Multi-Step Antibody Validation for nCounter High-Plex Multiomics Panels

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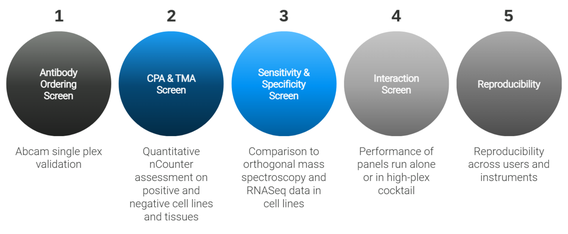
Introduction

Expanded development on the nCounter® Analysis System now enables simultaneous quantification of over 500 proteins and up to 800 transcripts from a single FFPE tissue sample. With the introduction of two high-plex protein panels used in combination with any prebuilt nCounter® RNA Panel this new capability brings a novel non-destructive FFPE assay with new depth, simplicity and reliability for immuno-oncology multiomics research. The nCounter® platform has long been known as the gold standard for oncology and immunology studies including diagnostic development. Confirming performance in support of translational and clinical applications is essential part of this capability

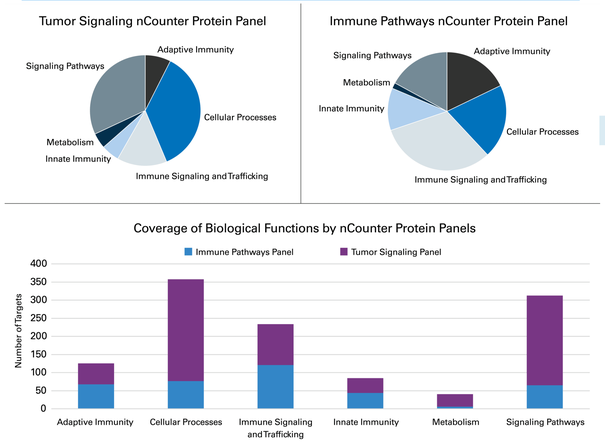
To ensure high data integrity in this complex environment, we developed a rigorous multi-step antibody validation pipeline. The process begins with an initial IHC screening, performed by our antibody partner AbCam, to confirm specificity in known positive and negative tissues. These single-plex IHC validated antibodies are individually conjugated to nCounter-compatible UV-cleavable oligonucleotides. Functional performance of the high-plex protein panels was assessed using more than 90 cell lines and more than 100 tissue cores from over 30 tissue types featuring cancerous and healthy FFPE samples. Sensitivity and specificity are benchmarked against available orthogonal RNA-Seq and mass spectrometry datasets for the cell lines. The specificity of phospho-specific antibodies was also tested in a phosphatase treatment assay, confirming that >90% of signal is reduced with treatment.

To demonstrate the compatibility of these antibodies in a high-plex panel, we show antibody performance remains robust when our protein panels are stacked together or run independently. Finally, reproducibility is confirmed across users and runs. This validation framework ensures high specificity, sensitivity, and reproducibility, enabling confident multiomic profiling of complex tissue samples and accelerating translational insights in cancer biology.

Antibody Validation Workflow



Stackable Panel Content with up to 550 Targets



Functional Screen Across Diverse FFPE Cell Lines and Tissue Microarrays on GeoMx DSP

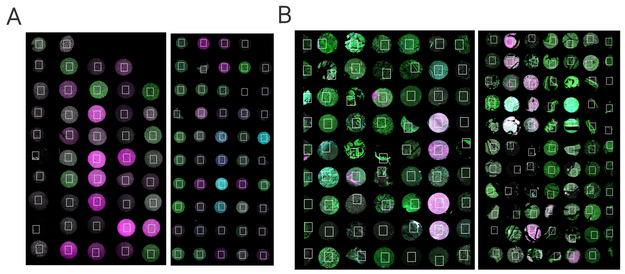


Figure 1 | GeoMx Digital Spatial Profiler (DSP) experimental design for high-throughput discovery applications. GeoMx exported images of scanned slide with 660 x 785 um area-of-interest (AOIs) on 5 µm thick section of FFPE cell pellet or tissue microarrays. (A) CPA46 and CPA49 representing 89 cell lines from 23 human tissue types. (B) FDA999 (normal tissues) and FDA800 (tumor tissues) representing 38 profiled human tissues types. Slides stained with fluorescent morphology antibody markers to guide ROI selection, PanCK+ for epithelial cells (green), CD45+ for immune cells (magenta), CD68+ for macrophages (cyan), and DNA stain (gray).

Phosphatase Treatment Demonstrate Specificity of Phospho-Specific Antibodies

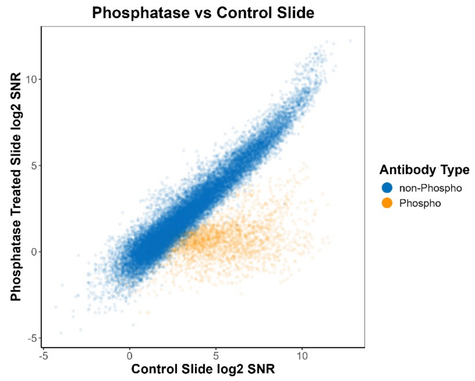


Figure 2 | Phosphatase treatment confirms specificity of phospho-specific antibodies. Matched FFPE cell pellet arrays (CPAs) were profiled in parallel with or without phosphatase pre-treatment to remove phosphate groups. Log2 SNR values measured on the untreated control slide (x-axis) are plotted against log2 SNR values measured on the phosphatase-treated slide (y-axis). Non-phospho antibodies (blue) show strong concordance between conditions, whereas phospho-specific antibodies (orange) show marked signal loss after phosphatase treatment, consistent with loss of the phosphorylated epitope. A phospho-specific antibody was considered validated when it produced strong signal in the untreated (phospho-present) condition and exhibited a >90% reduction in signal following phosphatase treatment (phospho-absent), supporting high-fidelity discrimination of phosphorylated targets.

Targets Demonstrate High Specificity in Various Tissue Types and Diverse Cell Lines

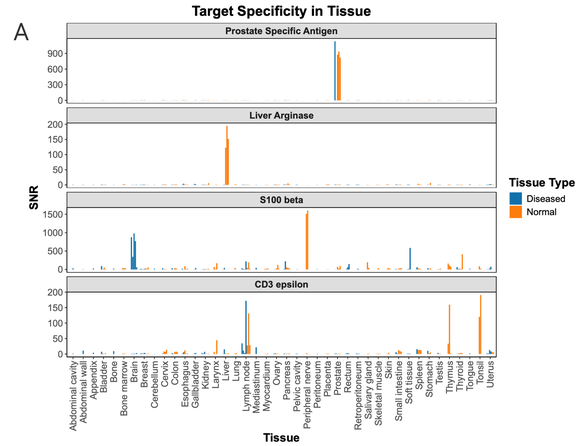


Figure 3A | Tissue microarray (TMA) screening confirms target specificity across normal and diseased tissues. FFPE tissue microarrays (TMAs) spanning normal and diseased tissues were profiled to assess antibody specificity in biologically relevant contexts. Representative examples illustrate tissue specific expression patterns consistent with known biology, including high tissue-enriched signal for prostate-specific antigen in prostate tissue, liver arginase in liver tissue, and S100 beta in nerve tissue, with low signal in non-matching tissues; CD3 epsilon is additionally enriched in lymphoid tissue. Bars are stratified by tissue category (normal vs. diseased) to show specificity across disease states.

Figure 3C | Cell pellet array (CPA) screening demonstrates robust signal-to-noise separation between positive and negative controls. FFPE cell pellet arrays (CPAs) were profiled using the nCounter Multiomics workflow to evaluate antibody performance in a controlled context across diverse expression states. For each target, cell lines expected to express the antigen (positive controls) were compared to cell lines lacking expression (negative controls) using signal-to-noise ratio (SNR), with minimum screening thresholds of SNR ≥ 10 and signal dynamic range ≥ 5 used to ensure strong signal above background and meaningful variation across samples.

Bulk Sensitivity and Specificity Screen using Orthogonal Datasets

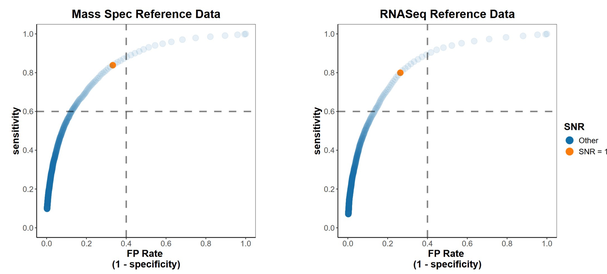
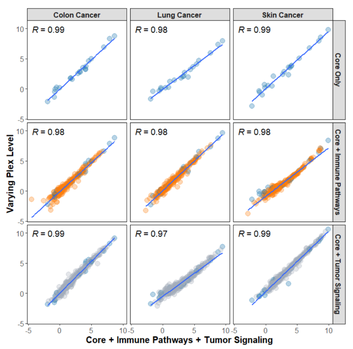


Figure 4 | Bulk sensitivity and specificity of nCounter high-plex protein measurements benchmarked to orthogonal reference datasets. Receiver operating characteristic (ROC) curves were generated by comparing nCounter protein data to orthogonal mass spectrometry (left) and RNA-seq (right) reference datasets across overlapping cell lines and varying the signal-to-noise ratio (SNR) threshold used to call targets as detected. Sensitivity (y-axis) is plotted versus false positive (FP) rate = 1 – specificity (x-axis). Each point represents performance at a different SNR threshold (blue), with the SNR = 10 operating point highlighted (orange). At SNR = 10, benchmarking yielded bulk sensitivity/specificity of 83.8% / 66.7% versus mass spectrometry and 79.9% / 73.6% versus RNA-seq, supporting good overall assay performance relative to orthogonal baselines.

Interaction Screen (Multiplex Compatibility) and Reproducibility

Figure 5 | High concordance across plex levels confirms multiplex compatibility. FFPE serial sections from colon, lung, and skin cancers were stained with Core only, Core + Immune Pathways, Core + Tumor Signaling, or the full combined panel (Core + Immune Pathways + Tumor Signaling; ~550-plex) to assess whether increasing plex introduces systematic interference. Log2 signal-to-noise ratio (SNR) expression values for overlapping targets were compared between each reduced-plex condition (y-axis, "Varying Plex Level") and the full panel reference (x-axis). Each point represents an overlapping target measurement, colored by panel, demonstrate strong agreement across cancer types and panel configurations (R = 0.97–0.99), indicating reproducible quantification and minimal multiplex interference when panels are used alone or in combination.

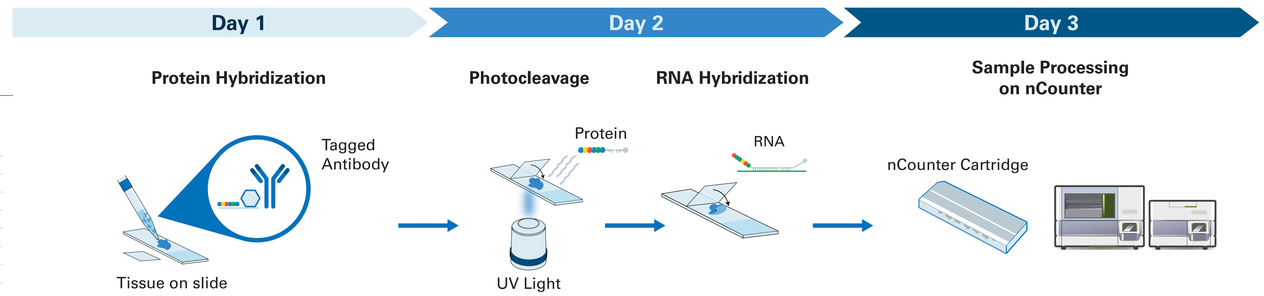


Conclusion

1. **Rigorous validation for every antibody:** All antibodies included in the high-plex nCounter Immune Pathways and Tumor Signaling Protein Panels undergo a multi-stage validation pipeline to ensure assay reliability in high-plex format
2. **Trusted input + standardized screening**: Partnership with Abcam provides a consistent pipeline of recombinant antibodies that enter the workflow with strong baseline vendor validation, then receive the same in-house evaluation in multiplex context.
3. **~90% of candidate antibodies successfully pass validation** criteria and are incorporated into the final panels.
4. **Pre-validated content accelerates translational research**: Using high-quality antibodies supports a smooth path from biomarker discovery to translational workflows, leveraging the same antibody content for targeted bulk or spatial follow-up

nCounter Multiomics Assay Workflow

Simple Bulk Multiomics Workflow for Protein and RNA readout from same FFPE section



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