WHITEPAPER

Selection and Validation of CosMx™ Custom-Labeled Antibodies

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NanoString Technologies, Inc., Seattle, WA 98109
MK5881 | July 2023

nanoString®

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To profile the biology of a tissue, it is critical to explore a broad set of protein targets. CosMx™ Protein Assays allow the user to perform high-plex analysis of protein targets at subcellular resolution within a single tissue slide. Profiling the target of interest is a key element to achieving meaningful results within a spatial experiment. Each antibody in the CosMx Protein Assay is conjugated to unique oligonucleotide barcodes that enable direct visualization and illumination of the proteins via imaging.

Commercially available CosMx protein panels allow for the addition of custom antibodies to meet individual project needs. While replacing existing antibodies in our profiling cocktails is not possible, it is now possible to add up to 8 custom targets to any CosMx protein assay. Adding custom labeled antibodies requires conjugation of our unique SMI barcode chemistry to your antibody. This conjugation step is available through the CosMx Protein Barcoding Service[1] for up to 8 targets per instrument run.

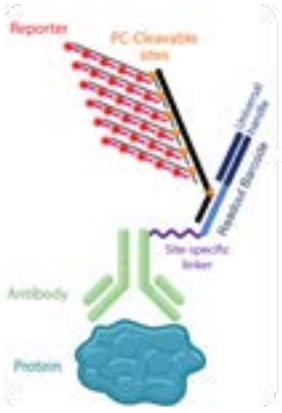

FIGURE 1: Protein probe chemistry

Unconjugated Antibody Selection and Validation

Criteria for antibody selection

Make sure to choose targets that are abundantly expressed to achieve a strong and specific signal. In addition, choose an antibody that has been recommended or has been validated for immunofluorescent (IF)/ immunohistochemistry (IHC) assays by the antibody provider, preferably using the same antigen retrieval buffer as used in CosMx Protein assays (citrate buffer, pH 6.0). Both monoclonal and polyclonal antibodies can work well with CosMx Protein assays, though each has its own set of pros and cons (see Table 2). It's best to weigh these pros and cons when sourcing an antibody for your experiment.

Antibodies must be provided in carrier-free buffer. Antibodies containing BSA, gelatin or cell culture supernatant are incompatible with protein labeling. While antibodies in glycerol-containing buffers can still be used for conjugation, overall conjugation efficiency may be affected. Addition of sodium azide is recommended to avoid potential contamination during storage. Note that the source of the antibody of choice (mouse, rat, or rabbit), should be compatible with the CosMx mouse or human Protein Assay being run. Align the antibody source with the included assay dependent IgG isotype controls, to ensure that the IgG isotype controls can be used for normalization of the custom labeled antibodies.

TABLE 1: Antibody format for barcoding

Criteria	Requirement
Concentration & Volume	>250 µg at a concentration of >0.5 mg/mL
Buffer	• 1x PBS with Sodium Azide at 0.02 – 0.05% (Azide recommended but optional) • Buffer must be free of carrier proteins (e.g. BSA, gelatin) and cryoprotectants (e.g. glycerol)
Source	Mouse, Rat, or Rabbit

TABLE 2: Comparison of monoclonal and polyclonal antibodies

	Monoclonal	Polyclonal
Pros	Specifically detects a defined epitope on an antigen, less likely to cross-react	Potentially gives a stronger signal as multiple antibodies may bind to multiple epitopes/ antigens on a target
	Returns reproducible results across antibody production lots	More stable over a broader range of experimental conditions
Cons	More vulnerable to loss of epitope through antigen retrieval process	More likely to return variable results across Ab production lots
	More sensitive to experimental conditions	More likely to cross-react

Pre-validation step before conjugation

Before protein barcoding, it's recommended to perform a standard IHC staining to ensure strong and specific antibody binding. For this, knowing the staining pattern to expect in the tissue of interest is essential. Consult the Human Protein Atlas (proteinatlas.org), Allen Brain Map for both human and mouse, or other online databases or publications for guidance.

Material needed in addition to CosMx SMI Slide Preparation reagents (consult the CosMx Slide Preparation User Manual):

- Antibody to test
- Slides from the tissue of interest with at least one serial section for each concentration to test
- Slides from the tissue of interest to be stained with the host isotype control antibody only for testing non-specific binding and background estimation
- Slides from tissue(s) known to be negative for the protein of interest (optional, but recommended)

Test for antibody staining efficiency and specificity

Barcoded antibodies should be screened via 3,3′-Diaminobenzidine (DAB) staining prior to use in a CosMx assay to determine the optimal antibody concentration and to confirm staining specificity. NanoString recommends the Abcam Mouse and Rabbit Specific HRP/DAB IHC Detection Kit (Abcam, Cat. Ab236466) for manual staining or Leica Biosystems' BOND Polymer Refine Detection (Leica Biosystems®, Cat. DS9800) for staining with the Leica BOND System, and, in general, starts with a range of dilutions around the antibody vendor IHC recommendations for concentration of the unconjugated antibody.

If there is no recommendation from the supplier, start with a test concentration for the initial range between 0.2 µg/mL- 4 µg/mL. If high background or non-specific staining is observed, it's recommended to test lower antibody concentrations; higher concentrations can be tested if there is no specific and strong signal observed on the tissue of interest. If after adjusting the concentration to 8 µg/mL, no specific or strong signal is present, a different antibody clone should be considered.

Optional (but recommended) Once the antibody concentration has been optimized, it's recommended to try this same concentration on a known negative control tissue to check for non-specific staining. In addition, testing the target antibody and its isotype control on the same tissue is recommended to determine background staining.

When using the CosMx Protein Barcoding Service, it is recommended to perform this IHC validation prior to completing the submission form[2] for shipping the antibody to NanoString for conjugation.

Post-Conjugation

CosMx Protein Barcoding Service

Conjugated (barcoded) antibodies are provided at a concentration of 200 µg/mL in a buffer of PBS and sodium azide. A test aliquot of 40 µL is provided for the purpose of post-conjugation testing.

Storage

Antibodies should be stored at 4°C for use within two weeks or aliquoted and kept at -80°C for long term storage. Avoid repetitive freeze thaw cycles during storage.

Probe Kit Files

When using custom barcoded antibodies, be sure to select the custom add-on probe kit that corresponds to your custom barcoded antibodies and experimental setup. These probe kits are selected when setting up the slide for a CosMx instrument run. The add-on probe kit is associated with a particular instrument and/or customer site and is generated based on the information provided during order submission. If there are changes to your experimental parameters from your original order, such as mixing and matching of targets or omission of certain targets, please request a new probe kit from AtoMxKitAdmin@nanostring.com. This process typically takes 1 business day, but can take up to 3 business days.

Post-Conjugation QC and Validation

Testing for a successful conjugation

When using the CosMx Protein Barcoding Service, testing of the conjugated antibody for presence of the SMI barcode tag and testing to exclude any contamination is included as part of the service.

Functional validation

NanoString recommends performing a post-conjugation, functional validation test to confirm the staining specificity of the conjugated antibody. This type of functional validation is not provided with the CosMx Protein Barcoding Service.

Use both unconjugated and conjugated antibody during this functional validation experiment to confirm the same antibody specificity is maintained post-conjugation (follow the same procedure outlined in the "Pre-validation step before conjugation" section within this guide). Since the modification with a barcode with naturally result in a decreased binding efficiency of the antibody to its antigen, a higher concentration of conjugated antibody than unconjugated may be required. NanoString recommends performing the DAB staining with the barcoded antibody at 1, 2 and 4 µg/mL. Antibody screening can be done up to 8 µg/mL, however, a concentration of 8 µg/mL is considered a low expressor and it may indicate a poor performing antibody.

In general, the concentration of antibody to use in the CosMx assay is half of the optimal concentration used in DAB staining (Table 3). However, signal intensity and sources of background will differ between the DAB assay and CosMx assay. DAB is an amplification-based method and relies on chromogenic detection, which differs from the fluorescent, amplificationfree detection method on CosMx.

Weak signal after antibody conjugation

After conjugation, a decrease in signal compared to the non-conjugated state is expected since the barcode may sterically hinder the polymer or secondary antibody from binding as efficiently to the primary antibody backbone. However, if the signal drops significantly after conjugation, consider the following troubleshooting steps:

- Increasing the concentration often can improve signal intensity. Generally, a range of 1-8 µg/mL works well for conjugated antibodies.
- Use NanoString's validated CosMx protocol, including the incubation time and retrieval conditions.
- Compare performance to unconjugated antibody for specificity and sensitivity.

If it is determined that the barcode is interfering with primary binding, alternative antibody clones should be considered for optimal performance. Please note, alternative barcoding methods might be available when using the CosMx Protein Barcoding Service.

Post-conjugation validation using CosMx DSP

Once the barcoded antibody is determined to function in IHC post-conjugation, it is recommended to optimize the custom antibodies using the CosMx Protein Assay panels of interest.

Post-conjugation IHC results can be used to determine the optimal amount of barcoded antibody to include with CosMx SMI Protein Assays. Custom antibodies will be diluted and then added to the working antibody solution according to the Protein Slide Preparation Protocol[3].

In the example shown in Figure 2, pre-conjugated and post-conjugated antibodies are screened in control tissues and IHC staining patterns are reviewed by a pathologist to ensure that conjugation has not affected the pattern of binding (top). Post-conjugation titration is performed to determine the optimal antibody concentration and to confirm staining specificity (bottom). Select the lowest concentration that gives specific measurable signal.

TABLE 3: Suggested conversion between chromogenic (DAB) staining results and concentrations used in the CosMx protein assay for custom conjugates. For illustration purposes only.

Assay	High Expressor	Medium Expressor	Low Expressor	
Post-conjugation chromogenic detection by DAB staining	1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml
CosMx Protein Assays	0.5 µg/ml	1 µg/ml	2 µg/ml	4 µg/ml

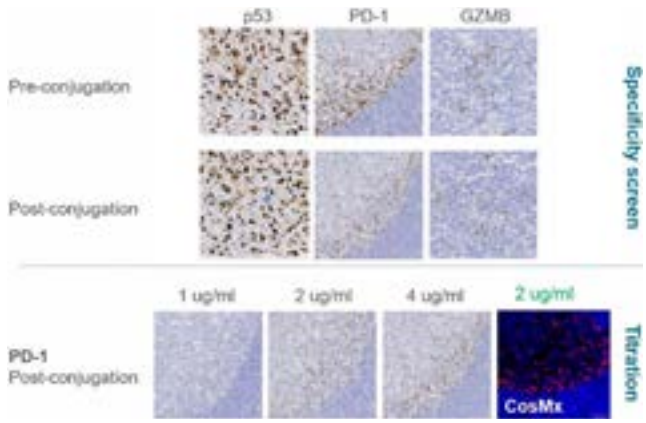

FIGURE 2: Pre- and post- conjugation antibody screen. Antibody specificity should be evaluated by examining serial sections for comparable staining patterns. Barcoded (post-conjugation) antibodies typically have weaker staining than unconjugated antibodies. If specificity is acceptable, then a titration should be performed to find an optimal antibody concentration.

References

1 Protein Barcoding Service: https://nanostring.com/cosmx-pbs/

2 Submission Form (under "Related Resources"): https://nanostring.com/cosmx-pbs/

3 CosMx SMI Manual Slide Preparation User Manual: https://university.nanostring.com/cosmx-smi-manual-slide-preparation-user-manual

For more information, please visit nanostring.com/cosmx-pbs

NanoString Technologies, Inc.
530 Fairview Avenue North
Seattle, Washington 98109
T (888) 358-6266
F (206) 378-6288
nanostring.com
info@nanostring.com

Sales Contacts
United States **us.sales@nanostring.com**
EMEA: **europe.sales@nanostring.com**
Asia Pacific & Japan **apac.sales@nanostring.com**
Other Regions **info@nanostring.com**

AUG 2023 MK5881