O-Glycan Array User Manual

Z BIOTECH

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For Research Use Only

Introduction

Glycans attached to cell membranes and other types of proteins are the primary determinants for binding activity and consecutive cellular function. The study of the function and characteristics of these sugars is a fundamental part of immunology research and is applicable in understanding a variety of intercellular interactions. New technologies such as microarray for glycan-binding applications allow researchers to investigate and reveal new information about this wide and developing field of glycoscience.

Tumor-associated carbohydrate antigens (TACAs) represent potential biomarkers for early detection of cancer as well as immunotherapeutic targets. For example, the extracellular glycoprotein MUC1 is overexpressed and aberrantly glycosylated in many types of cancers. The extracellular domain of the MUC-1 contains a variable number of tandem repeats (VNTR) of 20 amino acids residues with serine (Ser) or threonine (Thr) sites for O-glycosylation. Generally, aberrantly expressed O-Glycans aid in the metastasis of diseased cells, yet also distinguish diseased from healthy cells at the cell surface. Therefore, the O-Glycan array can serve as a research tool for determining O-glycan immunodominant epitopes in cancers or sensitively detect the glycan binding epitopes of autoantibodies that target unique O-glycan antigens.

This manual is provided as a comprehensive guide to help the researcher acquire clear results from the assay. Please read through carefully before starting your experiment.

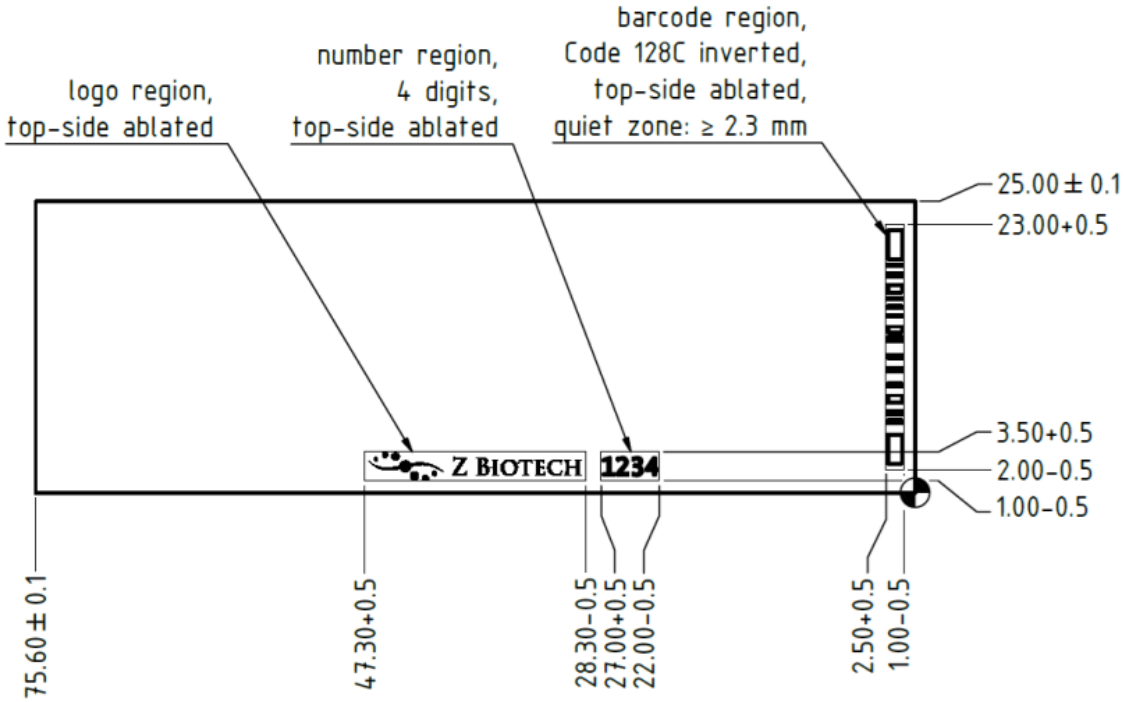
Handling and Storage

Store the bag of slides and any buffers in a 4°C refrigerator if they are to be assayed within 24 hours 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.

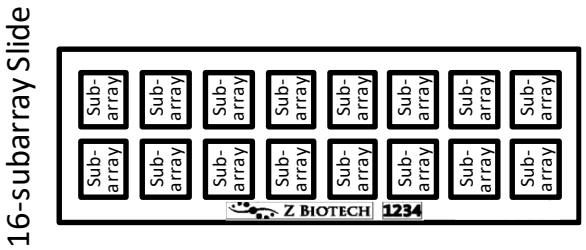
Allow the bag of slides to equilibrate to room temperature at least 20 minutes before opening. After opening, re-seal any unused slides in the moisture barrier bag with a desiccant inside and refreeze.

Array Map/Schematic

O-Glycan Array slides have either 8 or 16 subarrays. Arrays are printed on the side with the “Z Biotech” label and 4-digit number ID facing upward. The “Z Biotech” label is located on the bottom center from a landscape view. The number ID is consistent with the barcode ID on the bottom from a portrait view. Dimensions and array maps are shown below.



Array Map (16-subarray slides)



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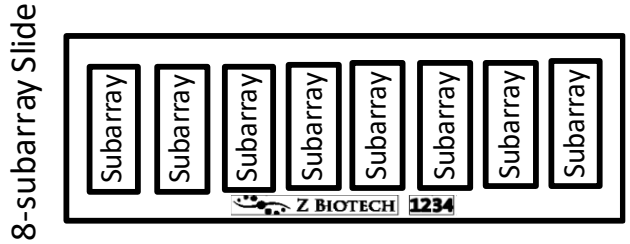
79	79	79	80	80	80	81	81	81	82	82	82	83	83	83	84	84	84
85	85	85	86	86	86	87	87	87	88	88	88	89	89	89	90	90	90
91	91	91	92	92	92	93	93	93	94	94	94	NC	NC	NC	PC1	PC1	PC1
PC2	PC2	PC2	PC3	PC3	PC3	PC4	PC4	PC4	Blank	Blank	Blank	Blank	Blank	Blank	M	M	M

Array Map (8-subarray slides):



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85	85	85	85	86	86	86	86	87	87	87	87	88	88	88	88	89	89	89	89	90	90	90	90	91	91	91	91	M	M

O-Glycan Identification List

ID	Structure
O1	GalNAcα
O2	GalNAcα
O3	Neu5Acα2-6GalNAcα
O4	Neu5Acα2-6GalNAcα
O5	Galβ1-3GalNAcα
O6	Galβ1-3GalNAcα
O7	Neu5Acα2-3Galβ1-3GalNAcα
O8	Neu5Gcα2-3Galβ1-3GalNAcα
O9	GalNAcβ1-4(Neu5Acα2-3)GalNAcα
O10	Fucα1-2Galβ1-3GalNAcα
O11	GalNAcβ1-3(Fucα1-2)Galβ1-3GalNAcα
O12	Galα1-3(Fucα1-2)Galβ1-3GalNAcα
O13	GlcNAcβ1-3Galβ1-3GalNAcα
O14	Galβ1-4GlcNAcβ1-3Galβ1-3GalNAcα
O15	Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-3GalNAcα
O16	Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-3GalNAcα
O17	GalNAcβ1-4(Neu5Acα2-3)Galβ1-4GlcNAcβ1-3Galβ1-3GalNAcα
O18	Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-3GalNAcα
O19	Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-3GalNAcα
O20	Fucα1-2Galβ1-4(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-3GalNAcα
O21	Neu5Acα2-6(Neu5Acα2-3(GalNAcβ1-4)Galβ1-3)GalNAcα
O22	GlcNAcβ1-6(Galβ1-3)GalNAcα
O23	GlcNAcβ1-6(Galβ1-3)GalNAcα
O24	Galβ1-4GlcNAcβ1-6(Galβ1-3)GalNAcα
O25	GlcNAcβ1-3GalNAcα
O26	GlcNAcβ1-3GalNAcα
O27	Galβ1-4GlcNAcβ1-3GalNAcα
O28	Galα1-3Galβ1-4GlcNAcβ1-3GalNAcα
O29	Neu5Acα2-3Galβ1-4GlcNAcβ1-3GalNAcα
O30	Neu5Acα2-6Galβ1-4GlcNAcβ1-3GalNAcα
O31	GalNAcβ1-4(Neu5Acα2-3)Galβ1-4GlcNAcβ1-3GalNAcα
O32	Galβ1-4(Fucα1-3)GlcNAcβ1-3GalNAcα
O33	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3GalNAcα
O34	Fucα1-2Galβ1-4GlcNAcβ1-3GalNAcα
O35	GalNAcα1-3(Fucα1-2)Galb1-4GlcNAcβ1-3GalNAcα
O36	Galα1-3(Fucα1-2)Galb1-4GlcNAcβ1-3GalNAcα
O37	Neu5Acα2-6(GlcNAcβ1-3)GalNAcα
O38	Neu5Acα2-6(Galβ1-4GlcNAcβ1-3)GalNAcα
O39	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcα
O40	GlcNAcβ1-6GalNAcα
O41	Galβ1-4GlcNAcβ1-6GalNAcα
O42	Galα1-3Galβ1-4GlcNAcβ1-6GalNAcα
O43	GalNAcβ1-4(Neu5Acα2-3)Galβ1-4GlcNAcβ1-6GalNAcα
O44	Galβ1-4(Fucα1-3)GlcNAcβ1-6GalNAcα
O45	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-6GalNAcα
O46	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-6GalNAcα
O47	Fucα1-2Galβ1-4GlcNAcβ1-6GalNAcα
O48	GalNAcα 1-3(Fucα1-2)Galβ1-4GlcNAcβ1-6GalNAcα
O49	GalNAcα-H2N-APGSTAPP-NH2
O50	GalNAcα-H2N-TSAPDTRPAP-NH2
O51	Neu5Acα2-3Galβ1-3GalNAcα
O52	Neu5Acα2-6Galβ1-3GalNAcα
O53	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-3GalNAcα
O54	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-3GalNAcα
O55	Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAcα
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O57	Neu5Acα2-3Galβ1-3(GlcNAcβ1-6)GalNAcα
O58	Neu5Acα2-3Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα
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O60	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Neu5Acα2-3Galβ1-3)GalNAcα
O61	Neu5Acα2-6Galβ1-3(GlcNAcβ1-6)GalNAcα
O62	Neu5Acα2-6Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα
O63	Neu5Acα2-3Galβ1-4GlcNAcβ1-6(Neu5Acα2-6Galβ1-3)GalNAcα

O64	Neu5Acα2-6Galβ1-4GlcNAcβ1-6(Neu5Acα2-6Galβ1-3)GalNAcα
O65	Neu5Acα2-3(GalNAcβ1-4)Galβ1-4GlcNAcβ1-6(Neu5Acα2-6Galβ1-3)GalNAcα
O66	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Neu5Acα2-6Galβ1-3)GalNAcα
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O68	Fucα1-2Galβ1-4GlcNAcβ1-6(Neu5Acα2-6Galβ1-3)GalNAcα
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O72	Neu5Acα2-3(GalNAcβ1-4)Galβ1-4GlcNAcβ1-6(Neu5Acα2-3(GalNAcβ1-4)Galβ1-3)GalNAcα
O73	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3)GalNAcα
O74	Fucα1-2Galβ1-3(GlcNAcβ1-6)GalNAcα
O75	Fucα1-2Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα
O76	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Fucα1-2Galβ1-3)GalNAcα
O77	Galα1-3Galβ1-4GlcNAcβ1-6(Galβ1-3)GalNAcα
O78	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3GalNAcα
O79	GalNAcβ1-4GlcNAcβ1-3GalNAcα
O80	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcα
O81	Galβ1-4GlcNAcβ1-3(Galβ1-3GlcNAcβ1-6)GalNAcα
O82	Galα1-3Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-3GlcNAcβ1-6)GalNAcα
O83	Neu5Acα2-6Galβ1-4GlcNAcβ1-3(Neu5Acα2-6Galβ1-3GlcNAcβ1-6)GalNAcα
O84	Neu5Acα2-3(GalNAcβ1-4)Galβ1-3GlcNAcβ1-3(Neu5Acα2-3(GalNAcβ1-4)Galβ1-3GlcNAcβ1-6)GalNAcα
O85	Galβ1-3(Fucα1-3)GlcNAcβ1-3 (Galβ1-4(Fucα1-3)GlcNAcβ1-6)GalNAcα
O86	Neu5Acα2-3Galβ1-3(Fucα1-3)GlcNAcβ1-3(Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-6)GalNAcα
O87	Fucα1-2Galβ1-3GlcNAcβ1-3(Fucα1-2Galβ1-4GlcNAcβ1-6)GalNAcα
O88	Fucα1-2(GalNAcα1-3)Galβ1-3GlcNAcβ1-3(Fucα1-2(GalNAcα1-3)Galβ1-4GlcNAcβ1-6)GalNAcα
O89	Fucα1-2(Galα1-3)Galβ1-3GlcNAcβ1-3(Fucα1-2(Galα1-3)Galβ1-4GlcNAcβ1-6)GalNAcα
O90	Fucα1-2Galβ1-3(Fucα1-3)GlcNAcβ1-3(Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-6)GalNAcα
O91	Neu5Acα2-3Galβ1-4GlcNAcβ1-6GalNAcα
O92	Neu5Acα2-6Galβ1-4GlcNAcβ1-6GalNAcα
O93	Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-6GalNAcα
O94	GalNAcβ1-4GlcNAcβ1-6GalNAcα

Controls

NC: Negative control, Print Buffer
PC1: Positive control 1, Biotinylated PEG (0.01 mg/mL)
PC2: Positive control 2, Human IgG (0.1 mg/mL)
PC3: Positive control 3, Mouse IgG (0.1 mg/mL)
PC4: Positive control 4, Rabbit IgG (0.1 mg/mL)
Marker: Anti-human IgG, Cy3 (0.01 mg/mL) and anti-Human IgG, Alexa647 (0.01 mg/mL)

Materials Required

- Arrayed glass slides
- 16 or 8 cassettes
- Glycan Array Blocking Buffer (GABB, Item #10106), add 1% BSA (10 mg/ml) if needed
- Glycan Array Assay Buffer (GAAB, Item #10107), add 1% BSA (10 mg/ml) if needed
- Wash Buffer: 50 mM Tris-HCl, 137 mM NaCl, 0.05% Tween 20, pH 7.6
- Laser fluorescence scanner (able to scan at the wavelength of your fluorophore)
- Coplin jar
- Adhesive slide cover film

Preparation of assay samples:

Prepare glycan-binding protein samples or secondary antibodies of interest in a centrifuge tube by diluting with the Glycan Array Assay Buffer. We recommend a range of 50 μg/ml to 0.1 μg/ml concentration for protein samples, although some experimentation may be required to establish the concentration that will provide the highest binding signals with the lowest background fluorescence. This can be accomplished by applying a different dilution of samples to different wells of the array. For the fluorescently labelled streptavidin we suggest a concentration of 1 μg/mL. Calculate the volume of sample needed depending on how many slides and subarrays are to be assayed. Use 100 μL volume of sample per well for 16

subarray cassettes and 200 µL for 8 subarray cassettes in order 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 120 µL for 8 subarrays. 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. Touch the pipette tip to the corner of the well of the cassette and tip the slide so that the sample pools to that corner and remove blocking buffer. 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. Touch the pipette tip to the corner of the well of the cassette and tip the slide so that the sample pools to that corner and remove it. 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. Analyze data with microarray analysis software. If there is specific binding the signal intensity should be significantly higher than the negative control signal. Our standard method of comparing signal intensities is to quantify the median signal intensity data and subtract the background intensity.

Interpretation of Control Signals:

Negative Controls (Print Buffer): The negative controls should produce little to no signal. Since there is no binding involved with the negative control, any other signals around the negative control's intensity are also not binding.

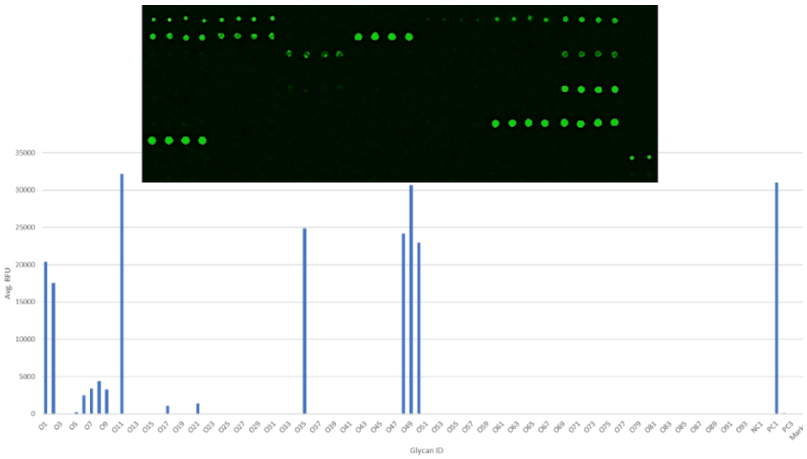
Marker: The array marker should show a strong fluorescence signal regardless of the assay. It is there primarily to aid with orientation of the array map during analysis.

Biotinylated PEG (PC1): This positive control will bind directly to the fluorescent labelled streptavidin. If your glycan-binding protein 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.

Typical Binding Assay Result from the O-Glycan Array

Example 1: A well on an O-Glycan Array slide (8-subarray) was assayed with glycan-binding protein biotinylated Helix pomatia agglutinin (HPA) lectin (10 µg/mL), followed by Streptavidin-Cy3. Array was scanned with InnoScan 710 microarray scanner at 2 PMT and low laser power at 532nm wavelength. There is very low non-specific binding for the negative control spots. Positive control 1 and the marker show binding as expected, as well as terminal-GalNAc-containing O-glycans.



Troubleshooting

Condition	Possible Causes	Potential Solutions
High Background	• Concentration of glycan-binding 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.	• Use a lower concentration range of samples. Consider a wider range if you are unsure where the detection limit is. • 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 • If you think that the protein is aggregating during incubation, try shaking at a higher speed
Signal Variation	• Slide drying out during assay. • Binding samples are not equally distributed in the wells • Glycan-binding protein aggregation during incubation • Bubbles during incubation	• 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	• Cross contamination between wells or other sources. • Sample contamination	• 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