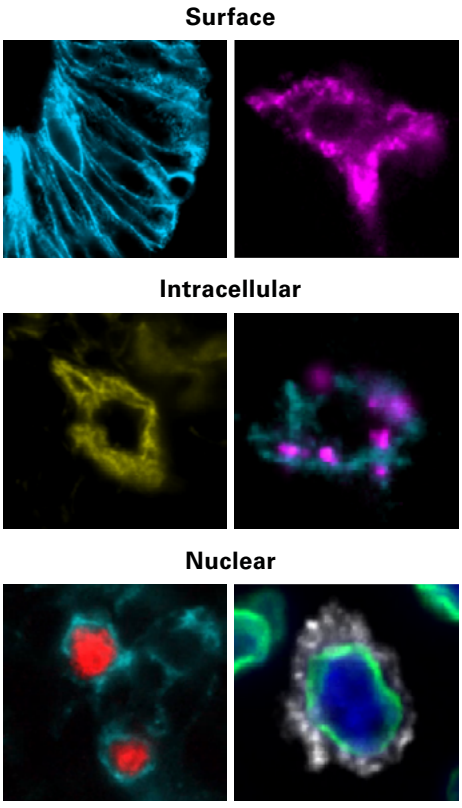

FIGURE 1: Candidate clones are evaluated for expected surface, intracellular, or nuclear staining. Surface staining is characterized by concentrated intensity along the cell periphery (top image set). Intracellular staining includes both cytoplasmic fills and punctate staining on intracellular membranes (middle image set). Nuclear staining is characterized by nucleus or nuclear envelope staining (bottom image set).

Antibody Titration

Using an optimal amount of each antibody is key to producing high quality results. Too little antibody yields a weak signal, while too much can increase background fluorescence and potential off-target binding **(FIGURE 2)**. Exhaustive titration is not always necessary with HDR imaging, but when an antibody exhibits high background signal or off-target binding, the lowest effective concentration of an antibody is identified and used for multiplex assays.

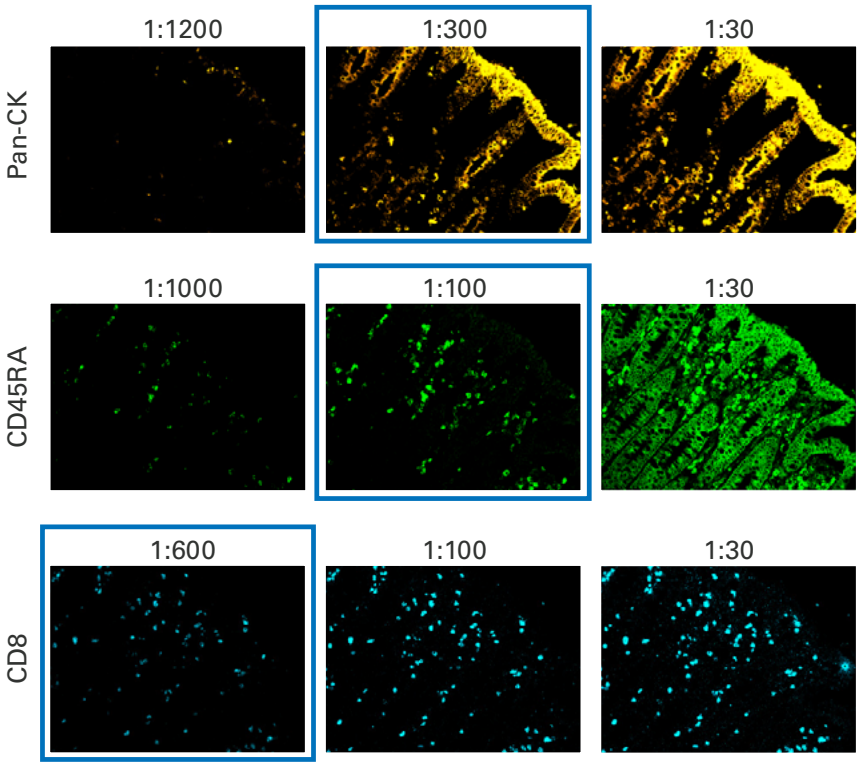

Figure 2: A human colorectal cancer FFPE tissue stained with Pan-CK, CD45RA, and CD8 antibodies at low, medium, and high concentrations. Blue boxes indicate recommended starting dilutions for use in VistaPlex multiplex assays.

Assay Validation

Following clone selection and assay design, the multiplex panels are validated for performance by specificity, sensitivity, reproducibility, and suitability measurements. Validation experiments are completed using serial tissue sections from multiple relevant tissues, on different CellScape instruments, in different laboratory sites, and with staining scored by multiple scientists **(FIGURE 3)**.

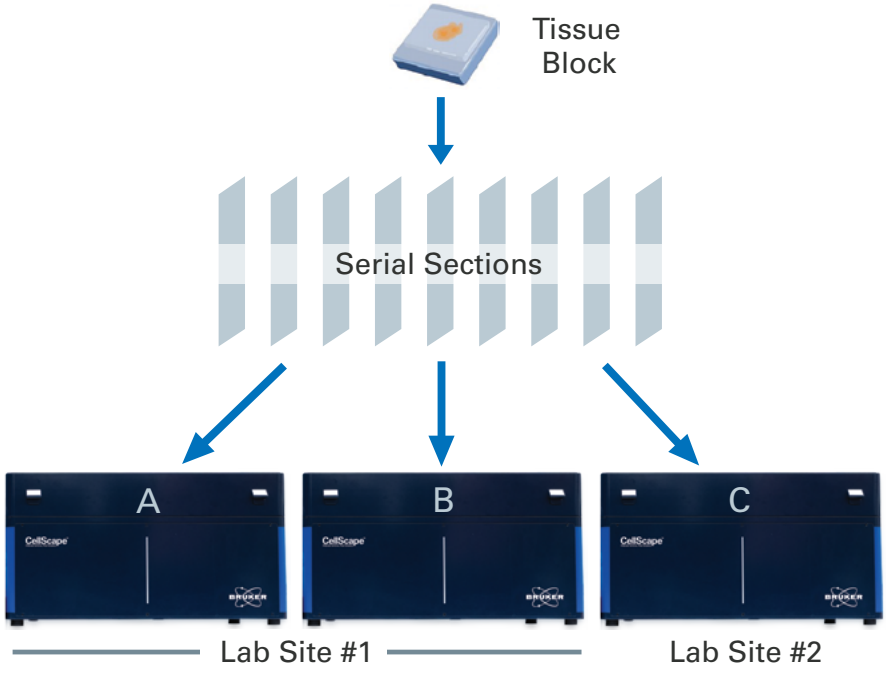

Figure 3: Schematic of the validation procedure using serial sections to complete both intra-run (technical replicates) and inter-run (different instruments) comparisons.

Specificity

In addition to localization confirmation, a qualitative specificity assessment confirms that antibodies bind only their intended biomarker targets. Specificity testing looks for expected staining of specific cell types based on counterstains and co-localization with known reference markers, as shown in the example in **FIGURE 4**. Staining patterns for each tissue are also evaluated semi-quantitatively, comparing expected expression scores of 0 to 2 with staining quality scores ranging from -2 to 2, as shown in **TABLE 1**. Staining scores of 2 or 1 are considered a pass for any stain, and a score of 0 passes if the marker is not expressed in the tissue type being analyzed. A score of -1 indicates the presence of moderate off-target staining, indicating that the antibody should be titrated and re-tested. A staining score of -2 indicates non-specific staining and would exclude an antibody from being used in VistaPlex assay panels.

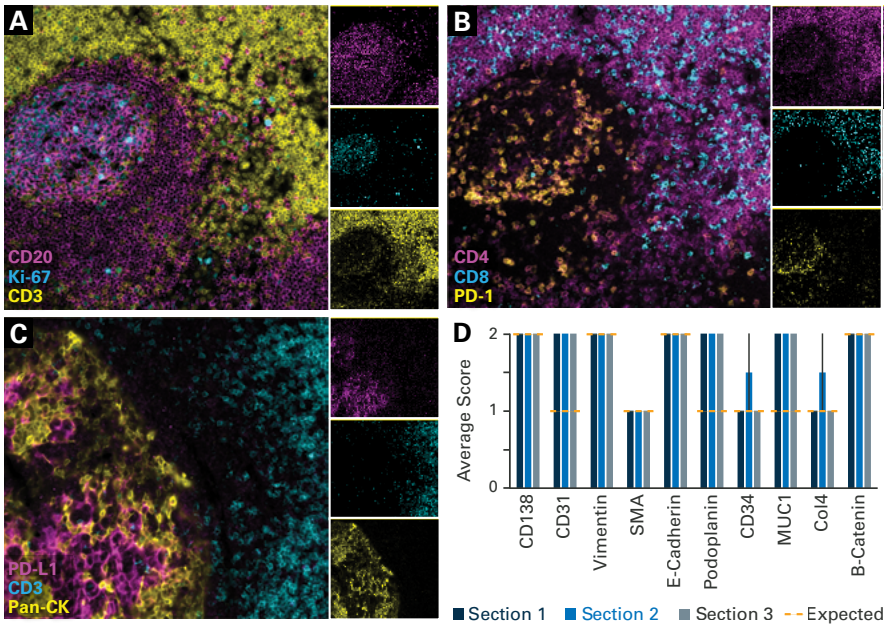

Figure 4. Assessments of staining quality. **A-C:** Colocalization assessment of eight antibodies from the VistaPlex Spatial Immune Profiling Kit. Distinct staining patterns confirm specificity of each antibody. **A:** CD3 and CD20 are expressed by T cells and B cells, respectively. Ki-67 is expressed by proliferating B cells. **B:** CD3-positive cells express either CD8 or CD4. CD4-positive follicular T cells express PD-1. **C:** PD-L1 colocalizes with cytokeratin-positive tonsillar epithelia. **D:** Example scoring of staining quality from select markers in the VistaPlex Tissue Architecture Kit, with the orange dotted line indicating the expected staining score based on the Human Protein Atlas.

Table 1. Scoring system used for qualitative stain evaluation.

Expected Expression Based on Protein Atlas		**Reviewer Stain Scores**	
Score	**Criteria**	**Score**	**Criteria**
2	High to medium expression level	2	Strong staining
1	Low expression or expression outside of functional compartments	1	Weak but acceptable staining
0	Not expressed	0	No staining
		-1	Moderate off-target staining or high background
		-2	Non-specific staining

Sensitivity

A quantitative sensitivity analysis is completed to ensure that detected signal is strong enough to differentiate positive cells from background fluorescence. An intensity ratio of ≥ 2 is required, though most antibodies exhibit staining intensity ratios of ≥ 4. Our standardized workflow to calculate an intensity ratio for each biomarker in a multiplexed panel is completed using at least 3 serial tissue sections and calculates mean fluorescence intensity (MFI) of stain and background as follows:

1. Identify at least 3 Regions of Interest (ROIs) per tissue section- ROIs should be matched between serial sections.
2. Perform cell segmentation to detect individual cells using StarDist, DeepCell, or similar pipelines.
3. Set threshold values for each marker based on the average intensity of the appropriate compartment (nucleus, cytoplasm or whole cell). Cells equal to or above the threshold value are classified as positive, while cells below the threshold are classified as negative.
4. Merge positive cell detections to create a single mask of the stained regions. Subtract the stained mask area from the original ROI to generate a second mask comprising the unstained regions **(FIGURE 5A)**.
5. Calculate Intensity Ratio = MFI stained / MFI unstained **(FIGURE 5B)**.

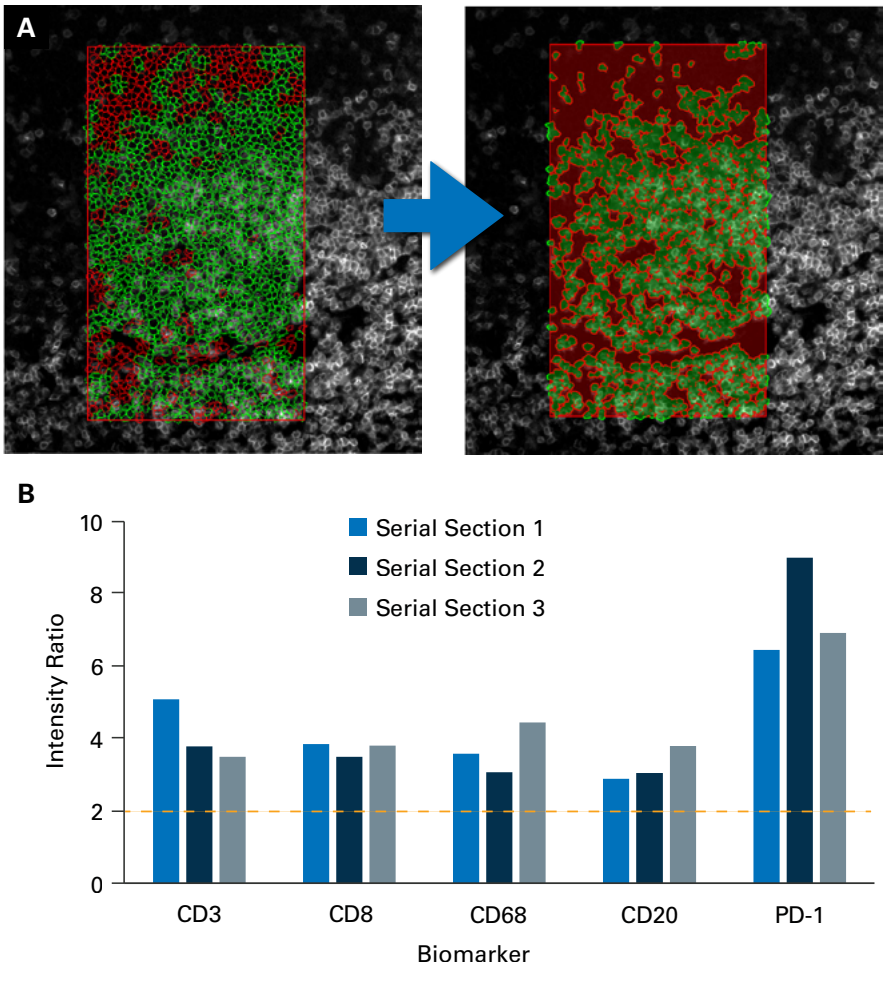

Figure 5. A, Thresholds (left), with positive cells identified in green and negative cells identified in red, are used to create two masks (right), with the stained mask displayed in green and unstained in red. **B**, Example data: Intensity ratios were compared across serial sections from the same run. Orange dotted line indicates minimum acceptable intensity ratio of 2.0.

Reproducibility

Additional measurements are taken of serial sections to determine intra-run and inter-run reproducibility of cell quantification enabled by VistaPlex panels. Quantitative readouts include mean fluorescence intensity and cell density (cells/mm²) of positive cells in addition to intensity ratio, and reproducibility is determined by comparing values obtained across instruments **(FIGURE 6)**.

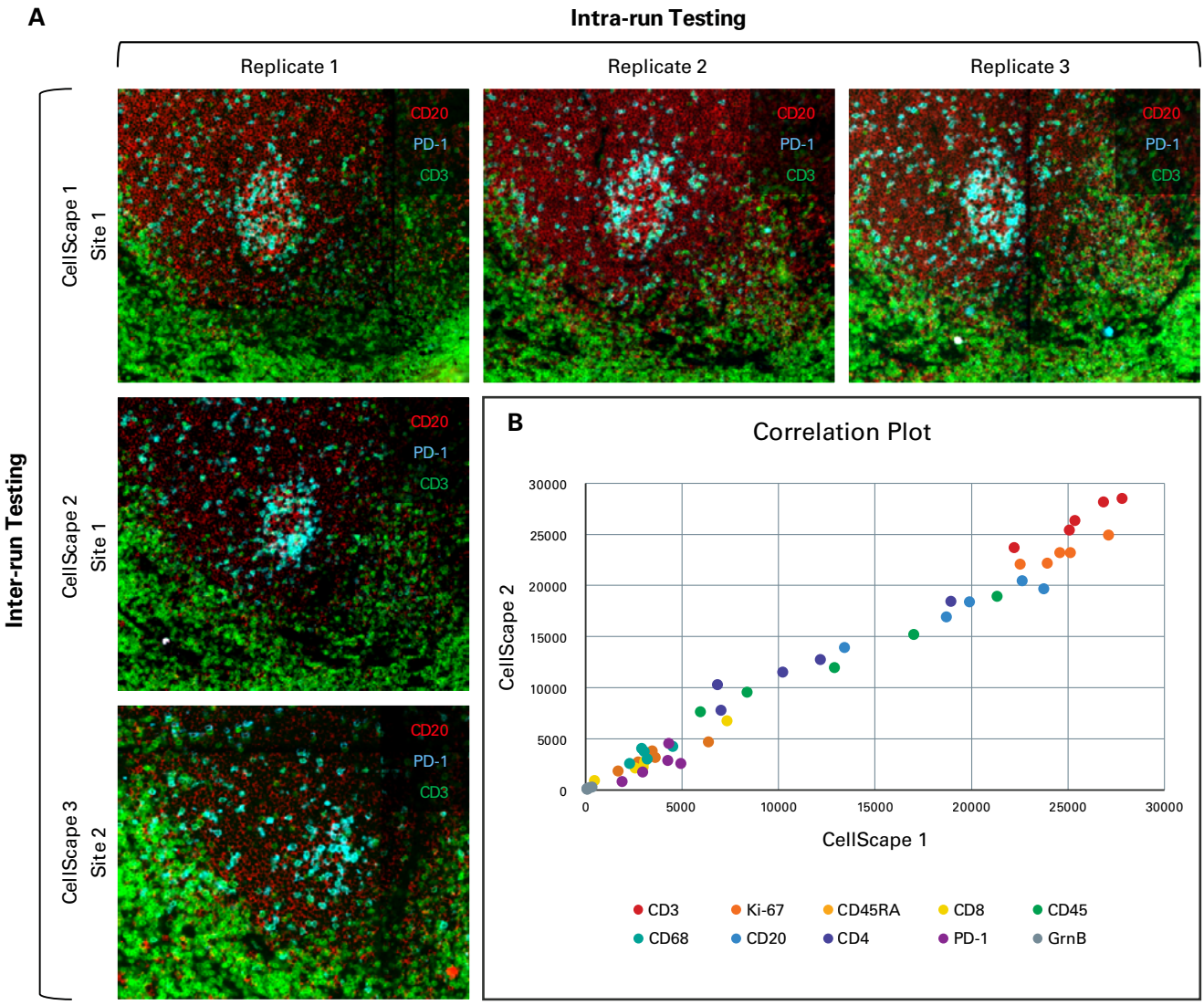

Figure 6. A, Illustration of intra-run, inter-run, and inter-site measurements of three serial tonsil sections stained with antibodies from the VistaPlex Spatial Immune Profiling Kit. Matched ROIs contained at least one germinal center in each. **B,** Correlation plot comparing cell density (cells/mm2) results of VistaPlex Spatial Immune Profiling markers between instruments.

Suitability

VistaPlex Assay Kits are designed to be suitable across a broad range of tissue and sample types for easy adoption into nearly any spatial biology workflow. Qualitative suitability assessments for each kit are generated using a range of tissue panels and/or tumor microarrays (TMAs), as illustrated in **FIGURE 7.** The panel is considered suitable for broad use when ≥70% of the tested tissues yield successful staining as determined by the scoring metrics described in **TABLE 1**.

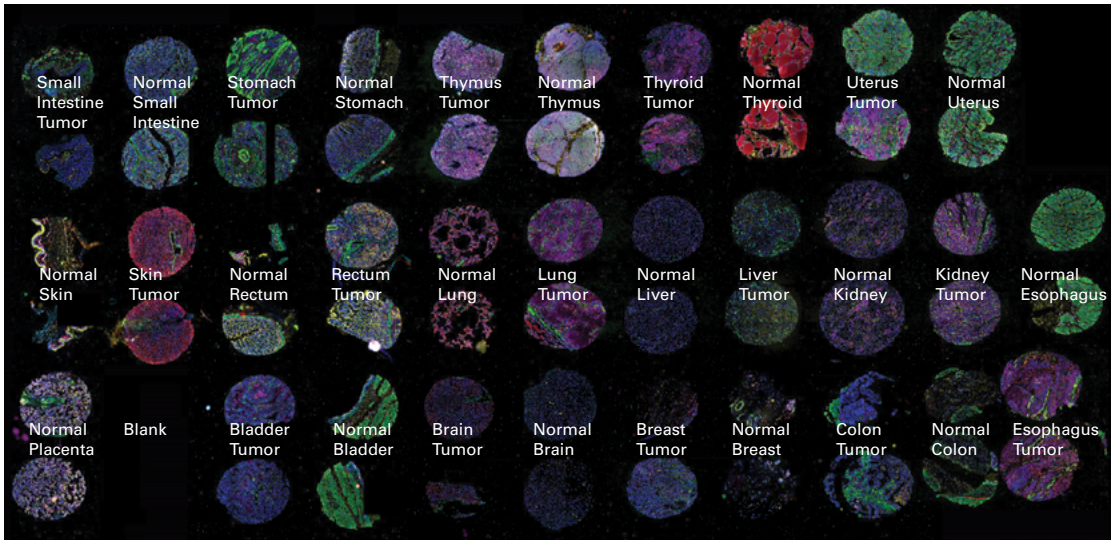

Figure 7. TMA with matched cancerous and non-cancerous (normal) tissue cores, stained with the VistaPlex Tissue Architecture kit, demonstrating suitability across sample and tissue types.

Summary

The CellScape platform with EpicIF technology enables simultaneous detection of multiple protein biomarkers on a single tissue section for deep phenotyping. VistaPlex panels contain carefully selected and optimized primary antibodies and are rigorously validated for specificity, sensitivity, reproducibility, and suitability across a range of sample types. Using these panels streamlines a spatial biology workflow, aids in the generation of consistent and reliable results, and provides a solid foundation on which larger customized assays can be built.

References

1. Du et al. (2019). Qualifying antibodies for image-based immune profiling and multiplexed tissue imaging. Nature Protocols, 14(10), 2900–2930.
2. Hickey et al. (2022). Spatial mapping for protein composition and tissue organization: a primer for multiplexed antibody-based imaging. Nature Methods, 19, 284–295.

VistaPlex panels are tested for use in a broad range of tissues; however, every biological sample is different, and panel performance may vary or need additional optimization based on sample type, integrity, age, and preparation. Quantitative validation metrics also vary between kits; see individual kit Validation Reports for more specific method information and data outputs.

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