


## Module 2. Glycan Array Printing & Analysis

### Goals

1. Create a print layout to analyze siglec ligand binding of sialylated glycans, both “natural” and “high-affinity”.
  2. Print slides on contact microarray printer, perform post-printing immobilization and blocking.
  3. Ensure samples are printed efficiently using plant lectins for detection. Assay Siglec-F and Siglec-9 at varying concentrations on the array, taking into account multivalency effects (i.e. pre-complexing of Fc-chimera protein VS 2-step incubation).
  4. Perform scanning/image analysis (Innoscan scanner and Mapix software) and data analysis (Excel macro/graphic output).
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1. Create a print layout to analyze siglec ligand binding of sialylated glycans, both “natural” and “high-affinity” (DEMONSTRATION)
    - Basic design of a comparative array which allows side-by-side analysis and is easily interpretable visually.
      - 6 compounds
        - a) (6S)LacNAc (-ve control)
        - b) 3'sialo-Lac(6S)NAc (sialylated -ve control)
        - c) 3'sialo-(6S)LacNAc (native +ve control)
        - d) High affinity Siglec-F ligand
        - e) New generation Siglec-9 ligand
        - f) Original Siglec9 ligand
      - Layout design that allows comparison of concentration dependent binding and sample to sample binding affinity.
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      - Basics of plate layouts, printing from low through high concentrations, basic grid design and array design.
  2. Print slides on contact microarray printer, perform post-printing immobilization and blocking (DEMONSTRATION).
    - Demonstration of printer and running a print.
    - Immobilization/humidification step and then desiccation.
    - Demonstrate array gridding and slide blocking.

3. Assay 2 biotinylated plant lectins to assure printing efficiency. Assay 2 siglecs (Siglec-F/Siglec-9), taking into account multivalency effects (i.e. pre-complexing of Fc-chimera protein VS 2-step incubation).

- Slides with multiple arrays/slide will allow the participants to assay Siglecs in 2 fashions: pre-complexed vs 2-step assay.
- Observe selectivity of proteins for high affinity analogues.

4. Scan arrays and perform image analysis (Innoscan and Mapix) and data analysis (Excel macro/graphic output)

- Multiple seats of Mapix software will allow us to demonstrate the analysis of one array and then each participant can analyze an array individually and create the output graph for “their” array
- Combine assay results into a summary table/PPT that shows the complete experiment

#### Selected References

*Production and application of glycan microarrays.* Busch J, McBride R, Head SR. *Methods Mol Biol.* 2010;632:269-82. PMID: 20217584

*Printed covalent glycan array for ligand profiling of diverse glycan binding proteins.* Blixt O, Head S, Mondala T, Scanlan C, Huflejt ME, Alvarez R, Bryan MC, Fazio F, Calarese D, Stevens J, Razi N, Stevens DJ, Skehel JJ, van Die I, Burton DR, Wilson IA, Cummings R, Bovin N, Wong CH, Paulson JC. *Proc Natl Acad Sci U S A.* 2004 Dec 7;101(49):17033-8. PMID: 15563589

*Microarray method for the rapid detection of glycosaminoglycan-protein interactions.* Rogers CJ, Hsieh-Wilson LC. *Methods Mol Biol.* 2012;808:321-36. PMID: 22057535

## Detailed Methods

### 1. Create a print layout to analyze siglec ligand binding of sialylated glycans, both “natural” and “high-affinity” (DEMONSTRATION)

#### Materials

- microbalance/scale, scale paper and weighing “straws”
- 1.5mL Eppendorf/sample microtubes
- pipettors and pipetting tips (recommend at least P10 and P1000)
- printing buffer (150mM sodium phosphate buffer + 0.005% Tween-20, pH 8.2)
- benchtop centrifuge
- -20C freezer
- 150uL microtubes (PCR tubes)
- 96 well microtiter plate (Nunc, Cat# 276002)
- 384 well microtiter plate and lids (ThermoFisher, Plate Cat#4361; Lid Cat#4950)
- Sealing film (USA Scientific, Cat#2921-0000)
- Centrifuge equipped with swinging baskets capable of holding microtiter plates

#### 1a. Library stock preparation for glycan samples

- Weigh out lyophilized sample to nearest ug and place into a 1.5mL microtube.
- Using MW calculate the volume of printing buffer necessary to create a 1mM stock solution.
- Pipette appropriate volume of printing buffer directly into the microtube, cap the tube and vortex vigorously for 10-15sec to fully dissolve the material.
- Using a bench top centrifuge, spin down the microtube to collect all the volume at the bottom of the microtube – this is the library stock.
- NOTE – we generally allow the sample one freeze/thaw cycle, overnight, in a -20C freezer prior to sample use, but this step can be omitted.

#### 1b. Printing stock and plate preparation for glycan array printing

- Prepare a 1:10 dilution of the stock, in printing buffer to obtain a 100uM printing stock (e.g. for 100uL of printing stock, pipette 10uL of library stock glycan into 90uL of printing buffer).
- For creation of multiple concentrations of printed sample, all dilutions are performed directly into printing buffer.
- For this exercise, we will perform 10 1:2 serial dilutions of each glycan to obtain a concentration series of 100, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4 and 0.2uM.
- Dilutions are performed in 150uL microtubes by preparing 9 tubes, each with 10uL of printing buffer.
- Starting with 20uL of 100uM printing stock in a 150uL microtube, 10uL is transferred to a tube containing 10uL of printing buffer and is mixed by pipetting up and down and then sequentially transferred to each of the 9 tubes.

- The sample preparations and dilutions can be prepared in 96-well microtiter plates, if there is sufficient printing planned to necessitate several printing sessions (more than 400 arrays).
- 384-well microtiter plate (“Printing Plate”) preparation will depend on the printing tool (pin tool) used and the number of arrays to be printed.
- For the example print, we will use a 2x3 pin tool containing 6 pins, 1 pin for each sample.
- Each “block” of 2x3 wells will contain 1 concentration of each of the 4 samples to be printed and blocks will be sequentially prepared from low to high concentration.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	Blue	Green	Red	Blue	Green	Red																		
B	Red	Yellow	Blue	Red	Yellow	Blue																		
C	Blue	Green	Red	Blue	Green	Red																		
D	Red	Yellow	Blue	Red	Yellow	Blue																		
E	Blue	Green	Red																					
F	Red	Yellow	Blue																					
G	Blue	Green	Red																					
H	Red	Yellow	Blue																					
I	Blue	Green	Red																					
J	Red	Yellow	Blue																					
K	Blue	Green	Red																					
L	Red	Yellow	Blue																					
M	Blue	Green	Red																					
N	Red	Yellow	Blue																					
O	Blue	Green	Red																					
P	Red	Yellow	Blue																					

- Prepare the Printing Plate by pipetting 10uL of each sample into the appropriate well of the plate, covering each “block” of samples with a piece of sealing film to reduce evaporation as the plate is filled with the remaining samples.
- Once all samples have been loaded into the plate, remove the small pieces of sealing film, replace with a whole piece of film and spin down the samples in a centrifuge equipped with plate holding baskets.
- The printing plate may be used directly or stored on wet ice until ready for printing.

## 2. Print slides on contact microarray printer, perform post-printing immobilization and blocking (DEMONSTRATION)

### Materials

- Contact microarray printer (MicroGridII, Genomic Solutions)
- Contact capillary microarray pins, SMP4B (Telechem)
- NHS-ester activated microscope slides (SlideH, Schott/Nexterion Cat#1070936)
- Humidification chamber (Pyrex dish, 48-hole tube rack, Paper Towels, Cellophane film)
- Desiccator with Drierite (Drierite, Cat#23005)

### 2a. Printing and post-processing of glycan microarrays

- Turn on microarray printer according to manufacturer's specifications.
- Using the appropriate pin tool, design the layout of the desired spotting pattern and approximate placement on the slide surface.
- Load a 384-well microtiter plate with 10uL of printing buffer in one "block" of wells appropriate to the selected pin tool (pickup).
- Perform a layout/pin test to make sure the placement of the desired spotted array is correct and that all pins "fire" (make a spot) throughout the number of selected spots (generally ~50).
- If placement is wrong, adjust the software accordingly and retest.
- If one or more pins do not fire, remove it from the print head and examine under a light microscope or magnifying glass to see if there is any blockage and try to remove the material either by blowing with a strong stream of compressed nitrogen or by running deionized water through the capillary space to try and dislodge the particle.
- Assuming the placement is correct and all pins fire correctly, replace the test plate with the printing plate and place the appropriate number of slides on the printing deck.
- Close the printer cover and, using the humidity controller, bring the humidity up to 55-60% relative humidity, as measured by a standard weather hygrometer.
- Once the humidity has stabilized, begin the print run, paying close attention to that the pins are correctly dipping into the microtiter plate wells and are creating a visible spot when contacting the slide surface.
- Allow the print run to go to completion and remove the printed slides from the printing deck.
- Place each slide into a humidification chamber and allow the slides to rest for 1h.
- After 1h, remove the slides from the chamber, place into a microscope slide box and place into a desiccator overnight.
- NOTE – we generally allow an overnight desiccation of the printed arrays prior to performing the blocking step, but this can be skipped and proceed directly to blocking

## **2b. Blocking of printed arrays**

- Remove slides from the desiccator and, using a lab marker, indicate the placement of the printed arrays with “brackets” and number/name the slide accordingly.
- Place into a Coplin slide staining dish or staining rack with appropriate dish.
- Fully immerse the slides in blocking buffer by pouring the buffer directly over the slides.
- Place the dish on a rotary shaker and allow the slides to block 1h at low/medium rotation.
- At the end of 1h, remove the slides from the blocking buffer and wash off the remaining buffer by rapidly dipping the slides into deionized water 10X.
- Following washing, dry the slides in a centrifuge equipped with swinging baskets capable of accommodating microtiter plates.
- Dry, blocked slides can now be stored in the desiccator or used immediately for analysis.

### **3. Assay siglecs (Siglec-F and Siglec-9) on the array, taking into account multivalency effects. Also, perform a lectin incubation for quality control of printed spots (HANDS-ON)**

#### Materials

- printed glycan array slide
- PBS + 0.05% Tween-20 (PBS, Corning Cat#46-013-CM; Tween-20, Fluka Cat#93773)
- 1.5mL microtube
- recombinant SiglecF-Fc-chimera (R&D Systems Cat#1706-SF-050)
- anti-human IgG-R-PE (Jackson Cat#109-115-098)
- MAL-II lectin (Vector Labs)
- ECA lectin (Vector Labs)
- Streptavidin AlexaFluor555 (LifeTechnologies)
- hydrophobic marker or mask
- PBS (PBS, Corning Cat#46-013-CM)
- deionized water
- centrifuge equipped with swinging baskets

#### 1. Prepare Arrays for Incubations:

Using a hydrophobic marker or mask, separate each printed array, for this experiment we will use a hydrophobic marker appropriate to the printed area. To ensure the integrity of the hydrophobic marker throughout the incubation steps, allow to dry fully on the bench or dry with a stream of compressed nitrogen gas.

#### 2. Prepare a humidified chamber:

Place a couple of paper towels in the bottom of a Pyrex dish and soak them generously with water. Use a test tube rack or lid of some sort inside the dish to put the slides on during the incubation to raise the slides up off the wet towels. Cover with cellophane to close the chamber.

#### 3. Prepare Samples for 2-step/sequential binding:

A1) SiglecF-Fc (100ug/mL)

1ug/mL 148.5 PBS-T + 1.5uL SiglecF (150uL)

A2) Siglec9 (supernatant)

150uL

Vortex and spin down

A3) MAA-II lectin (2mg/mL)

10ug/mL = 199 PBS-T + 1uL of 2ug/mL MAA-II (200uL)

B3) ECA lectin (2mg/mL)

10ug/mL = 499 PBS-T + 1uL of 5ug/mL SNA (500uL)

4. Soak the arrays by pipetting 200uL PBS-T directly onto the array for 2 min to hydrate the print surface. This is an important step to eliminate nonspecific binding, which can cause high background.
5. Pipette 150uL of samples A1, A2, A3, B3 into each array.
6. Incubate in the sealed humidification chamber for 1 h.
7. Prepare the secondaries:  
A1/A2) Anti-human-IgG-RPE @ 10ug/mL  
298uL PBS-T + 2uL anti-human-IgG-RPE (300uL total)  
  
A3) Streptavidin-AlexaFluor555 @ 2ug/mL  
999uL PBS-T + 1uL AlexaFluor555 (1mL total)
8. Prepare the pre-complexed SiglecF and Siglec9  
B1) SiglecF (1ug/mL) + anti-human-IgG-RPE (0.5ug/mL)  
253uL PBS-T + 5uL of anti-human-IgG-R-PE (diluted 1:100) + 1.5uL SiglecF  
(150uL total)  
  
B2) Siglec9 (supernatant) + anti-human-IgG-RPE (10ug/mL)  
149uL PBS-T + 1uL of anti-human-IgG-R-PE (150uL total)
9. At the end of the first incubation period, wash the arrays, by dipping 3X in PBS-Tween.
10. Add 150uL of secondary to each of the “2-step” arrays (A1/A2), 150uL of the “pre-complexed” samples to fresh arrays (B1/B2) and 150uL to each of the lectin control arrays (A3/B3).
11. Incubate in the sealed humidification chamber for 1 h.
12. After 1h, wash the slides and dip 4X in PBS/0.05% Tween-20, 4X in 1× PBS, and lastly 4X in ddH<sub>2</sub>O.
13. Following washing, dry the slides in a centrifuge equipped with swinging baskets capable of accommodating microtiter plates.

#### **4. Perform scanning/image analysis (Innoscan/Mapix) and data analysis (Excel macro/graphic output) (HANDS-ON)**

##### Materials

- Confocal fluorescent microscope slide scanner (Innoscan, Innopsys)
- Mapix software (Mapix, Innopsys)
- Microsoft Excel
- Graph creation macro



- Using the scanner software, set up the instrument to scan with the desired parameters. We will scan 3 repetitions, each at an increasing strength of both laser power (LP) and photo-multiplier tube enhancement (PMT).  
1) LowLaser(5mW)/10PMT; 2) LowLaser/25PMT; and 3) LowLaser/50PMT
- Using a previously constructed GAL file (Gene Axon List - spot mapping file), measure the array and save the data results to a folder, noting the slide number, sample and scanner settings in the title. The placement of a sample marker known to react upon analysis is important in order to accurately place the grid file. We will be using spotted dyes to accurately place the grid file in the correct location.
- The output, a tab-delineated text file, can then be opened in Microsoft Excel and, with the use of a multistep macro, the data can be quickly sorted, organized, and graphed.
- Save the output Excel file, noting the slide number, sample and scanner settings in the title.