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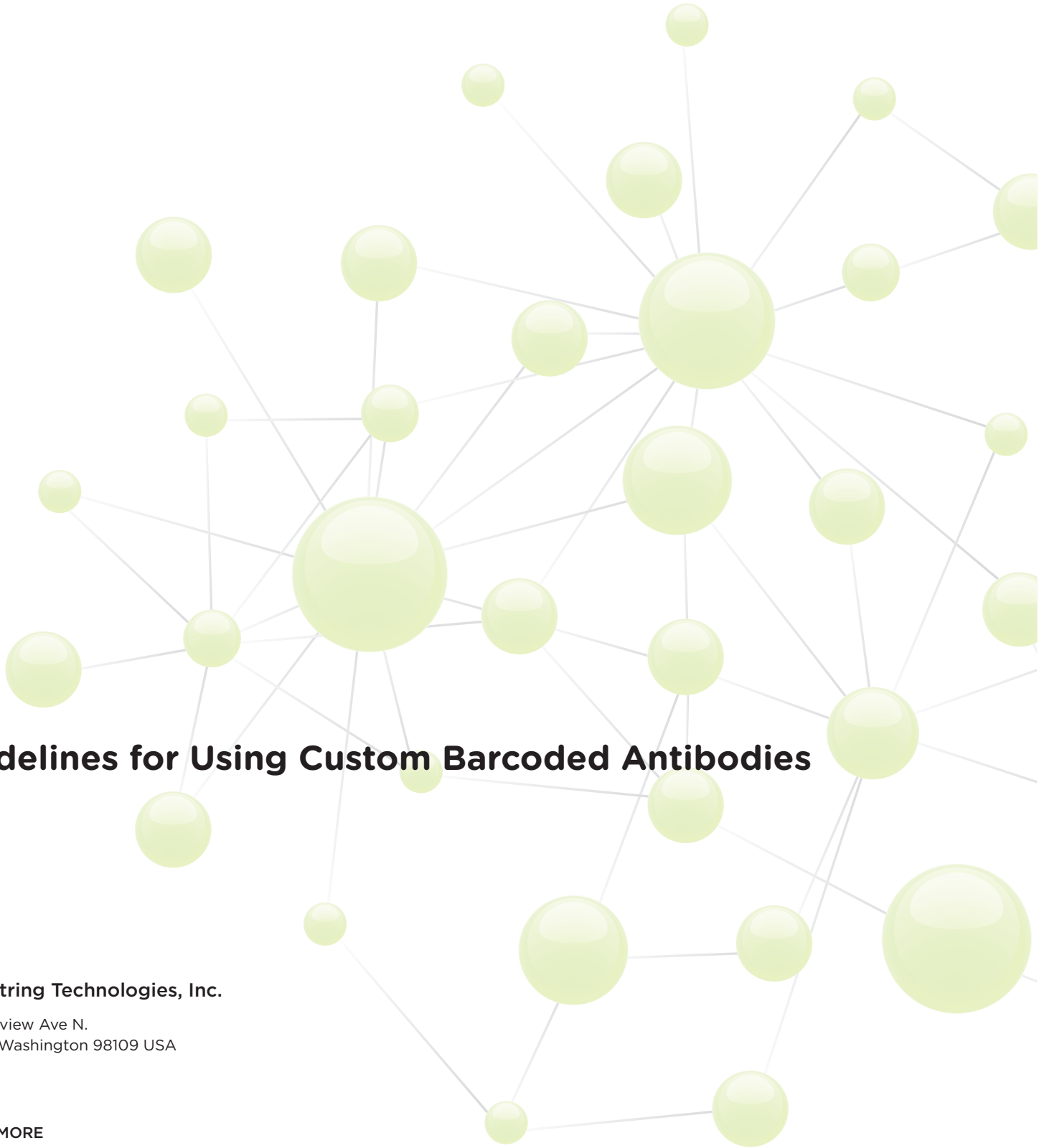

Guidelines for Using Custom Barcoded Antibodies

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FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

Guidelines for Using Custom Barcoded Antibodies

Up to three custom barcoded antibodies may be added as a spike-in to the nCounter Vantage 3D Protein Assays, in addition to the antibody mix supplied with the Vantage 3D Assay. Alternatively, custom barcoded antibodies can be combined and used as a custom antibody mix. Custom barcoded antibodies are supplied by NanoString's Vantage 3D Protein Barcoding Service at a concentration of 200 µg/mL and are supplied for use in NanoString assays. These guidelines refer specifically to preparation of your antibody mix. Sample preparation should be performed as documented in your nCounter Vantage 3D manual with modifications only to the preparation and addition of antibody mix to your sample.

The custom barcoded antibody is supplied as a tester aliquot and master aliquot(s) in separate tubes. The tester aliquot may be used to optimize assay conditions for your specific custom barcoded antibody without repeated freeze-thawing of the master aliquot(s). The tester aliquot is provided as a 20 µL volume. Your master aliquot(s) should be further aliquoted and stored in volumes optimized for your desired NanoString assay. Note that there may be more than one vial of the master aliquot per antibody depending on overall yield after conjugation.

NanoString recommends preparing custom barcoded antibodies as described below.

Use the following guidelines to dilute and then measure the performance of your custom barcoded antibody. These recommendations are provided as a starting point when determining the optimal concentration of antibody to be used in your desired NanoString assay. For cell suspension-based NanoString assays, a working range between 0.02 µg/mL–0.5 µg/mL final concentration of each antibody should be used, and the following guidelines result in a **0.1 µg/mL final concentration** for these assays. For lysate and FFPE-based NanoString assays, a working range between 0.05 µg/mL–2 µg/mL final concentration of each antibody should be used, and the following guidelines result in a **0.05 µg/mL final concentration**, which is the recommended starting point.

Note: Optimization of individual antibody concentrations beyond that described in **Step 1** may be necessary.

Recommended Use for Cell Suspension-based NanoString Assays

Advanced Preparation

For one 12-sample cartridge, prepare 154 µL of a 2 µg/mL custom antibody mix as follows:

Perform a 1:100 dilution of the 200 µg/mL stock custom barcoded antibody by adding 1.5 µL of each custom barcoded antibody to a final volume of 154 µL in 1X PBS (pH 7.4). Keep at 4°C until use.

Protein Sample Preparation

1. Modify the addition of antibody during protein sample preparation specific to your panel or sample/target type (i.e., refer to MAN-10031).
   - For spike-in applications, add 10 µL of the custom antibody mix prepared above *in addition* to the 10 µL of the antibody mix supplied with your Vantage 3D Assay to each well.
   - For fully-custom protein analysis, add 10 µL of the custom antibody mix prepared above *instead of* the 10 µL of the antibody mix to each well.
2. Proceed with the Vantage 3D Assay protein sample preparation (i.e., according to MAN-10031).

Recommended Use for Lysate-based NanoString Assays

Advanced Preparation

For one 12-sample cartridge, prepare 100 µL of 2 µg/mL working solution as follows:

Perform a 1:100 dilution of the 200 µg/mL stock custom antibody mix by adding 1 µL of each custom barcoded antibody to a final volume of 100 µL in blocking buffer (BioLegend cell staining buffer, dextran sulfate (200 kDa) (5 mg/mL), salmon sperm DNA (0.1 mg/mL); see MAN-10033 or -10034 Advanced Preparation for details). Keep at 4°C until use.

Protein Sample Preparation

1. Modify the addition of antibody during protein sample preparation specific to your panel or sample/target type (i.e., MAN-10033).
    - For spike-in applications, add 16 µL of the custom antibody mix prepared above *in addition* to the 16 µL of the antibody mix supplied with your Vantage 3D Assay to 625 µL of the blocking buffer prepared above.
    - For fully-custom protein analysis, add 16 µL of the custom antibody mix prepared above *instead of* the 16 µL of the antibody mix supplied with your Vantage 3D Assay to 625 µL of the blocking buffer prepared above.
2. Add 50 µL of the working antibody solution to each well and proceed with the Vantage 3D Assay protein sample preparation (i.e., according to MAN-10033 or -10034).

Note: All unused antibody should be discarded after dilution in blocking buffer.

Recommended Use for FFPE-based NanoString Assays

Advanced Preparation

For one 12-sample cartridge, prepare 154 µL of 5 µg/mL working solution as follows:

Perform a 1:40 dilution of the 200 µg/mL stock custom antibody mix by adding 2 µL of each custom barcoded antibody to a final volume of 80 µL in antibody diluent (signal stain antibody diluent, salmon sperm DNA (0.1 mg/mL), dextran sulfate (200kDa); see MAN-10035 and -10036 Advanced Preparation for details). Keep at 4°C until use.

Protein Sample Preparation

1. Modify the addition of antibody during protein sample preparation specific to your panel or sample/target type (i.e. refer to MAN-10035).
    - For spike-in applications, add 64 µL of the custom antibody mix prepared above *in addition* to the 64 µL of the antibody mix supplied with your Vantage 3D Assay to 2.5 mL of the antibody diluent prepared above.
    - For fully-custom protein analysis, add 64 µL of the custom antibody mix prepared in Step 1 *instead of* the 64 µL of the antibody mix supplied with your Vantage 3D Assay to 2.5 mL of the antibody diluent prepared above.
2. Proceed with the Vantage 3D Assay protein sample preparation (i.e., antibody incubation according to MAN-10035 and -10036).

Note: All unused antibody should be discarded after dilution in antibody diluent.

Data Analysis

All custom barcoded antibodies are accompanied by an add-in library file (ALF), which specifies the association between each custom barcoded antibody and its target. Information from the ALF must be merged with the reporter library file (RLF) from the original nCounter Vantage 3D Assay prior to scanning on the Digital Analyzer. Failure to merge an ALF with the original RLF will result in no count information being collected for targets of custom barcoded antibodies. Merging an ALF with the original nCounter Vantage 3D RLF will generate a new RLF that contains all probe information for both the custom barcoded antibodies and the original nCounter Vantage Assay. **To merge an ALF or to convert an ALF into an RLF for custom protein only assays, please contact bioinformatics@nanostring.com. A new RLF will be generated and e-mailed to you directly.**

Once the optimal final concentration for your custom barcoded antibody has been determined, the master aliquot can be further aliquoted into volumes suitable for single use. Antibodies are pre-formulated with 10% rabbit serum and 2 mM sodium azide for long-term storage. Freeze aliquots at -80°C.

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