Bruker Spatial Biology

Multi-Step Antibody Validation for High-Plex nCounter® Immune Pathways & Tumor Signaling Protein Panels

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WHITEPAPER

Background

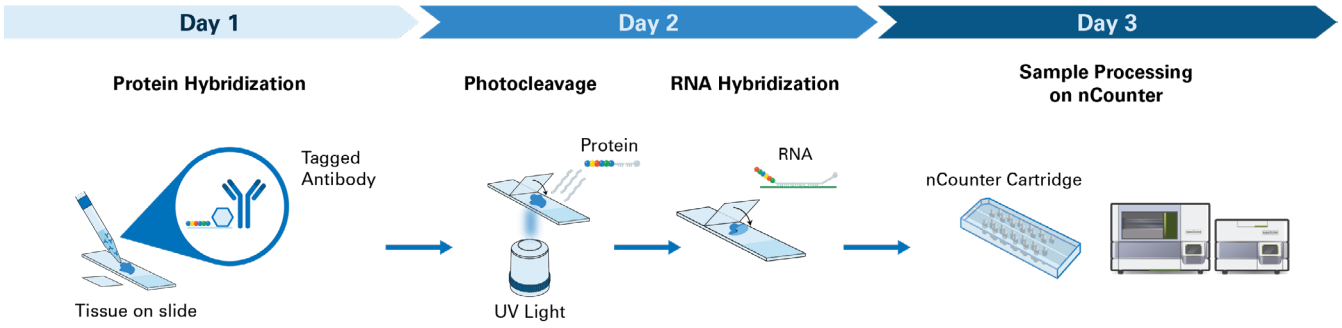
The complex interplay between genes and proteins driving disease biology requires a systems-level approach that unites transcriptomic and proteomic data. The new nCounter Multiomics Solutions achieve this by simultaneously detecting protein and RNA from the same sample, bridging the gap between gene expression and functional output to reveal the true phenotypic state of the tissue. By pairing our new high-plex protein panels (200–500+ targets) with any gene expression panel, the platform delivers actionable results from challenging samples, including FFPE tissues, in approximately three days with minimal hands-on time. To complete the workflow, the integrated Data Analysis Pipeline eliminates cross-platform harmonization issues, providing an intuitive, automated solution for seamlessly visualizing multi-dimensional RNA and protein data together.

To perform an nCounter High-Plex Multiomics assay, slide-mounted FFPE tissue is first stained with the protein panels of interest. The panels contain mixtures of target-specific antibodies, each one conjugated to a unique photocleavable oligo tag (DNA barcode).

UV illumination releases the oligo tags from the entire tissue section (**Figure 1**), which are then collected. Afterwards, mRNA is purified from the same tissue section. The oligo tags from the protein readout and purified mRNA are independently hybridized to fluorescently-labeled probes and digitally counted on the nCounter system enabling parallel protein and gene expression profiling. This unified approach yields quantitative data for hundreds of proteins alongside up to 800 RNA transcripts, all from a single tissue section.

Creating high-plex protein panels that bring the same highly reproducible results as the nCounter gene expression panels requires thoroughly validated antibodies. Every target must be measured with accuracy, specificity, and reproducibility to ensure proper binding, minimal cross-reactivity, and consistent performance across samples—so scientists can trust the biology reflected in their data. This level of validation provides confidence and assurance that discoveries are real, comparable, and ready for deeper investigation or translational follow-up.

FIGURE 1: Diagram illustrating the nCounter multiomics workflow.



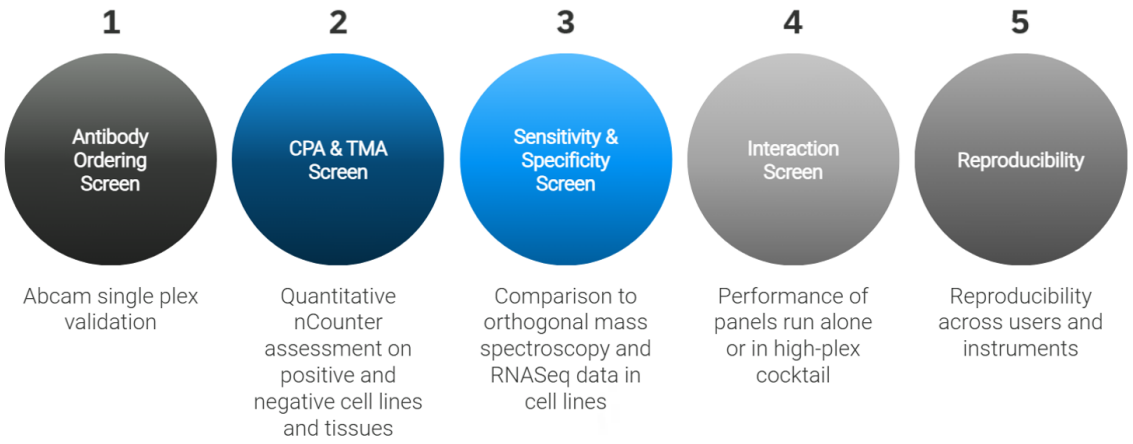
High-plex nCounter Protein Panels and the Need for Antibody Validation

Modularized protein panels currently available for multiomic assays include:

- Protein Core: 21 targets for Quality Control [QC], normalization, and high-value biological targets)
- Immune Pathways panel: 204 targets covering adaptive and innate pathways
- Tumor Signaling panel: 325 targets covering tumor signaling pathways
- Modular Panels can be used separately or combined (Core + Immune Pathways + Tumor Signaling) for 550-plex protein readout.

High-plex panel content demands rigorous antibody validation to ensure assay reliability and support data quality. Low-specificity antibodies could produce false positives by off-target binding, and antibodies with low signal could produce false negatives. These risks are addressed by a robust multi-step validation pipeline for every antibody before including it in a high-plex panel. The antibody validation pipeline involves multiple sequential stages, each designed to remove suboptimal performers and assess antibody performance (**Figure 2**).

FIGURE 2: Process for validating high-plex nCounter protein panels.



Each step has defined criteria and if an antibody fails at any stage, the antibody is removed or optimized (commonly by testing another clone or optimizing antibody concentration) and then re-tested. By the end of this rigorous validation pipeline, every included antibody has proven to be highly specific, sensitive, and reliable in the multiplexed assay. Below, each step is discussed in detail.

1. Antibody Ordering Screen

Antibodies in the nCounter human protein panels are sourced from Abcam, a vendor known for their rigorous validation process. Each recombinant monoclonal antibody includes IHC data and images, plus extensive application testing across up to 10 methods (e.g., Western blot, ICC/IF, IHC, ELISA, IP, ChIP, and/or flow cytometry). Leveraging these data provides high confidence in baseline specificity and performance, and chosen antibodies are conjugated with oligo tags and combined into an antibody mix before proceeding through the next steps in the validation pipeline.

2. Cell Pellet Array and Tissue Microarray Screening

To quantitatively assess antibody performance under both controlled and biologically relevant conditions, we applied the nCounter Multiomics workflow on FFPE cell pellet arrays (CPAs) and tissue microarrays (TMAs). For each target, minimum thresholds of SNR ≥10 and signal dynamic range ≥5 were defined to ensure strong signal above background and detection of meaningful variation across samples. CPAs captured a wide range of expression states across 23 tissue types, while TMAs contributed additional biological diversity by incorporating normal and diseased tissues across 38 tissue types (**Figure 3 A**).

For each antibody, some cell lines and tissues serve as positive controls by expressing the antigen, and others as negative controls by lacking expression (**Figure 3 B and 3 C**). When no suitable cell line was available, engineered cells or tissues were used. In TMAs, observed expression patterns matched known biology; for example, prostate-specific antigen, liver arginase, and S100 beta show high counts and strong tissue specificity in prostate, liver, and nerve tissues, respectively, with low counts elsewhere, and CD3 epsilon is additionally enriched in lymphoid tissue (**Figure 3C**).

Each antibody had to meet the minimum thresholds in either CPAs or TMAs to proceed to the next screening step. Antibodies that failed to produce signals in tissues where expression was expected were excluded from the panels.

Phospho-specific antibodies required additional scrutiny due to the dynamic nature of phosphorylation and the potential for non-specific binding. To confirm specificity, we performed phosphatase treatment assays using matched sets of CPAs. One set was treated with phosphatase to remove phosphate groups, while the other remained untreated. A phospho-specific antibody was considered valid if it showed strong signal in the untreated (phospho-present) condition and a significantly reduced signal >90% in the treated (phospho-absent) condition (**Figure 4**). These results provided strong evidence that the phospho-specific antibodies included in the panel can distinguish phosphorylated targets with high fidelity, further enhancing the biological relevance of the assay.

FIGURE 4: Phosphatase treatment demonstrates specificity of phospho-specific antibodies.

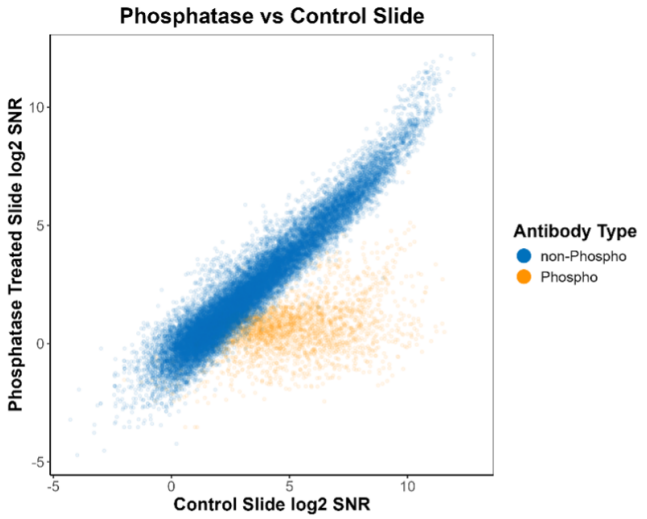
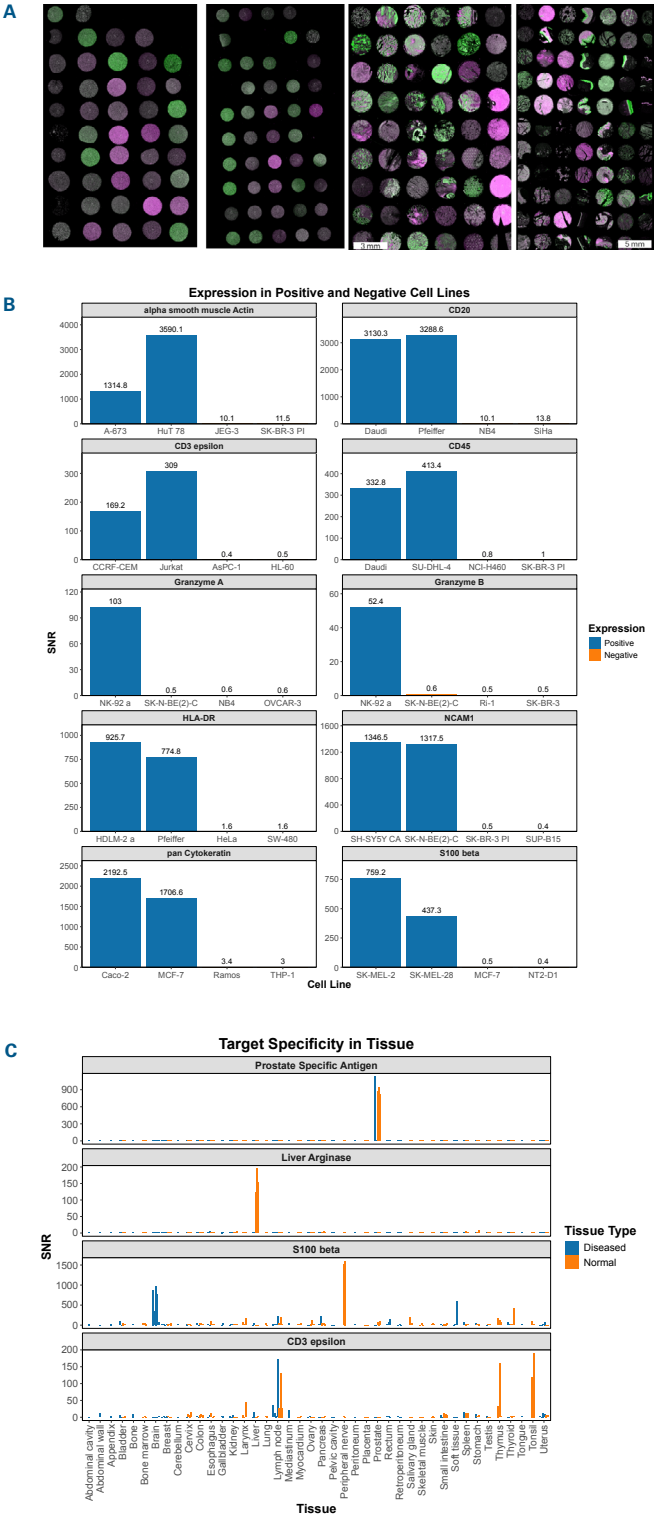


FIGURE 3: A) CPAs and TMAs profiled on GeoMx to access antibody performance. **B**) Signal-to-noise (SNR) in positive and negative cell lines. **C**) Target specificity in tissues.

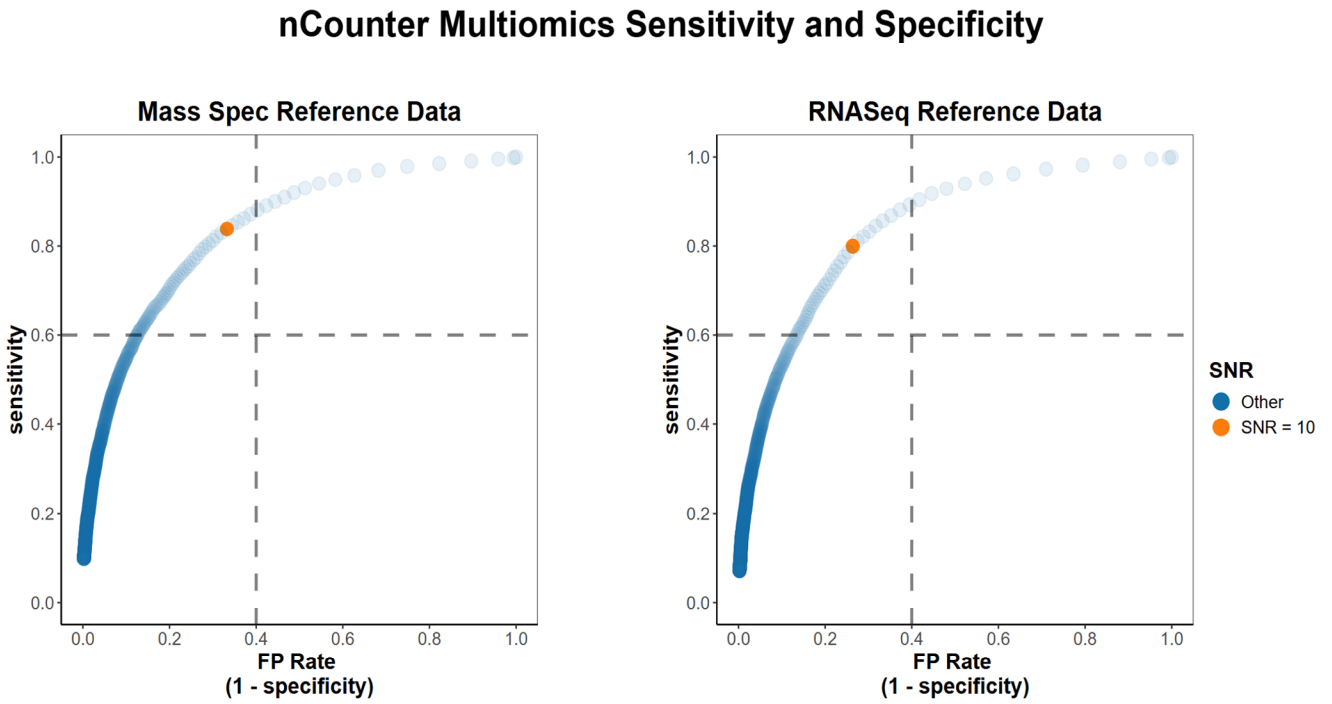


3. Sensitivity and Specificity using orthogonal datasets

To assess the overall performance of the nCounter High Plex Multiomics assay, we benchmarked the panel against orthogonal datasets from mass spectrometry and RNA sequencing (RNAseq) using overlapping cell lines (Gonçalves E, et al., 2022; Jin H, et al., 2023). While orthogonal comparisons provide valuable context, high concordance between antibody-based protein measurements and transcriptomic or proteomic data is not expected for all targets. Mass spectrometry is not ideal for detection of low-abundance proteins, membrane-bound proteins, or proteins with poor ionization efficiency. RNAseq, on the other hand, provides high sensitivity for transcript detection but does not account for translational control, protein degradation, or post-translational modifications (Vogel & Marcotte, 2012).

Despite these limitations, orthogonal datasets offer a useful baseline for evaluating antibody-based assay performance. By generating receiver operating characteristic (ROC) curves at varying SNR thresholds, we calculated bulk sensitivity and specificity across the protein panel (**Figure 5**). Benchmarking against mass spectrometry and RNASeq confirmed a bulk sensitivity of 83.8% and 79.9% and specificity of 66.7% and 73.6%, respectively, at an SNR = 10. Together, these results demonstrate good sensitivity and specificity between the nCounter High Plex Multiomics assay and orthogonal reference datasets.

FIGURE 5: ROC curves with mass spectroscopy and RNASeq reference datasets



4. Interaction Screen (Multiplex Compatibility)

When multiplexing hundreds of antibodies in a single assay, it is essential to ensure that individual reagents do not interfere with one another. To evaluate multiplex compatibility, we conducted a series of interaction studies using FFPE serial sections of colon, lung, and skin cancer samples, which express a broad range of immune and stromal markers. These sections were stained with the Core alone, Core plus one panel, or the full combination of Core, Immune Pathways, and Tumor Signaling Protein Panels.

The log2 SNR transformed protein expression values for all overlapping targets were compared by calculating the Pearson correlation coefficient (R) of overlapping targets between each dataset and the full panel (Core + Immune Pathways + Tumor Signaling) across the three cancer types (**Figure 6**). Pearson correlation coefficients (R) indicate strong agreement ($R \geq 0.97$) across all comparisons, demonstrating high reproducibility and consistency of measurements regardless of plex level or cancer type, which confirmed the full ~550-plex panel and its subsets can be used interchangeably without introducing systematic bias or interference.

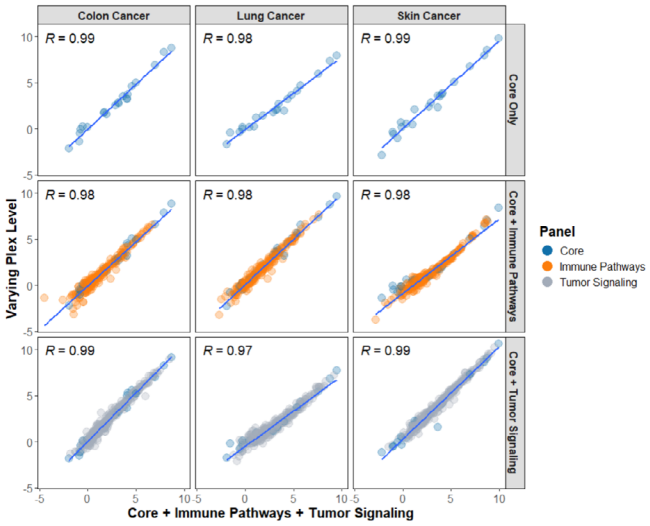
5. Reproducibility Testing

The final step in the validation process is to ensure that the assay delivers consistent results across different users and experimental runs. Even well-validated antibodies must demonstrate reproducibility to ensure the reliability of the assay in diverse laboratory settings. To assess user-to-user variability, three independent users performed the assay following the nCounter Multiomics User Manual using the same set of samples. Pearson correlation coefficients between any two users' datasets exceeded 0.90, demonstrating consistency, robustness and ease of use of the nCounter assay. As a result, researchers can expect reproducible performance in their own laboratories.

Conclusion

All antibodies included in high-plex nCounter Immune Pathways and Tumor Signaling Protein Panels undergo the rigorous validation process described above. Collaboration with trusted vendors such as Abcam provides a reliable pipeline of high-performance antibodies, each subjected to the same stringent evaluation. Approximately 90% of candidates pass all validation stages and are incorporated into the final product, underscoring the value and reliability of using pre-validated antibodies from trusted sources. Use of high-quality Abcam antibodies also facilitates a smooth transition from biomarker discovery to more translational studies, as the same antibodies can be leveraged for targeted bulk or spatial approaches. Bruker delivers these pre-validated antibodies in its high-plex nCounter Immune Pathways and Tumor Signaling Protein Panels, enabling researchers to focus on their scientific questions and move directly to experiments without the burden of antibody screening or optimization.

FIGURE 6: Interaction screen to demonstrate concordance. Each row represents a different panel configuration: Core Only (top), Core + Immune Pathways (middle), and Core + Tumor Signaling (bottom).



References

1. Gonçalves E, et al. (2022). Pan-cancer proteomic map of 949 human cell lines. *Cancer Cell*, 40(8):835–849.e8.
2. Jin H, et al. (2023). Systematic transcriptional analysis of human cell lines for gene expression landscape and tumor representation. *Nat Commun*, 14:5417.
3. Vogel C & Marcotte EM. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet*, 13(4):227–232.

For more information, please visit brukerspatialbiology.com/products/ncounter-assays-panels/ncounter-protein-assays/

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