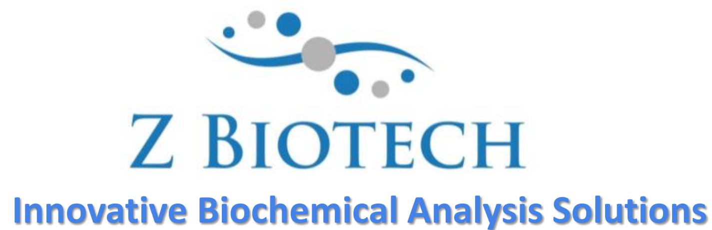
HMO Glycan Microarray User Manual



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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.

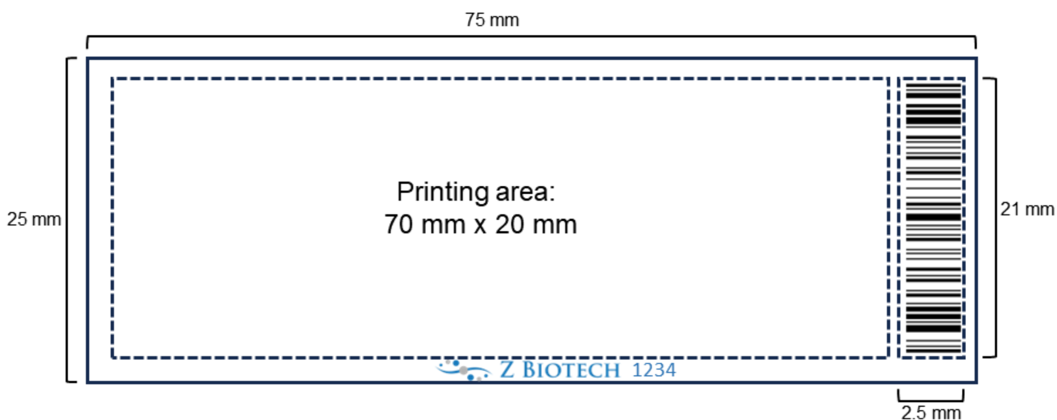
Handling and Storage

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):

16-subarray Slide

Z BIOTECH 1234

HMO1	HMO1	HMO1	HMO1	HMO2	HMO2	HMO2	HMO2	HMO3	HMO3	HMO3	HMO3	HMO4	HMO4	HMO4	HMO4
HMO5	HMO5	HMO5	HMO5	HMO6	HMO6	HMO6	HMO6	HMO7	HMO7	HMO7	HMO7	HMO8	HMO8	HMO8	HMO8
HMO9	HMO9	HMO9	HMO9	HMO10	HMO10	HMO10	HMO10	HMO11	HMO11	HMO11	HMO11	HMO12	HMO12	HMO12	HMO12
HMO13	HMO13	HMO13	HMO13	HMO14	HMO14	HMO14	HMO14	HMO15	HMO15	HMO15	HMO15	HMO16	HMO16	HMO16	HMO16
HMO17	HMO17	HMO17	HMO17	HMO18	HMO18	HMO18	HMO18	HMO19	HMO19	HMO19	HMO19	HMO20	HMO20	HMO20	HMO20
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HMO41	HMO41	HMO41	HMO41	HMO42	HMO42	HMO42	HMO42	HMO43	HMO43	HMO43	HMO43	HMO44	HMO44	HMO44	HMO44
HMO45	HMO45	HMO45	HMO45	HMO46	HMO46	HMO46	HMO46	NC1	NC1	NC1	NC1	NC2	NC2	NC2	NC2
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Array Map (8-subarray slides):

8-subarray Slide

Z BIOTECH 1234

HMO1	HMO1	HMO1	HMO1	HMO1	HMO1	HMO2	HMO2	HMO2	HMO2	HMO2	HMO2	HMO3	HMO3	HMO3	HMO3	HMO3	HMO3	HMO4	HMO4	HMO4	HMO4	HMO4	HMO4	HMO5	HMO5	HMO5	HMO5	HMO5	HMO5	HMO6	HMO6	HMO6	HMO6	HMO6	HMO6
HMO7	HMO7	HMO7	HMO7	HMO7	HMO7	HMO8	HMO8	HMO8	HMO8	HMO8	HMO8	HMO9	HMO9	HMO9	HMO9	HMO9	HMO9	HMO10	HMO10	HMO10	HMO10	HMO10	HMO10	HMO11	HMO11	HMO11	HMO11	HMO11	HMO11	HMO12	HMO12	HMO12	HMO12	HMO12	HMO12
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PC1	PC1	PC1	PC1	PC1	PC1	PC2	PC2	PC2	PC2	PC2	PC2	PC3	PC3	PC3	PC3	PC3	PC3	PC4	PC4	PC4	PC4	PC4	PC4	Blank	Blank	Blank	Blank	Blank	Blank	Marker	Marker	Marker	Marker	Marker	Marker

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
- 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 is often accomplished by applying a different dilution of samples to different wells of the array. For the fluorescently labelled streptavidin we recommend a concentration of 1 µg/mL. 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 for every step of the assay.** 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 subarray cassettes. 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. Let the arrayed slides equilibrate to room temperature (20-30 minutes) before opening the moisture barrier bag.
2. Add blocking buffer to each subarray well.
3. Cover the wells with adhesive film to prevent evaporation and incubate slide on shaker at 80 rpm for 30 min. 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 and detection.

Part 2 – Binding Assay

1. Unless the glycan binding protein 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 a pipette tip to the corner of the well, tipping the slide so that the sample pools to that corner, and pipetting off buffer. Avoid touching the array surface. Have the replacement buffer ready before removing the old buffer to ensure the array does not dry out.

3. Wash the wells three times by adding GAAB to each well and shaking the array at 80 rpm for 5 min. Remove the buffer and repeat.
4. Immediately apply the glycan binding protein sample of interest to each well. Avoid leaving air bubbles.
5. 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 hour at 80 rpm. If the samples can easily aggregate, shake at higher speed to prevent protein 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 protein samples are fluorescently labelled, go directly to Part 6 – Final wash and dry.

Part 3 – Wash

1. Remove buffer or sample from each well by gently touching a pipette tip to the corner of the well, tipping the slide so that the sample pools to that corner, and pipetting off buffer. Avoid touching the array surface.
2. Immediately add GAAB to each well. Incubate on the shaker for 5 minutes at 80 rpm. Completely remove the buffer by pipette and repeat this step twice more. Avoid allowing the slide to dry out by having your next wash or sample ready before you remove the 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 third GAAB wash, immediately add the secondary biotinylated antibody to each well. Seal the wells with adhesive film and incubate on the shaker for 1 hour at 80 rpm. Shaking at a faster speed can prevent protein aggregation. 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 third GAAB wash, immediately add the fluorescently labelled streptavidin sample. Seal the wells with adhesive film and shield the wells from light with aluminum foil. Incubate on the shaker at 80 rpm for 1 hour. Longer incubation time is acceptable, but not necessary.

Part 6 – Final Wash and Dry

1. Remove sample from each well by gently touching a pipette tip to the corner of the well, tipping the slide so that the liquid pools to that corner, and pipetting off. Avoid touching the array surface.
2. Briefly rinse each well with GAAB.
3. Completely remove the 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 GAAB. 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 a shaker at 80 rpm for 10 minutes.
7. Decant the 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 shaker at 80 rpm for 2 minutes.
9. Decant the water from the jar or beaker. Repeat once more with fresh de-ionized water.
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:

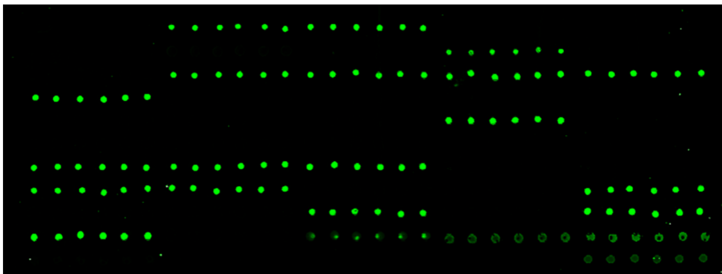
Negative Control (Print Buffer): The negative control should produce a signal close to the intensity of the background. Since there is no binding involved with the negative control, any other signals around the negative control's intensity are also not binding.

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.

Biotinylated Mannose (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 fluorescently labelled streptavidin to the array was not performed (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 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