WHITEPAPER

Multi-step Antibody Validation for the GeoMx® Digital Spatial Profiler

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Background

Understanding protein expression patterns within tissue compartments is imperative to investigating a wide range of biological questions, from examining the tissue specificity of disease etiology to elucidating cell fate determination or predicting patient response to pharmacological intervention. Historically, low plex immunohistochemical (IHC) approaches have been employed to assess the spatial heterogeneity of protein expression in tissue slices, but these techniques are of limited utility due to the challenge of measuring multiple targets in parallel, including additional tissue requirements, potential auto-fluorescence issues, and image processing capabilities across multiple slides.

The GeoMx Digital Spatial Profiler (DSP) enables investigation of high-plex, spatially resolved protein targets from a single slide mounted formalin-fixed paraffin-embedded (FFPE) or fresh frozen sample. Digital Spatial Profiling technology employs antibodies that are covalently linked to a DNA indexing oligo with a UV-cleavable linker. These affinity reagents are used to stain tissue sections and then UV light focused with the aid of micro-mirrors is used to precisely liberate indexing oligos from any region of interest on the tissue (**FIGURE 1A, 1B**). These oligos are then collected and digitally quantified to assess expression levels in a spatially resolved manner.

Product Highlights

GeoMx DSP capabilities and system validation studies were recently published by Merritt et al.[1] and Gupta et al.[2] See these for a more detailed review of GeoMx validated antibodies and for a comparison of GeoMx DSP to quantitative immunofluorescence (QIF) and immunohistochemistry (IHC) results.

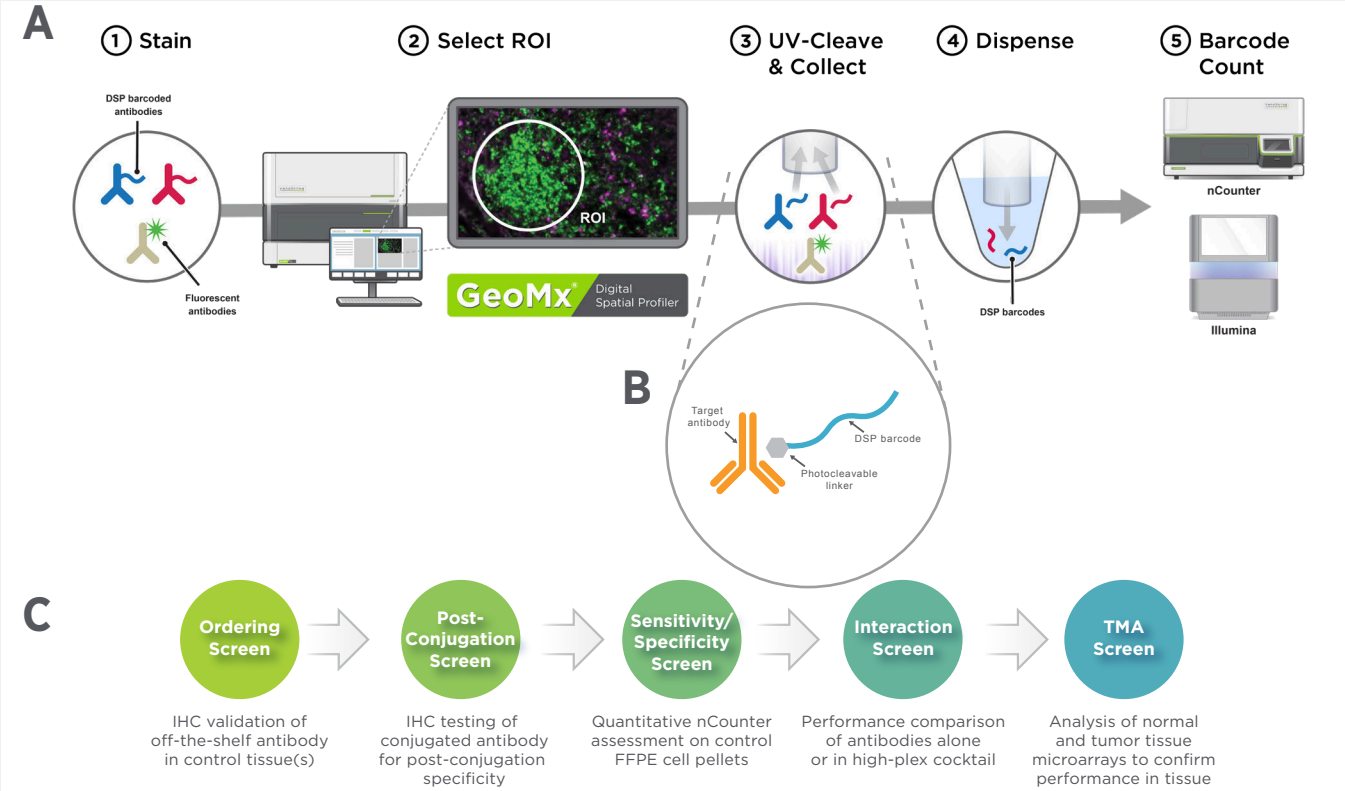

FIGURE 1: Validation of GeoMx antibodies. A) GeoMx workflow with a protein assay. B) Unconjugated antibodies are covalently conjugated to countable indexing oligos with a UV-cleavable moiety. C) All commercially-available GeoMx antibodies undergo a thorough antibody validation process.

NanoString follows a robust process to screen, conjugate, and validate oligo-tagged antibodies prior to incorporation into commercial panels (**FIGURE 1C)**. Antibodies with poor quality can lead to wasted time and resources in a highly dynamic research world, including false positives and non-reproducible results.[3,4] To enable customers to focus on research, every antibody in NanoString's commercial panels undergoes rigorous testing to ensure appropriate specificity, sensitivity, and overall performance. In this white paper, we review the multistep process for validating the antibodies used for protein analysis on GeoMx® DSP. NanoString's protein validation process includes confirming antibody specificity through IHC staining patterns, and through performance in cell pellet arrays and tissue microarrays with positive and negative controls (in line with recent suggestions for antibody validation from the Society for Immunotherapy of Cancer.[5]

For any GeoMx protein experiment, users must run a 20-plex core antibody panel (plus 6 controls) and can add additional 10-plex modules to increase plex. The core panel includes key targets of interest as well as IgG controls and housekeeping proteins which can be used for background assessment and data normalization. Users can add up to six 10-plex modules focused on different areas of biology to the 20-plex core. Every module is validated to ensure consistent performance with the core and other modules. Users can further customize their set of antibodies with up to 10 custom targets provided through a conjugation service by Abcam, enabling inclusion of their 6,000+ recombinant monoclonal antibodies, or by NanoString. In total, up to 96 protein targets can be profiled using the GeoMx DSP with nCounter® system readout. Larger plex protein assays, compatible with next-generation sequencing (NGS) are in development and will also be validated following the approach described in this white paper.

Antibody Ordering Screen

The first step in the antibody validation process is an ordering screen, in which NanoString screens the performance of off-the-shelf antibodies in FFPE tissue (**FIGURE 2A**). We use multi-organ, species-appropriate (e.g., human or mouse) tissue microarrays (TMAs) for assessment. Target-specific tissues are procured and used in lieu of a TMA as required (e.g., Alzheimer's brain tissue to assess amyloid plaque targeting antibodies). Tissues are sectioned, stained with the antibody of interest and a secondary antibody, and visualized with 3′-Diaminobenzidine (DAB). Stained sections are digitized on a Zeiss AxioScan and reviewed by a medical pathologist. Qualitative pathologist review includes an assessment of the intensity, compartment-specific expression, and cellular localization of each marker relative to its expected expression pattern as described by the vendor, review of the Human Protein Atlas[6], and consideration of peer-reviewed literature.

Conjugation Purification and Post-conjugation Screen

After we screen an antibody for appropriate staining in FFPE, it is conjugated with an indexing oligo for downstream collection on GeoMx DSP. To properly differentiate antibodies within the core or module, each antibody receives a unique oligo sequence. Unconjugated oligo and excess IgG are purified out from the mixture using high-performance liquid chromatography (HPLC), ensuring a pure product with maximum sensitivity. Purity and degree of labeling of each conjugated antibody are also established by spectroscopy and additional SDS-PAGE and DNA gel electrophoresis analysis.

We then conduct a post-conjugation antibody staining assessment to ensure oligo-conjugation has not adversely affected antibody performance (**FIGURE 2B**). Antibody concentrations are varied in order to achieve specific staining

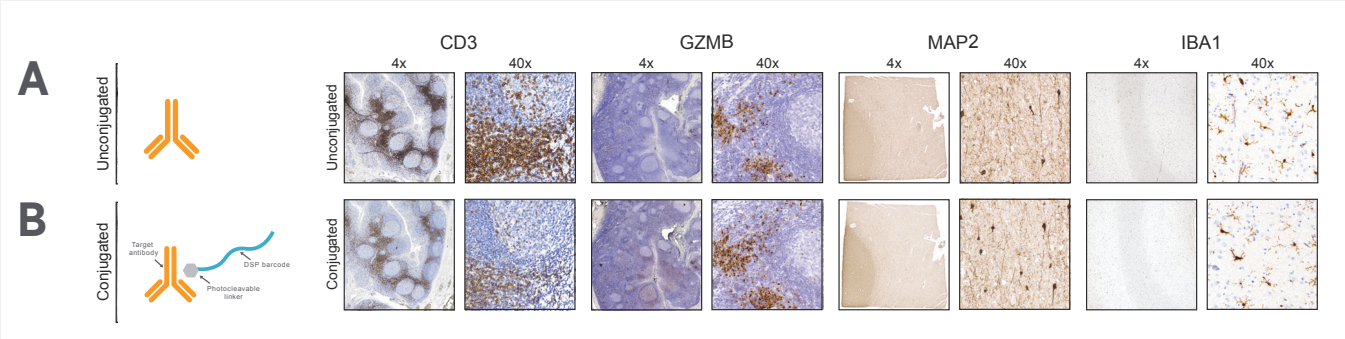

FIGURE 2: Pre- and post-conjugation antibody screen. Unconjugated antibodies (A) are screened in control tissues and reviewed by a pathologist. Unconjugated and oligo-conjugated (B) CD3 and GZMB antibody staining specificity on normal human tonsil, and MAP2 and IBA1 antibody staining specificity on normal human brain visualized with DAB IHC are compared. IHC staining patterns are reviewed by a pathologist to ensure that conjugation has not affected the pattern of binding.

intensity while minimizing the background stemming from non-specific binding. Since oligos may block access of secondary antibodies to conjugated antibodies' Fc regions, higher concentration of conjugated antibodies are used in comparison to unconjugated ones. Typically, the test concentration for the unconjugated antibody is 0.2-4 µg/mL and 1-12 µg/mL for the conjugated antibody. A medical pathologist compares the pre- and post-conjugation DAB IHC as described above.

Sensitivity/Specificity screen

Antibodies that pass the post-conjugation assessment must then demonstrate appropriate signal on the GeoMx® DSP system. Using FFPE cell pellet arrays that contain positive and negative control pellets stained with the antibody of interest, we run three geometric ROIs sizes (32 µm, 50 µm, and 300 µm diameter circles) in duplicate on the GeoMx DSP. Positive and negative cell lines for each antigen are identified using RNA expression levels measured on the nCounter with 100 ng of total RNA isolated from matching FFPE cell lines. Public databases such as Human Protein Atlas are also utilized to identify positive and negative controls. When no positive cell lines are identified, we screen analytes using healthy or diseased tissue.

Phospho-specific antibodies are validated with established models, which include phosphatase inhibitor treatment of cell lines prior to formalin fixation. These slides are then tested with and without phosphatase treatments before antibody incubation to assess specificity. Phospho-specific antibodies are also tested with a peptide competition assay. Unlike the phosphatase treatment which targets the antigen, the peptide competition assay targets the antibody. This assay utilizes synthetic phospho-peptides that selectively bind to their respective antibody, preventing the antibody from binding to the epitope of the full-length protein target found within the cells or tissue. Slides with and without the blocking peptide are compared to assess specificity.

We assess specificity based on a lack of signal in negative control pellets and a robust signal in positive control pellets (**FIGURE 3**). When there is no negative control available, we assess the

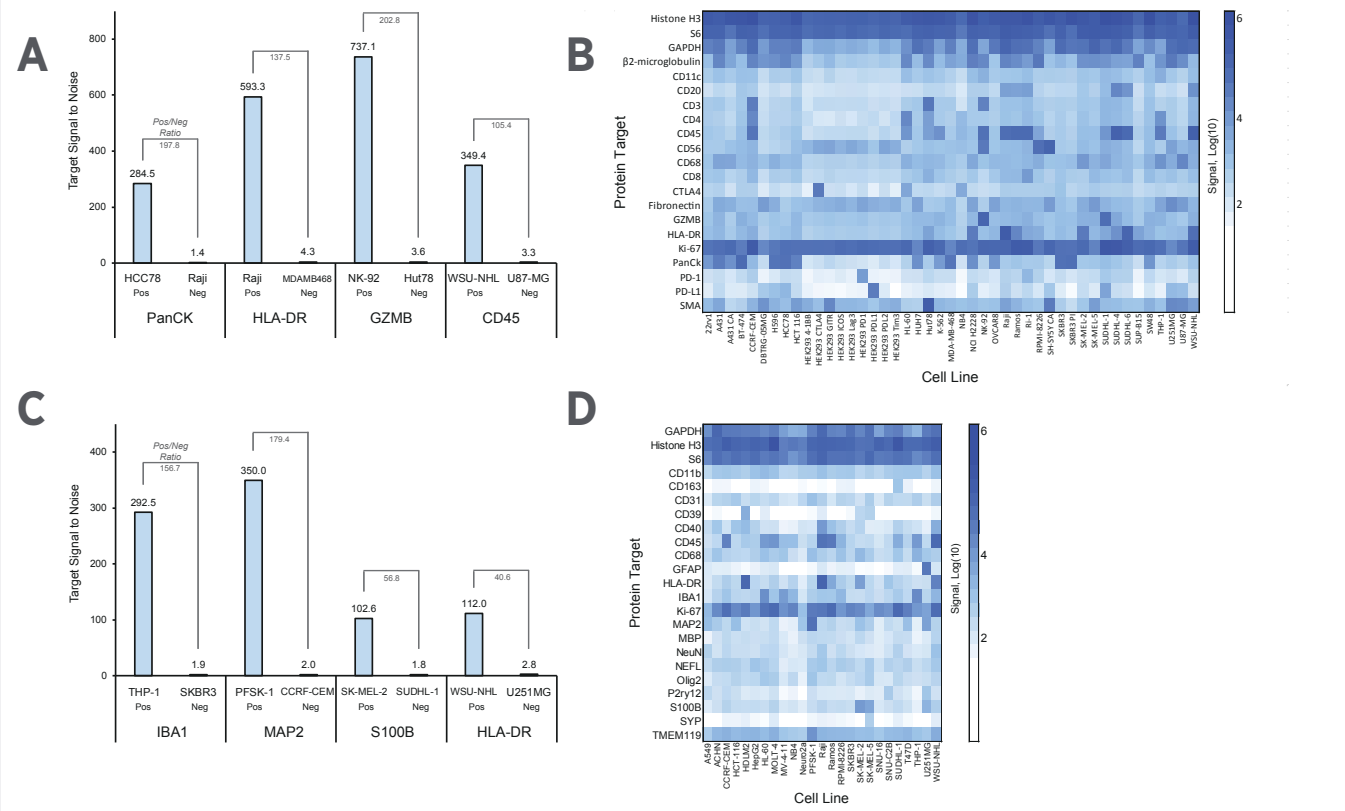

FIGURE 3: GeoMx antibody specificity screen. A) Ratios of signal to noise from several GeoMx Immune Cell Profiling Core antibodies between positive and negative FFPE cell pellets. B) Heatmap shows differential expression of the GeoMx Immune Cell Profiling Core antibody targets across 40+ cell lines (FFPE cell pellet array). Protein counts were normalized to housekeeping proteins and $\log_{10}$-transformed. C) Ratios of signal to noise from several GeoMx Neural Cell Profiling Core antibodies between positive and negative FFPE cell pellets. D) Heatmap shows differential expression of the GeoMx Neural Cell Profiling Core antibody targets across 20+ cell lines (FFPE cell pellet array). Protein counts were normalized to housekeeping proteins and $\log_{10}$-transformed.

dynamic range of the antibody in the cell lines, checking for a minimum of one log base 10 range between the low and high expressors. The low expressor threshold for the dynamic range assessment is calculated as the average counts of the three lowest expressing cell lines. Antibodies with acceptable commercial sensitivity exhibit a maximum positive signal divided by the limit of detection (for those with a negative control), or divided by the low expressor threshold (for those without a negative control), plus two standard deviations (SD) that is greater than or equal to 5. We perform a false positive test for each assay to confirm that with a threshold of 5 we will have a false positive rate of less than or equal to 10%.

Interaction Screen and Interference Assay

After an antibody passes functional validation it will be incorporated into a core or module. Thus, we assess the performance of antibodies in the experimental context of a core or a core plus module(s). We compare the correlation of target expression in serial sections incubated with either the core or a combination of the core and modules (**FIGURE 4**). Core and module combinations must have a Pearson's correlation >0.9 to pass.

Additionally, we perform an interference assay for each individual antibody. During tissue incubation with the oligo-tagged antibody we spike in a 40x concentration of an individual unlabeled antibody. We expect to observe a decrease in the signal of the corresponding conjugated antibody only, suggesting that the antibody is not interfering with any of the other antibodies. If the antibody in question is interfering with the binding of the other antibodies in the assay, then we will observe a decrease in signal in one or more of those antibodies. A greater than 2-fold drop in the signal to noise ratio of any of the other antibodies is considered to be potential interference and the antibody in question is removed from the panel. We assess the potential interference in three FFPE cell lines for each antibody.

TMA Screen

Finally, we test antibodies in a TMA screen to ensure that they will perform as expected in real samples and yield sufficient signal over background. We use multiple healthy and diseased tissues with at least four geometric regions of interest (two ROIs between 50-300 µm in duplicate) per cell line or tissue type to assess the signal on the GeoMx® DSP (**FIGURE 5**). Passing antibodies exhibit a maximum positive signal divided by the limit of detection plus two standard deviations (SD) greater than or equal to 5. When there is no negative control available, we assess the dynamic range of the antibody and calculate the maximum positive signal divided by the low expressor threshold, as described above.

Reproducibility testing

During antibody panel validation, functional performance of the GeoMx core and module antibodies is conducted by three different users to assess user-to-user variability. For each

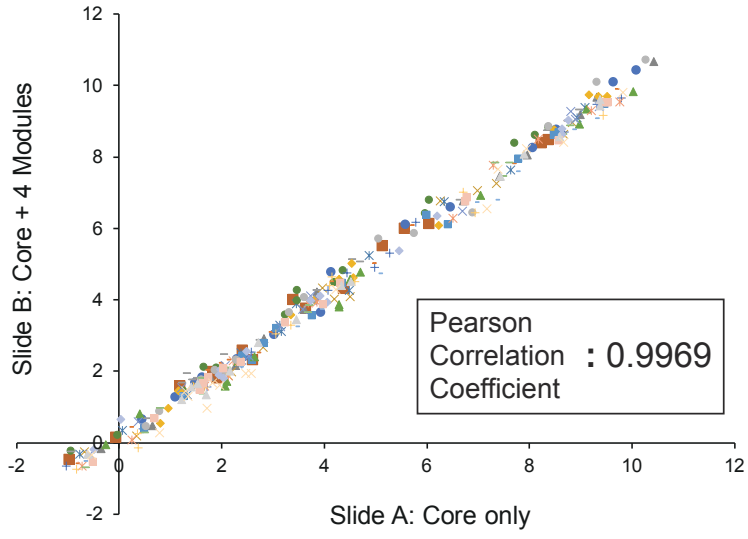

FIGURE 4: Interaction Screen. Antibody core and modules are evaluated to demonstrate consistent performance regardless of the number of modules used. Here the GeoMx Immune Cell Profiling Core was run alone (Slide A) and in combination with four modules (Slide B). Signal to noise log_2-transformed were obtained for each of Immune Cell Profiling Core antibody targets across 20+ cell lines (FFPE cell pellet array). The average Pearson correlation coefficient of the two datasets is >0.99.

antibody, the coefficient of variation (CV) is calculated for $\log_2$ histone H3 normalized counts across cell lines with an average CV ≤ 30% considered passing.

Every new GeoMx® core or module is tested between two lots to ensure reproducibility between lots. Replicate 300 µm geometric circular ROIs are selected on two slides of a cell pellet array each with a unique lot. For each antibody within a lot, signal is averaged across replicate ROIs for each cell pellet. The average signal for each antibody is then compared across lots for each cell pellet type (**FIGURE 6**). The commercial lot passes if it has a Pearson's correlation of the $\log_2$-normalized signal of R ≥ 0.8.

Conclusion

All of the antibodies included in GeoMx Protein Assays go through the extensive validation process described above. In total, approximately 60% of off-the-shelf antibodies tested for use in GeoMx assays pass the entire validation process and are put into commercial assays. This underscores the importance of antibody validation prior to incorporating a new antibody into an experiment when using any antibody-based technology.[5] By using NanoString's validated antibody content on the GeoMx DSP, researchers can get directly to their research without spending valuable time on antibody validation.

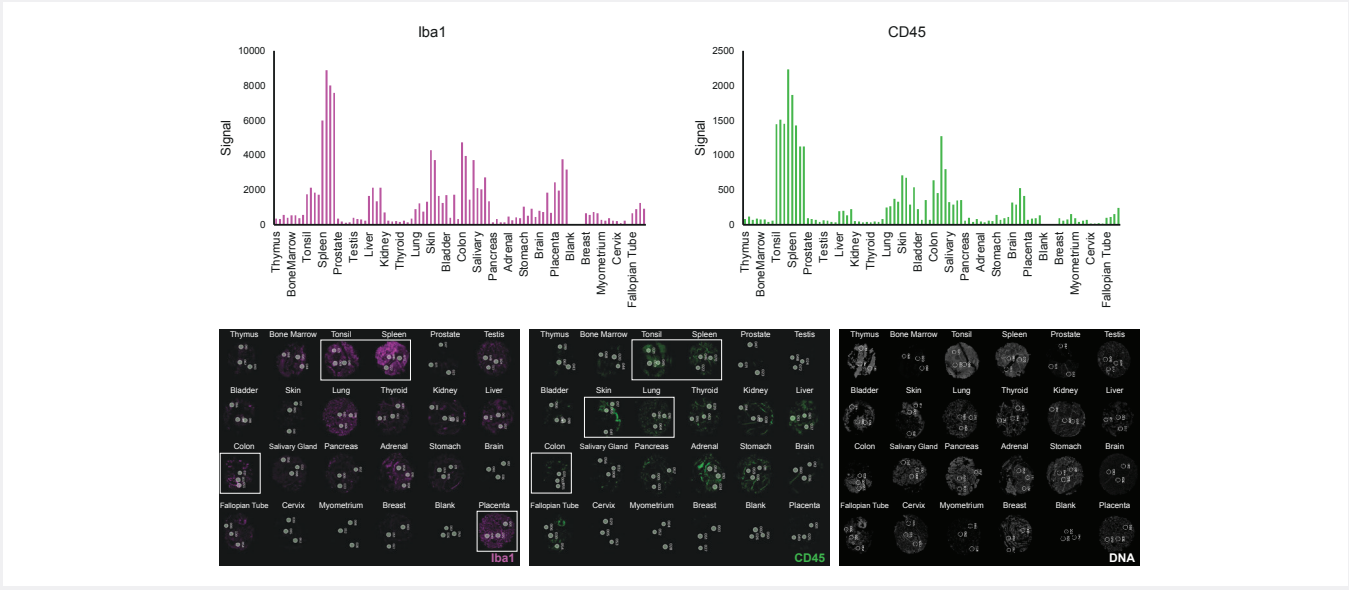

FIGURE 5: TMA Screen. We use tissue microarrays (TMAs) to assess differential expression of the GeoMx Neural Cell Profiling Core across tissue types and ensure antibodies will perform as expected in real samples. A) Differential expression of Iba1 and CD45 were assessed across at 23 core TMA. B) The 23 core TMA was labeled with fluorescent Iba1 and CD45 to provide correlative visual confirmation of expression patterns. Obtained protein expression within tissue types by GeoMx DSP was compared to expected expression within tissue types as described by the Human Protein Atlas..

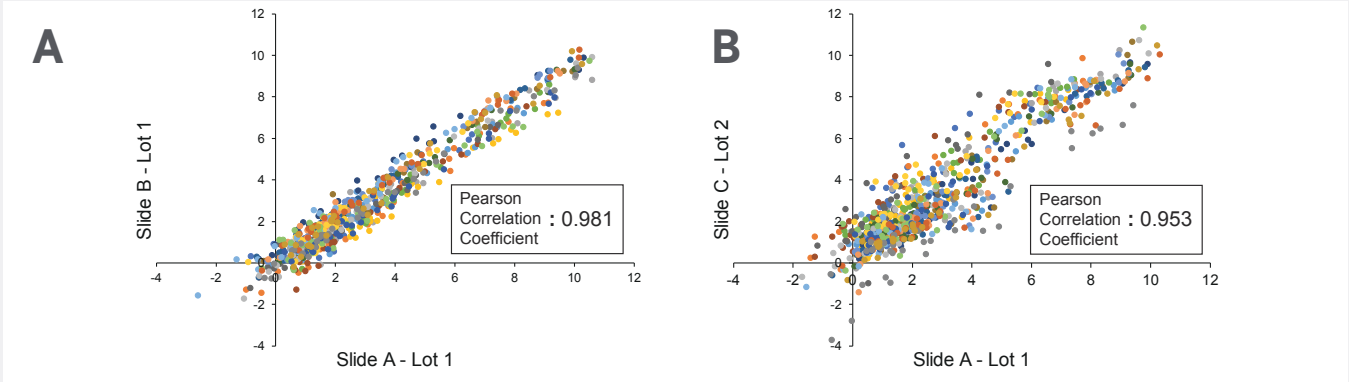

FIGURE 6: GeoMx slide-to-slide and lot-to-lot reproducibility. Each new GeoMx protein assay is evaluated for reproducibility between slides and between two lots. A) The signal to noise $\log_2$-transformed of two slides with the same lot of GeoMx Immune Cell Profiling Core and 4 modules are assessed across 20+ cell lines (FFPE cell pellet array). The average Pearson correlation coefficient of the two datasets is >0.98. B) The signal to noise $\log_2$-transformed of two slides with different lots of GeoMx Immune Cell Profiling Core and 4 modules are assessed across 20+ cell lines (FFPE cell pellet array). The average Pearson correlation coefficient of the two datasets is >0.955.

References:

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5 The Society for Immunotherapy of Cancer statement on best practices for multiplex immunohistochemistry (IHC) and immunofluorescence (IF) staining and validation. Taube et al. 2020, Journal for ImmunoTherapy of Cancer.

6 Human Protein Atlas available from proteinatlas.org

For more information, please visit nanostring.com

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