HMO Glycan Microarray User Manual

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For Research Use Only

Introduction

Human milk oligosaccharides (HMOs) are the third most abundant solid ingredient in breast milk. These sugars - unique to human milk - protect newborns from disease and infection, establish healthy gut bacteria, and are essential for infant brain, intestinal, and immune system development.

Inaugural studies of HMOs have found many potential benefits for the incorporation of HMOs in infant formula as well as medications for adults. They have been found not only to restore beneficial microorganisms to the gut, but also function as a natural antiviral, inhibit growth of pathogenic bacteria, and inhibit tumor cell growth. In addition, HMO profiling has been used to evaluate infant digestive development, diarrhea incidence, morbidity and mortality, body composition, and risk of developing certain disorders.

Upon discovering these highly beneficial roles for HMOs, biopharmaceutical companies have begun to incorporate 1 or 2 of the abundant HMOs in their infant formula, and it is anticipated that more HMOs will be added in the future to better mimic the complete nutrition provided by natural breastmilk. However, the specific functions and interactions of these carbohydrates are still largely unstudied and there remains much potential for their application as biomarkers or in the development of drugs and infant formula.

Z Biotech's HMO Glycan Microarray is designed to make the investigation of HMOs easy and efficient. As leaders in the glycan microarray industry we have developed sensitive, high density arrays for the research of glycan-binding proteins and antibodies. Using minimal sample volume and only a few hours, these arrays can be tested with cells, antibodies, or other proteins to determine which HMOs interact and give insight into the functions of these HMOs and their binding partners.

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.

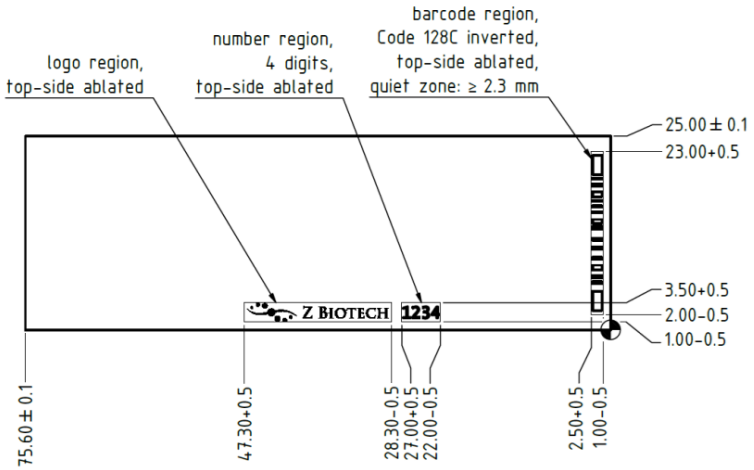
Handling and Storage

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

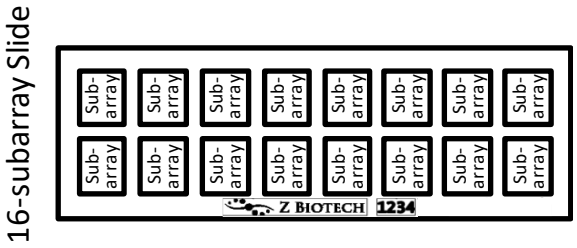
Allow the bag of slides to equilibrate to room temperature at least 20 minutes before opening. After opening, re-seal any unused slides in the moisture barrier bag with a desiccant inside and refreeze.

Array Map/Schematic

HMO Glycan Microarray 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):



HMO1	HMO1	HMO1	HMO2	HMO2	HMO2	HMO3	HMO3	HMO3	HMO4	HMO4	HMO4
HMO5	HMO5	HMO5	HMO6	HMO6	HMO6	HMO7	HMO7	HMO7	HMO8	HMO8	HMO8
HMO9	HMO9	HMO9	HMO10	HMO10	HMO10	HMO11	HMO11	HMO11	HMO12	HMO12	HMO12
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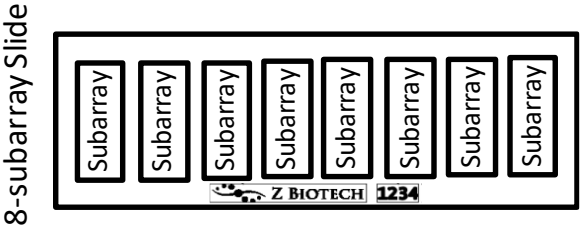
HMO41	HMO41	HMO41	HMO42	HMO42	HMO42	HMO43	HMO43	HMO43	HMO44	HMO44	HMO44
HMO45	HMO45	HMO45	HMO46	HMO46	HMO46	NC	NC	NC	PC1	PC1	PC1
PC2	PC2	PC2	PC3	PC3	PC3	PC4	PC4	PC4	MARKER	MARKER	MARKER

Array Map (8-subarray slides):



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HMO46	HMO46	HMO46	HMO46	HMO46	HMO46	NC	NC	NC	NC	NC	NC	PC1	PC1	PC1	PC1	PC1	PC1	PC2	PC2	PC2	PC2	PC2	PC2	PC3	PC3	PC3	PC3	PC3	PC3
PC4	PC4	PC4	PC4	PC4	PC4																			M	M	M	M	M	M

HMO Identification List:

ID	Structure	Common Name
HMO1	Galβ1-4Glc	Lactose
HMO2	Galβ1-4(Fucα1-3)Glc	3-FL
HMO3	Fucα1-2Galβ1-4Glc	2'-FL
HMO4	Neu5Acα2-3Galβ1-4Glc	3'-SL
HMO5	Neu5Acα2-6Galβ1-4Glc	6'-SL
HMO6	Galα1-4Galβ1-4Glc	Gb_3 (P^k antigen)
HMO7	GalNAcβ1-3Galα1-4Galβ1-4Glc	Gb_4
HMO8	Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc	Gb_5 (SSEA-3)
HMO9	Fucα1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc	Globo-H
HMO10	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	LNT
HMO11	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	LNnT
HMO12	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	LNFP-I
HMO13	Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc	LNFP-II
HMO14	Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	LNFP-III
HMO15	Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glc	Blood group H antigen
HMO16	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	LeY
HMO17	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	LsTc-I
HMO18	Galβ1-4GlcNAcβ1-3(Neu5Acα2-6)Galβ1-4Glc	LsTc-II
HMO19	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	LsTa
HMO20	Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	Ac3-LNnT
HMO21	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	Ac6-LNnT
HMO22	Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc	DSLNT
HMO23	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	Lacto-N-neoPentaose (LNnP)
HMO24	GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	Fuc-LNnP
HMO25	GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO26	GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO27	GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO28	GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO29	GlcNAcβ1-6(Neu5Acα2-6Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO30	GlcNAcβ1-6(Neu5Acα2-3Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO31	GlcNAcβ1-6(Galβ1-4(Fucα1-3)GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO32	GlcNAcβ1-6(Fucα1-2Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO33	GlcNAcβ1-6(Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO34	Galβ1-4GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO35	Neu5Acα2-6Galβ1-4GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO36	Galβ1-4(Fucα1-3)GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO37	Fucα1-2Galβ1-4GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO38	Galβ1-4GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO39	Galβ1-4GlcNAcβ1-6(Neu5Acα2-6Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO40	Galβ1-4GlcNAcβ1-6(Fucα1-2Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO41	Neu5Acα2-6Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO42	Neu5Gcα2-6Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO43	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO44	Neu5Gcα2-6Galβ1-4GlcNAcβ1-6(Neu5Gcα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO45	Fucα1-2Galβ1-4GlcNAcβ1-6(Fucα1-2Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	N/A
HMO46	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-6(Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3)Galβ1-4Glc	N/A

Controls

NC: Negative control, Print Buffer
PC1: Positive control 1, a biotinylated probe (0.01 mg/ml)
PC2: Postitive control 2, Human IgG (0.1 mg/ml)
PC3: Postitive control 3, Mouse IgG (0.1 mg/ml)
PC4: Postitive control 4, Rabbit IgG (0.1 mg/ml)
Array Marker: Anti-Human IgG, Cy3 (0.01 mg/ml) and anti-Human IgG, Alexa555 (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 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 μg/mL. For detection antibodies, we suggest a concentration around 1-10 μg/ml. A range of 100 μg/ml to 0.1 μg/ml concentration for glycan-binding samples works, 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. In addition to testing a dilution range for your glycan-binding protein 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 protein of interest. A fluorescent signal due to specific binding to your protein of interest should be dose-dependent within the dynamic range of your protein dilution, and should have a positive binding signal after a signal from control assays has been subtracted. 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 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. 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, and there are no dry spots on the subarray after the sample is added.

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 the 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 (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 saturates the spots), 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.

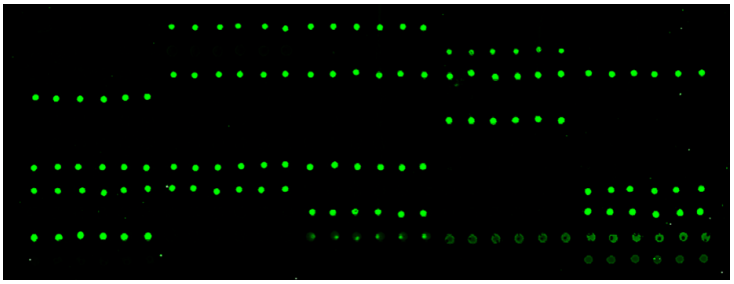
Positive Control (a biotinylated probe): This positive control will bind directly to the fluorescent labelled streptavidin. If your glycan-binding sample is already fluorescently labelled, or in any case where the addition of fluorescent labelled streptavidin to the array was not preformed (Part 5 – 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 Result from the HMO Glycan Microarray

Example: HMO Glycan Microarray on 8 subarray formats. A subarray assayed with biotinylated AAL lectin (10 μg/ml), followed by streptavidin-Cy3 (1 μg/ml). The array was scanned with GenePix scanner at 450 PMT and 100% laser power at 532nm wavelength. The positive control shows binding as expected. All HMOs containing fucose show fluorescent binding signal.



Troubleshooting

Condition	Possible Causes	Potential Solutions
High Background	• 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.	• 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	• 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