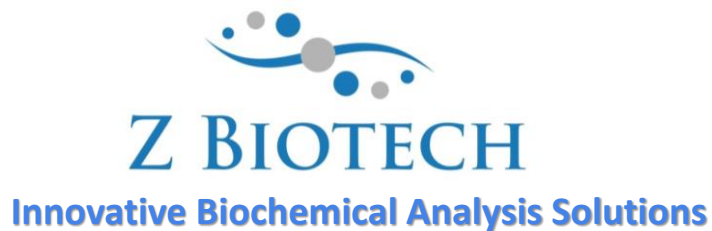


Neu5Gc/Neu5Ac N-Glycan Array User Manual



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Introduction

Neu5Gc is a major mammalian sialic acid synthesized from Neu5Ac by an enzyme no longer present in humans. Regardless, nonhuman, diet-derived Neu5Gc is metabolically integrated into a variety of human tissues. Human cells can biochemically recognize Neu5Gc and incorporate it into cell-surface glycoconjugates, however the immune system recognizes Neu5Gc-containing glycans as alien and produces anti-Neu5Gc antibodies resulting in inflammation. Diseases relating to chronic inflammation such as cancer, cardiovascular diseases, and thyroiditis are associated with higher levels of anti-Neu5Gc antibodies and Neu5Gc antigens, and it is suspected to be involved in other diseases that are exacerbated by a red meat, Neu5Gc-rich diet.

Z Biotech's Neu5Gc/Neu5Ac N-glycan Array represents a broad range of N-glycans found on cell surfaces that have incorporated Neu5Gc. These arrays can be investigated with glycan-binding proteins, antibodies, or cells in order to determine their specific interaction with these Neu5Gc xenoantigens. For comparison, each array includes the precursory, non-xenogenic Neu5Ac sialic acid form of each glycan.

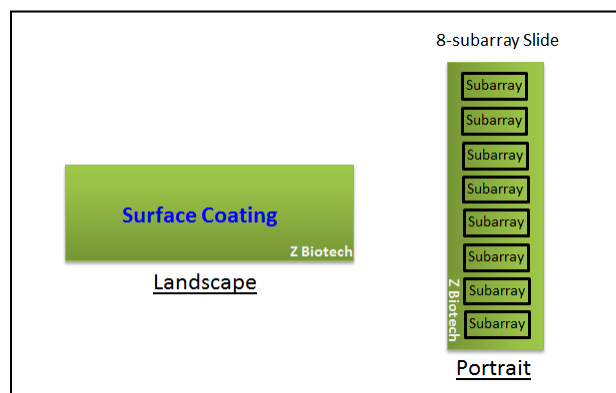
Handling and Storage

Store the bag of slides and any buffers in a 4°C refrigerator if they are to be assayed within 3 weeks upon receipt. For long term storage keep the bag of slides at -20°C. Avoid freezing and thawing multiple times. Purchased slides and buffers should be used within 6 months.

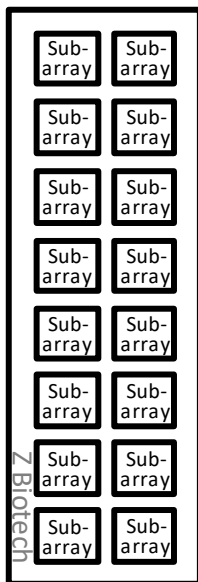
Allow the bag of slides to equilibrate to room temperature at least 20 minutes before opening. After opening, re-seal any unused slides in the moisture barrier bag with a desiccant inside and refreeze. Handle the slides in a clean, dust free environment. Wear gloves and hold the slides on the edges. When adding sample do not touch the pipette tip to the array surface. When removing sample gently touch the pipette tip at the corner of the well of the cassette and tip the slide so that the sample pools to that corner. Avoid contact with the surface of the slides.

Array Map/Schematic

Neu5Gc/Neu5Ac N-glycan Array slides have 8 or 16 subarrays depending on the customer's request. Arrays are printed on the side with the "Z Biotech" label facing upward. The "Z Biotech" label is located on the bottom right corner from a landscape point of view, or the bottom left corner in a portrait point of view (see image on right). The orientation of the printed array is consistent with the portrait slide orientation. Dimensions and array map are shown on the following pages.



16-subarray Slide



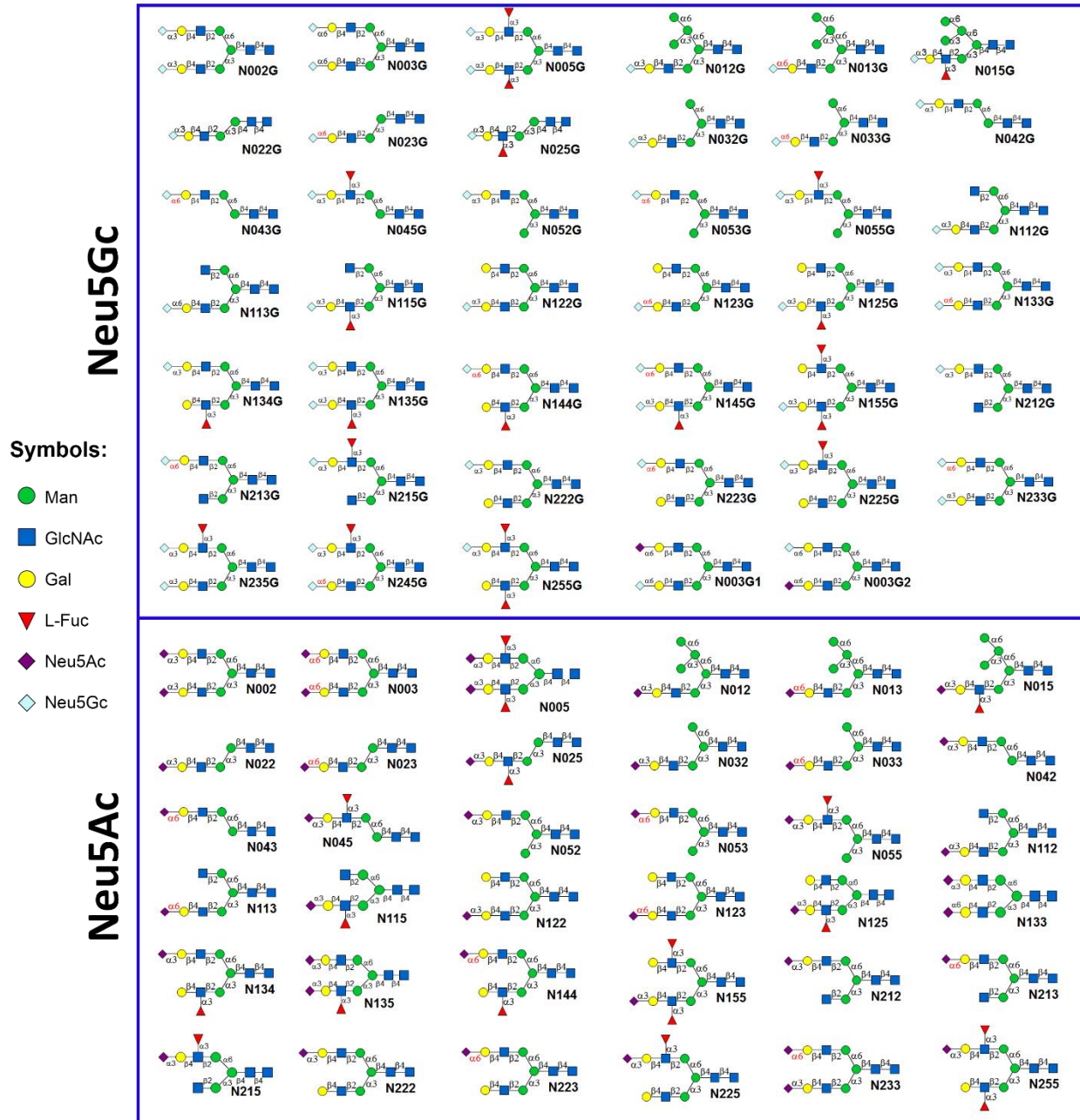
Array Map:

GC-1	GC-1	GC-1	GC-2	GC-2	GC-2	GC-3	GC-3	GC-3	GC-4	GC-4	GC-4	GC-5	GC-5	GC-5	NC1
GC-6	GC-6	GC-6	GC-7	GC-7	GC-7	GC-8	GC-8	GC-8	GC-9	GC-9	GC-9	GC-10	GC-10	GC-10	NC1
GC-11	GC-11	GC-11	GC-12	GC-12	GC-12	GC-13	GC-13	GC-13	GC-14	GC-14	GC-14	GC-15	GC-15	GC-15	NC1
GC-16	GC-16	GC-16	GC-17	GC-17	GC-17	GC-18	GC-18	GC-18	GC-19	GC-19	GC-19	GC-20	GC-20	GC-20	PC1
GC-21	GC-21	GC-21	GC-22	GC-22	GC-22	GC-23	GC-23	GC-23	GC-24	GC-24	GC-24	GC-25	GC-25	GC-25	PC1
GC-26	GC-26	GC-26	GC-27	GC-27	GC-27	GC-28	GC-28	GC-28	GC-29	GC-29	GC-29	GC-30	GC-30	GC-30	PC1
GC-31	GC-31	GC-31	GC-32	GC-32	GC-32	GC-33	GC-33	GC-33	GC-34	GC-34	GC-34	GC-35	GC-35	GC-35	PC2
GC-36	GC-36	GC-36	GC-37	GC-37	GC-37	GC-38	GC-38	GC-38	GC-39	GC-39	GC-39	GC-40	GC-40	GC-40	PC2
AC-1	AC-1	AC-1	AC-2	AC-2	AC-2	AC-3	AC-3	AC-3	AC-4	AC-4	AC-4	AC-5	AC-5	AC-5	PC2
AC-6	AC-6	AC-6	AC-7	AC-7	AC-7	AC-8	AC-8	AC-8	AC-9	AC-9	AC-9	AC-10	AC-10	AC-10	PC3
AC-11	AC-11	AC-11	AC-12	AC-12	AC-12	AC-13	AC-13	AC-13	AC-14	AC-14	AC-14	AC-15	AC-15	AC-15	PC3
AC-16	AC-16	AC-16	AC-17	AC-17	AC-17	AC-18	AC-18	AC-18	AC-19	AC-19	AC-19	AC-20	AC-20	AC-20	PC3
AC-21	AC-21	AC-21	AC-22	AC-22	AC-22	AC-23	AC-23	AC-23	AC-24	AC-24	AC-24	AC-25	AC-25	AC-25	PC4
AC-26	AC-26	AC-26	AC-27	AC-27	AC-27				AC-29	AC-29	AC-29	AC-30	AC-30	AC-30	PC4
AC-31	AC-31	AC-31	AC-32	AC-32	AC-32	AC-33	AC-33	AC-33	AC-34	AC-34	AC-34	AC-35	AC-35	AC-35	PC4
AC-36	AC-36	AC-36							AC-39	AC-39	AC-39	GC-41	GC-41	GC-41	Marker

N-Glycan Identification List:

Gc Glycan ID	Neu5Gc Glycans	Ac Glycan ID	Neu5Ac Glycans
GC-1	N002G	AC-1	N002
GC-2	N003G	AC-2	N003
GC-3	N005G	AC-3	N005
GC-4	N012G	AC-4	N012
GC-5	N013G	AC-5	N013
GC-6	N015G	AC-6	N015
GC-7	N022G	AC-7	N022
GC-8	N023G	AC-8	N023
GC-9	N025G	AC-9	N025
GC-10	N032G	AC-10	N032
GC-11	N033G	AC-11	N033
GC-12	N042G	AC-12	N042
GC-13	N043G	AC-13	N043
GC-14	N045G	AC-14	N045
GC-15	N052G	AC-15	N052
GC-16	N053G	AC-16	N053
GC-17	N055G	AC-17	N055
GC-18	N112G	AC-18	N112
GC-19	N113G	AC-19	N113
GC-20	N115G	AC-20	N115
GC-21	N122G	AC-21	N122
GC-22	N123G	AC-22	N123
GC-23	N125G	AC-23	N125
GC-24	N133G	AC-24	N133
GC-25	N134G	AC-25	N134
GC-26	N135G	AC-26	N135
GC-27	N144G	AC-27	N144
GC-28	N145G		
GC-29	N155G	AC-29	N155
GC-30	N212G	AC-30	N212
GC-31	N213G	AC-31	N213
GC-32	N215G	AC-32	N215
GC-33	N222G	AC-33	N222
GC-34	N223G	AC-34	N223
GC-35	N225G	AC-35	N225
GC-36	N233G	AC-36	N233
GC-37	N235G		
GC-38	N245G		
GC-39	N255G	AC-39	N255
GC-40	N003G1		
GC-41	N003G2		

Neu5Gc and Neu5Ac N-Glycans



Materials Required

- Arrayed glass slide
- 16 or 8 subarray cassette
- Blocking Buffer (Item #10109): 1% BSA in PBST (PBS with 0.05% (v/v) Tween-20, pH 7.4)
- Glycan Array Assay Buffer (GAAB Item #10107)
Materials above are included for purchase in our Lectin Microarray Kit (<http://www.zbiotech.com/products.html>)
- Wash Buffer: 20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.6
- Glycan-binding protein samples of interest.
- Biotinylated secondary antibodies (for sandwich assay format only)
- Fluorescent labeled streptavidin (for biotin labeled glycan-binding samples)
- Sterile de-ionized water
- Orbital shaker
- Laser fluorescence scanner (able to scan at the wavelength of your fluorophore)
- Centrifuge
- Pipette and sterile pipette tips
- Sterile centrifuge tubes
- Coplin jar or 250 mL beaker
- Adhesive slide cover film
- Aluminum foil

Preparation of assay samples:

Prepare glycan-binding samples or detection antibodies in a centrifuge tube by diluting with the GAAB buffer. For the fluorescently labelled streptavidin we recommend a concentration of 1 $\mu\text{g}/\text{mL}$. For detection antibodies, we recommend a concentration around 10-1 $\mu\text{g}/\text{ml}$. We generally recommend a range of 100 $\mu\text{g}/\text{ml}$ to 0.1 $\mu\text{g}/\text{ml}$ concentration for glycan-binding samples, although some experimentation may be required to establish the concentration that will provide the highest binding signals with the lowest background fluorescence. This is often accomplished by applying a different dilution of samples to different wells of the array. In addition to testing a dilution range for your sample of interest, we recommend setting up control assays for any additional detection or secondary antibodies to ensure that any binding observed is specific to your sample of interest. Fluorescent signal due to specific binding to your sample of interest should be dose-dependent within the dynamic range of your sample dilution, and should have positive binding signal after signal from control assays has been subtracted. Calculate the volume of sample needed depending on how many slides and subarrays are to be assayed. We recommend using 100 μL volume of sample per well for 16 subarray cassettes and 200 μL for 8 subarray cassettes to ensure full and even coverage of the printed area throughout incubation. If necessary, the assay can be done successfully with a minimal volume of 60 μL per well for 16 subarray cassettes and

80 μL for 8 subarrays. We caution that using a minimal volume in the wells has an increased risk of the array drying out during the assay, and may also cause unequal distribution of the sample across the arrayed surface which may result in signal variation. Please ensure each sample is homogeneous and thoroughly mixed.

Assay Protocol

Part 1 – Blocking

Handle the slide in a clean, dry environment. Use gloves and avoid touching the slide surface

1. Allow the arrayed slides to equilibrate to room temperature (20-30 minutes) before opening the moisture barrier bag.
2. Add blocking buffer to each subarray well. We recommend using 100 μL per well for 16 subarray cassettes and 200 μL for 8 subarray cassettes to ensure full and even coverage of the printed area throughout incubation.
3. Cover the wells with adhesive film to prevent evaporation and incubate slide on shaker at 85 rpm for 1 hour. Longer incubation time is acceptable, but not necessary.

Make sure the orbital shaker is completely flat. If the slide is sloped in any direction during incubation it can cause variation in binding.

Part 2 – Binding assay

1. Unless the glycan-binding sample of interest is bacteria or cells, centrifuge samples briefly to avoid adding irrelevant particles to the array.
2. Remove blocking buffer from each well by gently touching the pipette tip to the corner of the well of the cassette and tip the slide so that the sample pools to that corner. Avoid touching the array surface.
3. Immediately apply the glycan-binding sample of interest to each well. We recommend using 100 μL per well for 16 subarray cassettes and 200 μL for 8 subarray cassettes to ensure full and even coverage of the printed area throughout incubation. Avoid leaving air bubbles.
4. Seal the wells with adhesive film to prevent evaporation. If the sample is fluorescently labelled, cover with aluminum foil to keep it in the dark. Incubate on the shaker for 1-3 hours at 100 rpm. If the samples can easily aggregate, shake at higher speed to prevent aggregation. Longer incubation time may increase binding signal, especially for weakly binding samples.

Avoid allowing the slides to dry out at any point during the assay, especially during long incubation times. Make sure the adhesive film is sealed around each well.

If your glycan-binding samples are fluorescently labelled, go directly to Part 6 – Final wash and dry.

Part 3 – Wash

1. Remove glycan-binding samples from each well by gently touching the pipette tip to the corner of the well of the cassette and tip the slide so that the sample pools to that corner. Avoid touching the array surface, but a gentle touch is okay to ensure no sample is left pooled in the corners.
2. Add wash buffer to each well. We recommend using 100 μL per well for 16 subarray cassettes and 200 μL for 8 subarray cassettes. Cover the wells with adhesive film and incubate on the shaker for 5 minutes at 85 rpm. Completely remove the wash buffer by pipette and repeat this step. Avoid allowing the slide to dry out and have your next wash or sample ready before you remove the wash buffer.

If your glycan-binding sample is biotinylated, go directly to Part 5 – Fluorescent staining.

Part 4 – Binding of biotinylated antibody (Sandwich Assay Format)

1. Unless the secondary biotinylated antibody sample is bacteria or cells, centrifuge samples briefly to avoid adding irrelevant particles to the array.
2. After completely removing the wash buffer immediately add the biotinylated antibody to each well. We recommend using 100 μL per well for 16 subarray cassettes and 200 μL for 8 subarray cassettes. Seal the wells with adhesive film and incubate on the shaker for 1 hour at 100 rpm. Longer incubation time is acceptable, but not necessary.
3. After incubation repeat Part 3 – Wash

Part 5 – Fluorescent staining

1. Centrifuge fluorescent-labeled streptavidin samples briefly to avoid adding irrelevant particles to the array.
2. After completely removing the wash buffer immediately add the fluorescently labelled streptavidin sample. 100 μL per well is recommended for 16 subarray cassettes and 200 μL for 8 subarray cassettes. Seal the wells with adhesive film and shield the wells from light with aluminum foil. Incubate on the shaker at 85 rpm for 1 hour. Longer incubation time is acceptable, but not necessary.

Part 6 – Final wash and dry

1. Remove the sample from each well by gently touching the pipette tip to the corner of the well of the cassette and tip the slide so that the sample pools to that corner. Avoid touching the array surface.
2. Briefly rinse each well with wash buffer. 100 μL per well is recommended for 16 subarray cassettes and 200 μL for 8 subarray cassettes.

3. Completely remove the wash buffer by pipette. Avoid touching the array surface. Repeat steps 2 and 3.
4. Disassemble the cassette from the slide. For the provided cassette this can be done by holding the slide with one hand at the top and bottom edges and sliding out the cassette clips one by one with the other hand. If your provided cassette has metal clips they can be removed by rotating the clip outwards from the bottom of the slide. When the clips have been removed place the slide on the table and hold a small outer edge of the slide to the table as you gently peel the cassette off.
5. Immediately immerse the slide in a coplin jar or beaker full of wash buffer. Do not touch the surface of the array or allow the array surface to touch the sides of the beaker or jar.
6. Place the jar or beaker on the 60 rpm shaker for 10 minutes.
7. Decant the wash buffer from the jar or beaker while holding the slide in place (only touch the edge of the slide) and then add sterile de-ionized water to immerse the slide.
8. Place the jar or beaker on the 60 rpm shaker for 2 minutes.
9. Decant the water from the jar or beaker.
10. Allow the slide to dry completely in a clean, dust free environment before scanning.

Analysis

Scan the slide in a laser fluorescence scanner at the wavelength of emission for the fluorophore used. Adjust the laser power and PMT to obtain the highest possible signals without any being saturated (saturated positive control signal is okay). Analyze data with microarray analysis software. If there is specific binding the signal intensity should be higher than the background signal (area where there are no printed spots). Fluorescent signal due to specific binding to your sample of interest should be both dose-dependent with your sample dilution (unless the sample concentration range is too high and glycan binding is saturated), and should have positive binding signal after signal from control assays has been subtracted. Our standard method of comparing signal intensities is to quantify the median signal intensity data and subtract the background intensity. Subtracting signal from negative control spots as well as the same spots on a negative control assay (assay with only detection antibodies and fluorophore) will give more accurate specific binding data.

Interpretation of Control Signals:

Negative Control (Print Buffer): The negative control should produce a signal close to the intensity of the background. Since there is no binding involved with the negative control, any other signals around the negative control's intensity are also not binding. Subtracting the negative control's signal from the other binding signals will give more accurate specific binding data.

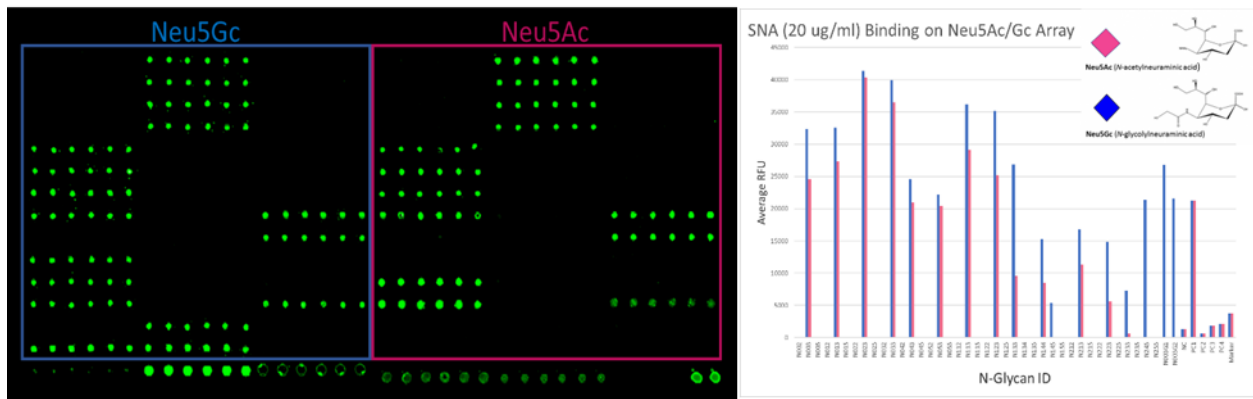
Positive Control 1 (biotinylated mannose): This positive control will bind directly to the fluorescent labelled streptavidin. If your glycoprotein sample is already fluorescently labelled, or in any case where the addition of fluorescent labelled streptavidin to the array was not preformed (Part 5 – Fluorescent staining) this positive control will not be reactive.

IgG (PC2, PC3, PC4): IgG is an antibody found in blood that is a primary component of humoral immunity. If the glycan-binding or secondary antibody sample is an anti-IgG from human, rabbit, or mouse it should bind to the respective IgG control.

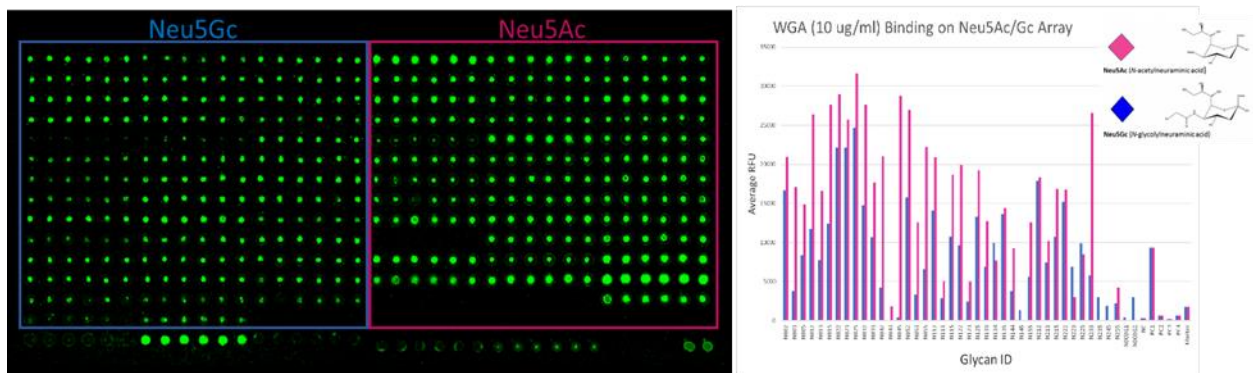
Marker: The array marker should show fluorescence signal regardless of the assay. It is there primarily to aid with orientation of the array map during analysis.

Typical Binding Assay Results from the Neu5Gc/Neu5Ac N-Glycan Array

Example 1: Neu5Gc/Neu5Ac array on 8 subarray format. A subarray assayed with a biotinylated SNA target (20 $\mu\text{g/ml}$), followed by streptavidin-Cy3 (1 $\mu\text{g/ml}$). The array was scanned with GenePix scanner at 500 PMT and 100% laser power at 532 nm wavelength. The positive control shows binding as expected. N-glycans containing α -2,6 Neu5Gc and α -2,6 Neu5Ac show binding as expected. Analysis of the fluorescence intensity reveals that Neu5Gc-sialylated glycans bind more strongly.



Example 2: Neu5Gc/Neu5Ac array on 8 subarray format. A subarray assayed with a biotinylated WGA target (10 $\mu\text{g/ml}$), followed by streptavidin-Cy3 (1 $\mu\text{g/ml}$). The array was scanned with GenePix scanner at 450 PMT and 100% laser power at 532 nm wavelength. The positive control shows binding as expected. Most N-glycans show binding as expected. Analysis of the fluorescence intensity reveals that Neu5Ac-sialylated glycans bind more strongly.



Troubleshooting

Condition	Possible Causes	Potential Solutions
High Background	<ul style="list-style-type: none"> • Concentration of protein samples is too high. • Concentration of fluorescent samples is too high. • Arrays are not thoroughly washed. • Slide drying out during assay. • Excessive particles in the samples due to sample aggregation, dust, etc. 	<ul style="list-style-type: none"> • Use a lower concentration range of samples. Consider a wider range if you are unsure where the detection limit is. Use control assays to determine which sample is causing high background. • Apply longer times for washing steps and use a higher shaking rate • Make sure wash buffer and sample is completely removed before the next step. • Make sure adhesive film fully seals the wells to avoid evaporation • Centrifuge the samples prior to assay to avoid adding irrelevant particles. Make sure buffers are filtered. • If you think that the protein is aggregating during incubation, try shaking at a higher speed
Signal Variation	<ul style="list-style-type: none"> • Slide drying out during assay. • Binding samples are not equally distributed in the wells • Glycan-binding protein aggregation during incubation • Bubbles during incubation 	<ul style="list-style-type: none"> • Make sure wells are sealed to prevent evaporation during incubation. • Apply a larger volume of sample to each well to ensure equal distribution • Use a higher shaking rate during incubation • Make sure samples are homogeneous, mixed thoroughly, and do not leave bubbles on the array surface
Unexpected Binding	<ul style="list-style-type: none"> • Cross contamination between wells or other sources. • Sample contamination 	<ul style="list-style-type: none"> • Make sure to use sterilized pipette tips and tubes used for sample application and preparation • Ensure cassette is pressed firmly to the slide so that there are no gaps to allow leaking between wells • Be careful not to cross contaminate samples when applying to the wells, even during wash steps