

Video Article

# Printed Glycan Array: A Sensitive Technique for the Analysis of the Repertoire of Circulating Anti-carbohydrate Antibodies in Small Animals

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## Abstract

The repertoire of circulating anti-carbohydrate antibodies of a given individual is often associated with its immunological status. Not only the individual immune condition determines the success in combating internal and external potential threat signals, but also the existence of a particular pattern of circulating anti-glycan antibodies (and their serological level variation) could be a significant marker of the onset and progression of certain pathological conditions. Here, we describe a Printed Glycan Array (PGA)-based methodology that offers the opportunity to measure hundreds of glycan targets with very high sensitivity; using a minimal amount of sample, which is a common restriction present when small animals (rats, mice, hamster, etc.) are used as models to address aspects of human diseases. As a representative example of this approach, we show the results obtained from the analysis of the repertoire of natural anti-glycan antibodies in BALB/c mice. We demonstrate that each BALB/c mouse involved in the study, despite being genetically identical and maintained under the same conditions, develops a particular pattern of natural anti-carbohydrate antibodies. This work claims to expand the use of PGA technology to investigate repertoire (specificities) and the levels of circulating anti-carbohydrate antibodies, both in health and during any pathological condition.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/57662/>

## Introduction

Antibodies play a central role in our defense against invading pathogens by directly neutralizing viruses<sup>1,2</sup> and bacteria<sup>2,3</sup>, by activating the complement system<sup>4,5</sup> and the enhancement of phagocytosis<sup>6</sup>. Additionally, they are essential elements in cancer targeting and elimination of malignant cells<sup>7</sup>, and homeostasis maintenance<sup>8,9</sup>.

Disorders of the immune system can result in autoimmune and inflammatory diseases<sup>10</sup> and cancer<sup>11</sup>. All these pathological conditions ideally demand a prompt diagnosis for an efficient treatment. In the case of autoimmune disorders, the serological presence of autoantibodies in most of the cases is a predictor for diagnostic of autoimmunity<sup>10,12</sup>. These antibodies react with the cell surface and extracellular autoantigens and, they are often present for many years before the presentation of autoimmune disease<sup>10,12</sup>. Immune deficiencies and cancer are also diagnosed with blood tests that either measure the level of immune elements such as antibodies, or their functional activity<sup>11</sup>.

The identification of the repertoire of circulating antibodies and their serological levels are paramount to set a prognosis and evaluate the progression of all of the mentioned pathological conditions. We have previously demonstrated the potential of PGA technique for the analysis of circulating antibodies in different animal species<sup>13-16</sup>, minimizing the use of large volumes of serological samples, avoiding the problem associated with antibodies cross-reactivity<sup>17</sup> and allowing high-throughput profiling of an extensive repertoire of antibodies<sup>15</sup>.

Glycan-based immunoassays are mainly conditioned, among other factors, by the origin and production of carbohydrates, which determine the affinity and binding of ligands<sup>15,18-21</sup>. Glycan-based immunoassays can be developed in suspension (microspheres)<sup>15,21,22</sup> or in flat-activated surfaces<sup>15,21-24</sup>. The last include ELISA (the most conventional of these methods) and PGA. There is not much data comparing these methodologies in the same experimental setting<sup>15,25-27</sup>. We have previously compared the efficacy and selectivity of these immunoassays to profile anti-glycan antibodies in individual human plasma samples<sup>15</sup>. For some antibodies such as those targeting anti-A/B blood group, all the immunoassays could detect them with statistical significance and they positively correlated with each other<sup>15,18,21</sup>. Meanwhile, anti-P1

antibodies were primarily detected by PGA with the highest discriminative power, and there was no correlation in the determinations made by the different glycan-based immunoassays<sup>15,18,21</sup>. These differences between methods were mainly related to the antibody/antigen ratio and glycan orientation<sup>15</sup>. ELISA and suspension arrays are more susceptible to unspecific binding than PGA because there is an excess of antigen over antibodies in these methods<sup>15</sup>. Additionally, the orientation of glycans in the PGA is more restricted than in ELISA and suspension arrays<sup>15</sup>. ELISA is convenient when the study includes a limited panel of glycans. Along with suspension arrays, ELISA offers broader flexibility regarding assay reconfiguration. PGA is exceptionally convenient for discovery approaches<sup>15,18,21,28</sup>. Despite these clear advantages and disadvantages, the three mentioned immunoassays could be used to study different aspects of glycan-antibody interactions. The final goal of the study is the one will guide the selection of the more suitable methodology.

The present work aims to extend the use of PGA technology for the analysis of the repertoire of circulating anti-glycan antibodies in small animals. As a representative result, we present here a detailed protocol to assess the repertoire of natural anti-carbohydrate antibodies in adult BALB/c mice by PGA.

## Protocol

### 1. Glyochips Production

#### 1. Microarray preparation

1. Print the glycans (50 mM) and polysaccharides (10 µg/mL) in 300 mM phosphate buffered saline (PBS, pH 8.5) at 6 replicates onto N-hydroxysuccinimide-derivatized glass slides, using non-contact robotic arrayer (drop volume ~900 pL). Each slide contains 4 different blocks of sub-arrays (**Figure 1A**, in colors) repeated 6 times. Every single sub-array is formed by 112 different glycan spots, including controls (8 rows × 14 columns) (**Figure 1B**).

NOTE: Glycan-related information is provided in the **Supplementary Table 1**. The glycan library used for printing microchips is the result of a long-term synthetic effort of the IBCh team; examples of synthesis are described in related publications<sup>29-38</sup>. The glycan library included blood group antigens and some of the most frequently occurring terminal oligosaccharides, as well as core motifs of mammalian N- and O-linked glycoproteins and glycolipids, tumor-associated carbohydrate antigens, and polysaccharides from pathogenic bacteria.

2. Incubate the slides in moisture box (relative humidity ~70%) at room temperature (25 °C) for 1 h.
3. Blocking microarrays: incubate the slides for 1.5 h with blocking buffer at room temperature (100 mM boric acid, 25 mM ethanolamine, 0.2% (v/v) Tween-20 in ultrapure water).
4. Wash the glycochip with ultrapure water and dry it by air.

#### 2. Glycochip quality control

1. Analyze two microarrays from each batch using 1mg/mL solution of complex immunoglobulin preparation (CIP, containing IgG, IgM and IgA), 10 µg/mL solution of biotinylated goat anti-human immunoglobulins as a secondary antibody (IgM + IgG + IgA), followed by 1 µg/mL solution of the corresponding fluorescent streptavidin conjugate (via protocol described below, see step 2).
2. Scan and analyze the glycochips (see step 3, analysis of glycan array).
3. Use microarray batches with intra- and inter-chip correlation higher than 0.9.

### 2. Glycan Array Technique

#### 1. Prepare the following aqueous solutions (in ultrapure water) and store them at room temperature (25 °C):

- Buffer-1: 1% (w/v) bovine serum albumin (BSA) in PBS, 1% (v/v) Tween-20 and 0.01% (w/v) NaN<sub>3</sub>
- Buffer-2: 1% (w/v) BSA in PBS, 0.1% (v/v) Tween-20 and 0.01% (w/v) NaN<sub>3</sub>.
- Buffer-3: 0.1% (v/v) Tween-20 in PBS.
- Buffer-4: 0.001% (v/v) Tween-20 in PBS.

#### 2. Glycochip and sample preparation

1. Put the storage box with the slides on the table until they reach room temperature (25 °C).  
NOTE: Use powder-free latex gloves. The glycochip must be manipulated by the bottom part of the glass slide, where the barcode is located. The barcode will help you to identify the right side, avoiding the contact with the surface where the glycans are printed.
2. Open the box, take the glycochip and place it in the incubation chamber (25 °C), already conditioned with wet filter paper to keep humidity constant inside the chamber.
3. Meanwhile, dilute the mice serum with Buffer-1 (1:20) in 1.5 mL tubes. Homogenize the serum solution (5 s) with a vortex mixer.  
NOTE: The volume needed to totally cover a single glycochip surface is approximately 1 mL.
4. After the homogenization, incubate the diluted serum at 37 °C for 10 min in a water bath to avoid immunoglobulin aggregation. Centrifuge the tubes for 3 min at 10,000 × g and 25 °C, collect the supernatant and discard any precipitated material.
5. Place the glycochip carefully in the incubation chamber. Incubate it for 15 min at 25 °C with 1 mL of Buffer-3 to eliminate any residual material on the surface of the glycochip.
6. Hold the glycochip in a vertical position and rewash it with some drops of Buffer-3 using a plastic Pasteur pipette. Carefully remove the buffer from the glycochip surface using filter paper.

#### 3. Reaction: antibodies binding

1. Place the glycochip in the incubation chamber. Spread the diluted serum sample over the glycochip surface using a micropipette. Incubate with orbital agitation (30 rpm) at 37 °C for 1.5 h. Ensure that all dry area of the glycochip surface is covered by the diluted serum sample using the tip of the pipette.

2. Remove any excess sample and immerse the glycochip for 5 min in Buffer-3 at 25 °C. Then, move the glycochip to a container with Buffer-4 (5 min) and finally wash (5 min) the glycochip with ultrapure water. Centrifuge the glycochip for 1 min at 175 x g and 25 °C to remove the liquid.

#### 4. Detection: secondary antibody

1. Place the glycochip in the incubation chamber. Spread over the glycochip surface a solution (5 µg/mL) of goat anti-mouse (IgG + IgM) conjugated to biotin in Buffer-2. Incubate with orbital agitation (30 rpm) at 37 °C for 1 h.
  2. Remove the unbound fraction and repeat the washing steps.
  3. After the centrifugation, incubate the glycochip in darkness at 25 °C for 45 min (30 rpm) with 2 µg/mL of the corresponding fluorochrome-labeled streptavidin solution (in Buffer-2).
  4. In darkness, remove the unbound fraction and repeat the washing steps.
  5. Dry the glycochip by air.
- Note: Glycochip should be scanned as soon as possible. But if it's impossible to do scan immediately after staining, glycochips can be stored in a cool and dry place in darkness.

### 3. Analysis of Glycan Array

#### 1. Scan the array

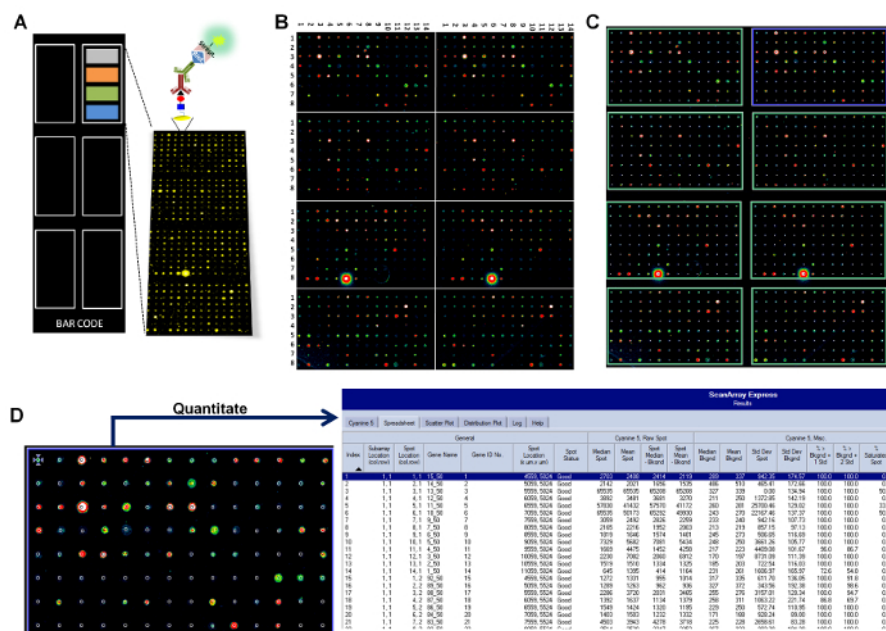
1. Leave the glycochip on the table until it reaches room temperature in dark. At the same time, turn on the slide scanner and the laser (excitation wavelength of 633 nm).
2. Holding the microarray, slide the glycochip into the slot until it touches the back.
3. Scan the glycochip ("run easy scan"), and save the scan as a ".TIFF" file.

#### 2. Array quantification

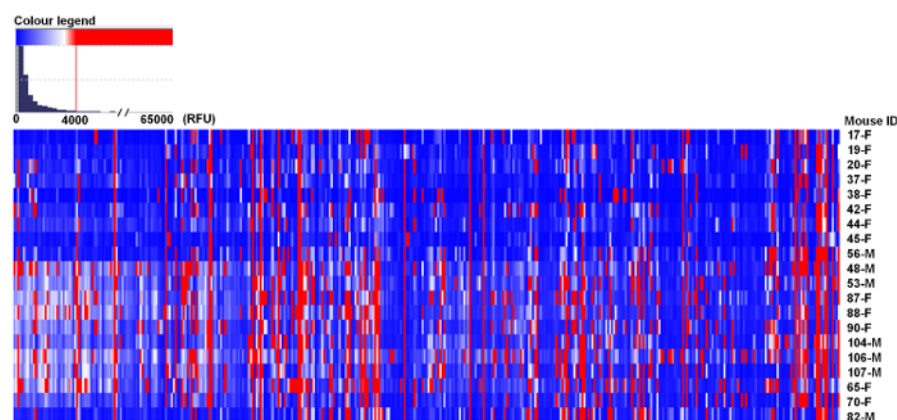
1. Quantify the array using a ScanArray Analysis System. Open previously scanned images, by clicking "File" in the "Configure & File group" on the Main Window (**Figure 1B-D**).
2. Load the corresponding array file template in GAL format (disposition of printed glycans on the glass slide) (**Figure 1C**).
3. Adjust GAL template by carefully aligning the array (grids) with the spots in the image and initiate quantification (**Figure 1D**).
4. Select the quantification parameters:
  - Quantification type: Run Easy Quant.
  - Quantification method: Fixed Circle
  - Auto find Spots: unclick all options
  - Normalization Method: LOWESS (Locally Weighted Scatter Plot Smoothing).
5. Save the quantified data as ".CSV" file (**Figure 1D**). Transfer this data into a common spreadsheet file using Microsoft Excel or another appropriate application.
6. Use the interquartile range (IQR) as the main statistical method: calculation of the median (Quartile 2) of all signals for each ligand and the interquartile deviation (75th and 25th percentiles, or upper and lower quartiles Q3 and Q1, respectively).
7. Perform interactive exploration of data by using a Hierarchical Clustering Explorer application.
8. Use clustering parameters: Average Linkage (UPGMA) and Euclidean distance as similarity distance measure. Perform hierarchical clustering by rows without normalization.

### Representative Results

Here, we present a summary of representative results obtained from the quantification of the repertoire of natural anti-glycan antibodies in a population of 20 BALB/c mice. The glycochips used in this study contained 419 different glycan structures. Most glycans were synthesized as -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub> spacer-armed O-glycosides, in several cases as -CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub> or -NHCOCH<sub>2</sub>NH<sub>2</sub> glycosides. All glycan structures were characterized by high resolution (700- or 800 MHz) NMR spectroscopy, purified and tested by HPLC, indicating their >95% purity. We have simultaneously determined IgM + IgG anti-glycan antibodies due to a restriction in the amount of mouse serum. In the PGA, we considered values above 4,000 RFU as a positive signal of antibody binding (this value is ~10% of the top glycans RFU). The results presented in this work follow most of the guidelines for reporting glycan microarray-based data<sup>39</sup>. Only 17% of carbohydrate structures demonstrated ≥4,000 RFU in the PGA (**Figure 2**, in red). Most of the glycan structures exposed in the glycochips were not recognized by the repertoire of circulating anti-glycan antibodies of BALB/c mice (**Figure 2**, in blue and white)<sup>28</sup>. The conserved pattern of natural anti-carbohydrate antibodies of BALB/c included 12 different glycan specificities, with very high median signal intensities of antibodies binding (≥10,000 RFU **Table 1**)<sup>28</sup>.



**Figure 1: Schematic representation (not at scale) of the glycan array configuration, printing, and analysis.** (A) Printed microchips are developed with a library of 419 different glycan structures, followed by the detection with an appropriate fluorescently labeled secondary antibody. Each slide contains 4 different blocks of sub-arrays (in colors), repeated 6 times. Every single sub-array is formed by 112 different glycan spots (8 rows  $\times$  14 columns), including controls. (B) A representative example of the images obtained from microchip scanning using a fluorescence scanner (third part of the image). (C) The process of aligning the "grid" to spots in every single sub-array (template adjustment during quantification). (D) The fluorescence is detected for each spot and results are transferred into a common spreadsheet file. [Please click here to view a larger version of this figure.](#)



**Figure 2: Repertoire of natural circulating anti-carbohydrate antibodies of BALB/c mice.** Mouse serum samples (1:20) were incubated with the glycochips and scanned using a ScanArray reader. Data were analyzed with a microarray analysis system and results were expressed in relative fluorescence units (RFU) as the median  $\pm$  median absolute deviation (MAD). Blue and white colors represent binding signals lower than 4,000 RFU (background); red color represents signals  $\geq 4,000$  RFU (positive binding). F: female; M: male (n = 20). This figure has been reproduced from Bello-Gil, D. *et al.*<sup>28</sup>. [Please click here to view a larger version of this figure.](#)

Glycan ID (#)	Structure	Common name	Median and MAD as RFU		Number of mice showing RFU $\geq 4000$ (%)
60	6-O-Su-Gal $\beta$ -sp <sup>b</sup>		61113	1156	100
271	Gal $\beta$ 1-6Gal $\beta$ 1-4Glc $\beta$ -sp		53622	1934	100
802	Gal $\beta$ 1-3GalNAc(fur <sup>c</sup> ) $\beta$ -sp		51348	2324	100
176	3-O-Su-Gal $\beta$ 1-4(6-O-Su)Glc $\beta$ -sp		43008	9342	100
166	GlcA $\beta$ 1-6Gal $\beta$ -sp		39105	2993	85
150	3-O-Su-Gal $\beta$ 1-3GalNAc $\alpha$ -sp		37943	3232	100
437	GalNAc $\alpha$ 1-3(Fuc $\alpha$ 1-2)Gal $\beta$ 1-3GalNAc $\beta$ -sp	A(type 4)	33886	3193	90
125	6-Bn-Gal $\beta$ 1-4GlcNAc $\beta$ -sp		32674	5389	95
154	3-O-Su-Gal $\beta$ 1-3GlcNAc $\beta$ -sp		32651	3954	100
177	3-O-Su-Gal $\beta$ 1-4(6-O-Su)GlcNAc $\beta$ -sp		32496	7215	100
287	3-O-Su-Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ -sp	SuLe <sup>a</sup>	20063	4962	95
234	Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ -sp	Le <sup>x</sup>	13573	2635	80

**Table 1: Top rank glycan structures recognized by natural antibodies of BALB/c mice.** Glycans with binding signals above 4,000 RFU in at least 80% of examined mice (n = 20). <sup>b</sup>sp means aminoethyl, aminopropyl or glycol spacer. <sup>c</sup>furanose; all other monosaccharides are in a pyranose form; Fuc residue has L-configuration, all other monosaccharides - D-configuration. This table has been modified from Bello-Gil, D. *et al.*<sup>28</sup>.

**Supplementary Table 1: List of glycans, their binding to natural circulating antibodies (IgM + IgG) of BALB/c mice (n = 20), expressed in relative fluorescence units (RFU) as median  $\pm$  MAD, and the number of animals exceeding cut off ( $\geq 4000$  RFU).** This table has been reproduced from Bello-Gil, D. *et al.*<sup>28</sup>. [Please click here to download this table](#)

## Discussion

Glycan microarrays have become indispensable tools for studying protein-glycan interactions<sup>40</sup>. The present work describes a protocol based on PGA technology to study the repertoire of circulating of anti-carbohydrate antibodies in BALB/c mice. Since PGA offers the possibility to screen large numbers of biologically unknown glycans, it is an exceptionally convenient discovery tool<sup>13,15,28</sup>. The proposed method offers the possibility to measure, in the same experimental setting, hundreds of glycan structures using a reduced amount of serological sample (50  $\mu$ L). This is especially critical in the case of small animals (little circulating blood volume), or when it's necessary to extract blood several times from the same experimental animal.

We demonstrated, as representative results, that genetically identical mice should not be considered as immunological equivalents; because they develop different patterns of natural anti-carbohydrate antibodies (only 12 glycan specificities were conserved). Serological levels for the rest of the repertoire of natural anti-carbohydrate antibodies varied considerably among the examined animals. Analysis of the gut microbiota of inbred animals<sup>41</sup> could explain this heterogeneity<sup>42-46</sup>. If the production of natural anti-glycan antibodies is mediated by the antigenic stimulation of microbiota, and this is different among inbred mice<sup>41</sup>, fine specificity of these antibodies will not be identical.

The main drawback for PGA development is the access to well-defined glycan structures<sup>40,47</sup>. Glycans produced in biological systems are heterogeneous<sup>40,47,48</sup>, and their biosynthesis relies on the differential expression of carbohydrate enzymes, resulting in heterogeneous mixtures of glycoforms, each with a distinct physiological activity<sup>47</sup>. The complex composition and configuration of the glycans present in the biological systems make their productions challenging<sup>40,47,48</sup>. Along with chemo-enzymatic synthesis, glycans isolated from natural sources will continue to be the major source of glycans for arrays development<sup>40</sup>. Low synthetic yields and the complex purification process from glycoproteins and glycosphingolipids make the efficient production of glycans at large scale difficult<sup>40,47,48</sup>. Hence, the availability and the prices of glycans continue being a very limiting condition to expand the use of PGA as a discovery tool.

Additionally, within the protocol, critical steps mostly relating to the correct distribution of solutions (serum, secondary antibodies) over the glycochip surface must be executed with caution. The methodology requires, at least, 1 mL of these solutions, to homogeneously soak all dry areas of the glycochip surface. This is crucial to obtain minimal differences between glycan replicates and also to avoid excessive background during quantification.

Despite the mentioned limitations, PGA is a very sensitive tool for approaches related to study protein-glycan interactions<sup>40</sup>, or to study the repertoire of anti-glycan antibodies in a particular experimental setting or condition<sup>13,15,28</sup>. This study can be extrapolated to different species (including human samples)<sup>13,15,23,28</sup>, providing a versatile methodology for identifying the repertoire of circulating anti-carbohydrate antibodies.

We also anticipate the potentiality that this approach may bring in the early diagnosis and derived treatment in some of the pathological conditions where antibodies directed to glycan structures seem to play an important role.



## Disclosures

Nailya Khasbiullina and Alexey Nokel are employees of Semiotik LLC, which is the supplier of the glycochips used in this study.

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