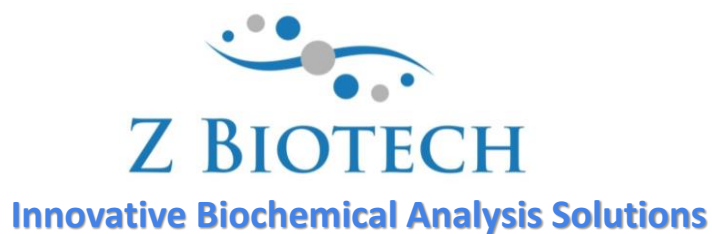


Heparan Sulfate Glycan Array User Manual



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Introduction

Heparan sulfate is a sulfated, unbranched polysaccharide found on cell surfaces as well as in the extracellular matrix. Heparan sulfates interact with a variety of proteins and are known to be important to the regulation of many physiological and pathophysiological functions. Because of the prevalence and multifunctionality of heparan sulfates, investigation of the binding roles of this type of glycan has become invaluable for understanding how to combat related diseases.

Z Biotech has expanded its microarray technology to provide a research platform specifically for the investigation of heparan sulfates. Our Heparan Sulfate Array can be used to determine the specific binding interactions between various types of heparan sulfates and their potential protein partners via microarray assays.

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.

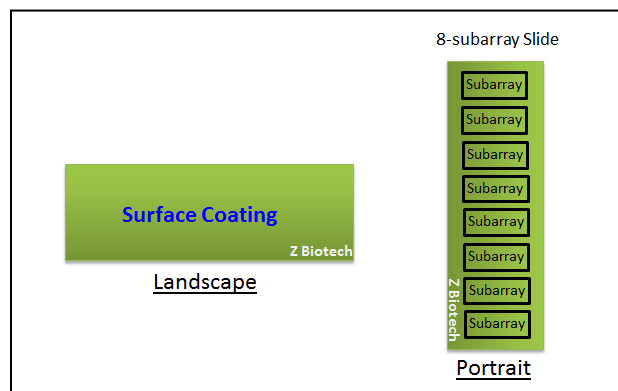
Handling and Storage

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. 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

24 Heparan Sulfate 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 (the "marker" spots will be on the lower right of each array when looking at the slide via the portrait orientation). Dimensions and array map are shown on the following pages.



24 Heparan Sulfate Glycan Identification List:

No.	Structure
HS001	GlcNAc-GlcA-GlcNAc-GlcA
HS002	GlcA-GlcNAc-GlcA-GlcNAc-GlcA
HS003	GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc-GlcA
HS004	GlcA-GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc-GlcA
HS005	GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc-GlcA
HS006	GlcA-GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc-GlcA
HS007	GlcNS-GlcA-GlcNS-GlcA
HS008	GlcA-GlcNS-GlcA-GlcNS-GlcA
HS009	GlcNS-GlcA-GlcNS-GlcA-GlcNS-GlcA
HS010	GlcA-GlcNS-GlcA-GlcNS-GlcA-GlcNS-GlcA
HS011	GlcNS-GlcA-GlcNS-GlcA-GlcNS-GlcA-GlcNS-GlcA
HS012	GlcA-GlcNS-GlcA-GlcNS-GlcA-GlcNS-GlcA-GlcNS-GlcA
HS013	GlcA-GlcNS-GlcA-GlcNS-GlcA-GlcNS-GlcA-GlcNS6S-GlcA
HS014	GlcA-GlcNS-GlcA-GlcNS-GlcA-GlcNS6S-GlcA-GlcNS6S-GlcA
HS015	GlcA-GlcNS-GlcA-GlcNS6S-GlcA-GlcNS6S-GlcA-GlcNS6S-GlcA
HS016	GlcA-GlcNS6S-GlcA-GlcNS6S-GlcA-GlcNS6S-GlcA-GlcNS6S-GlcA
HS017	GlcNS-GlcA-GlcNS-GlcA-GlcNS-IdoA2S-GlcNS-GlcA
HS018	GlcNS-GlcA-GlcNS-IdoA2S-GlcNS-IdoA2S-GlcNS-GlcA
HS019	GlcNAc-GlcA-GlcNS-IdoA2S-GlcNS-IdoA2S-GlcNS-GlcA
HS020	GlcNS6S-GlcA-GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS6S-GlcA
HS021	GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-GlcA
HS022	GlcNAc6S-GlcA-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-GlcA
HS023	GlcNS6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-GlcA
HS024	GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-GlcA

Controls

NC: Negative control, Print Buffer

PC1: Positive control 1, Biotinylated Mannose (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 slide
- 16 or 8 subarray cassette
- Blocking Buffer: 1% BSA and 1mM DTT in PBST (PBS with 0.05% (v/v) Tween-20, pH 7.4)
- Heparan Sulfate Glycan Array Assay Buffer (HSAB Item #10110)
Materials above are included for purchase in our 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 labelled streptavidin (for biotin labelled 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 protein samples or detection antibodies in a centrifuge tube by diluting with the Heparan Sulfate Glycan Array Assay Buffer. For the fluorescently labelled streptavidin we recommend a concentration of 1 $\mu\text{g}/\text{mL}$. We recommend a range of 50 $\mu\text{g}/\text{ml}$ to 0.1 $\mu\text{g}/\text{ml}$ 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. In addition to testing a dilution range for your 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. This may be especially important as heparin sulfates have been known to show weak non-specific binding to proteins. 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 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. Analyze data with microarray analysis software. If there is specific binding the signal intensity should be significantly higher than the background signal (area where there are no printed spots). Fluorescent signal due to specific binding to your protein of interest should be both dose-dependent with your protein dilution (unless the protein 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 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.

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

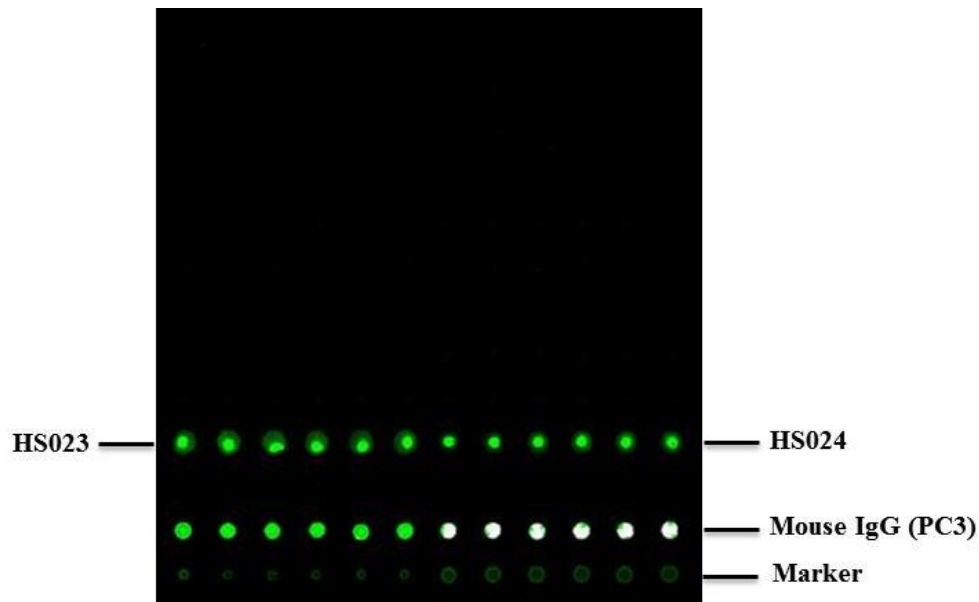
where the addition of fluorescent labelled streptavidin to the array was not preformed (Part 5 – Fluorescent staining) this positive control will not be reactive.

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 a 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 24 Heparan Sulfate Glycan Array

Example: 24 Heparan sulfate glycan array on 16 subarray format. A subarray assayed with protein antithrombin III (10 $\mu\text{g/ml}$), followed by anti-antithrombin III antibody (2 $\mu\text{g/ml}$), and then anti-mouse IgG antibody AlexaFluor555 (10 $\mu\text{g/ml}$). The array was scanned with GenePix scanner at 500 PMT and 100% laser power at 532nm wavelength. Positive controls 2-4 and the marker show binding as expected with the mouse IgG control being the strongest (PC3). HS023 and HS024 show specific binding to Antithrombin III.



Troubleshooting

Condition	Possible Causes	Potential Solutions
High Background	<ul style="list-style-type: none"> • 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. 	<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. • 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	<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