Supporting Information

for

Development of a Multiplex Glycan Microarray Assay and Comparative Analysis of Human Serum Anti-Glycan IgA, IgG, and IgM Repertoires

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Supplementary Materials and Methods

Anti-Human IgA: AlexaFluor488 Antibody Labeling Kit (ThermoFisher, A-20181) was used to conjugate the anti-human IgA reagents. To achieve lower dye-loading, the lyophilized AlexaFluor488 dye was reconstituted in 100 µL PBS (pH 7.4). 50 µL or 25 µL was then added to the unconjugated anti-IgA antibody (Jackson Immuno 109.005.011, 1.5 mg/mL) in PBS to achieve the 1.9 and 1.0 loadings, respectively. 0.10M sodium bicarbonate was added to promote the reaction of the dye’s ester and amine of the antibody. The rest of the conjugation and purification was performed following the manufacturer’s protocol. Absorbances at 280nm and 493nm were made with a NanoDrop2000 and the degree of labeling determined following the formula provided in the conjugation kit.

Anti-Human IgG: The AlexaFluor546 Antibody Labeling Kit (ThermoFisher, A-20183) was used to prepare the anti-human IgG secondary antibodies. For the 4.9-loaded anti-IgG, the manufacturer’s protocol was followed throughout the procedure. For the 1.52- and 1.6- loadings, the AlexaFluor546 dye was reconstituted in 100 µL of PBS (pH 7.4); 50 µL of this solution was then added to the unconjugated antibody (Jackson Immuno 109.005.098, 1.5 mg/mL) and 0.1M sodium bicarbonate solution. We used two unconjugated anti-IgG antibodies: whole (Jackson Immuno 109.005.098) and a F(ab’)2 (Jackson Immuno 109.006.170). The rest of the conjugation and purification process was performed following the manufacturer’s instructions. Absorbances at 280nm and 554nm were taken using the NanoDrop2000 and the degree of labeling calculated using the provided formula.

Anti-Human IgM: Initially, we used DyLight 649 labeled goat anti-human IgM. During the course of our studies, this secondary reagent was discontinued; we therefore had to select a new 635 fluorophore to detect IgM. While we first tested commercially-conjugated AlexaFluor647 anti-human IgM, we found this dye was unstable and prone to photobleaching; all 635 nm signal would be gone 24 hours after performing an assay experiment. Other dyes, such as Cy5, have chemical structures similar to AlexaFluor647; we wanted to find a dye that had a rhodamine core, as these dyes typically are more stable. We selected CF633 (Sigma Aldrich) because of its high signal and photostability. As this dye is not commercially conjugated to an anti-human IgM, we coupled the CF633 NHS-ester to a secondary
antibody with an average dye/antibody ratio of 2.20. The signal strength was comparable to the original DyLight649 secondary antibody after slight adjustment to the fluorescent scanner PMT settings.

CF633 succinimidyl ester dye (SCJ4600039) was purchased from Sigma Aldrich (St. Louis, MO). The dye was rehydrated in anhydrous DMSO (100 µL); unconjugated dye was stored at -20 °C. The anti-human IgM (Jackson Immuno, 109.005.129) was dissolved in PBS (pH 7.4). The reaction solution consisted of the anti-human IgM and rehydrated CF633 dye in an 8.88 dye-to-antibody molar ratio; the manufacturer recommended an antibody concentration of at least 2 mg/mL to optimize labeling. 0.1M sodium bicarbonate was also added to promote the amide linkage formation between the amine group of the antibody and ester of the dye. The reaction vial sat at room temperature for one hour and was inverted every 15 minutes. The procedure from the AlexaFluor Antibody Labeling Kits was used to purify the conjugated IgM antibodies from the free dye. Degree of labeling was calculated using absorbance readings at 280 and 630 nm via the NanoDrop2000 and the formula provided by Sigma-Aldrich. The secondary reagent used in our experiments had an average dye-loading of 2.20 CF633 per mole of antibody. The signal strength was comparable to the original DyLight 649 secondary after slight adjustment to the fluorescent scanner PMT settings.

All secondary antibodies were stored at 4 °C up to 6 months. We prepared a second batch of each dye-labeled secondary antibody and compared them with those used for the experiments in this paper. We evaluated two factors: signal correlation between the antibody batches and median signal for a subset of microarray glycans (termed the ‘normalization set’). For example, the second AlexaFluor488 conjugation gave us dye-loading of 0.90. When we compared the signal with the original 1.0 loading, the linear correlation was 0.99, showing that the secondary antibodies bound the same array components. We performed the same comparison between our original 1.5-loaded anti-IgG and a new 1.4 secondary antibody. The correlation between secondary antibodies was 0.96 with a linear slope of 0.96.

*Bleed-through:* Bleed-through with low loaded dyes was very low. The anti-IgG secondary reagent had no measurable bleed-through into the 635 nm channel. The anti-IgA secondary reagent
loaded with 1.0 dyes per antibody molecule had bleed-through into the 532 nm channel of only 2.5%. To account for this amount of bleed-through, we could apply a correction factor such that we would reduce each glycan’s 532 nm (IgG) signal by 2.5% of the IgA signal. From a practical point of view, this only becomes relevant if (A) the IgA signal for a particular array component is greater than 10,000 RFUs, and (B) the IgG signal is much lower than the IgA signal. This situation is rare, and to date we have not observed any instances where both criteria are satisfied. Therefore, for the vast majority of samples, no correction is necessary.

Testing other fluorophores: One approach for minimizing dye-dye interaction was to vary the spectral properties of the dyes to minimize spectral overlap. We performed reference serum profiling experiments with FITC and Atto465-labeled anti-IgA. Unfortunately, we did not observe any improvements in signal loss. In addition, these dyes had much lower stability and signal strength relative to AlexaFluor488. Therefore, we remained with the AlexaFluor488 dye. We also evaluated alternative fluorophores for detection using the 532 nm laser. With AlexaFluor594, we observed large amounts of spillover/bleed-through from the 635 nm lasers. Cy3 produced the strongest signals using the 532 nm laser but also gave the greatest IgA signal loss. AlexaFluor546 gave strong signals, showed good stability, and had slightly less IgA signal loss than the DyLight549 and Cy3. Therefore, AlexaFluor546 was selected for the multiplex assay. Overall, however, varying the dyes led to only minor improvements.
Table S1: Secondary antibody + fluorophore information

| Fluorophore     | Antibody Description                                                | Company, Catalogue # |
|-----------------|---------------------------------------------------------------------|----------------------|
| None            | AffiniPure Goat Anti-Human IgA, α chain specific                    | Jackson Immuno 109.005.011 |
| AlexaFluor647   | AffiniPure Goat Anti-Human IgA, α chain specific                    | Jackson Immuno 109.606.011 |
| Atto465         | AffiniPure Goat Anti-Human IgA, α chain specific                    | Dye: Atto-tech AD465-31  
|                 |                                                                     | Secondary: Jackson Immuno 109.005.011  |
| FITC            | AffiniPure Goat Anti-Human IgA, α chain specific                    | Jackson Immuno 109.095.011 |
| AlexaFluor488   | AffiniPure Goat Anti-Human Serum IgA, alpha Chain Specific          | Jackson Immuno 109-545-011 |
| None            | AffiniPure Goat Anti-Human IgG, Fcy fragment specific               | Jackson Immuno 109.005.098 |
| None            | AffiniPure Goat Anti-Human IgG, F(ab’)2 fragment specific          | Jackson Immuno 109.006.097 |
| DyLight549      | AffiniPure Goat Anti-Human IgG, Fcy fragment specific               | Jackson Immuno 109.505.008 |
| Cy3             | AffiniPure Goat Anti-Human IgG, Fcy fragment specific               | Jackson Immuno 109.165.098 |
| AlexaFluor594   | AffiniPure Goat Anti-Human IgG, Fcy fragment specific               | Jackson Immuno 109.585.098 |
| None            | AffiniPure Goat Anti-Human IgM, Fc5µ fragment specific             | Jackson Immuno 109.005.129 |
| AlexaFluor488   | AffiniPure Goat Anti-Human IgM, Fc5µ fragment specific             | Jackson Immuno 109.545.129 |
| AlexaFluor647   | AffiniPure Goat Anti-Human IgM, Fc5µ fragment specific             | Jackson Immuno 109.605.129 |
Table S2. Summary of Subjects and Samples Used in the Longitudinal Study

| Subject | Sex   | Race                  | Age | First draw | Second draw | Third draw |
|---------|-------|-----------------------|-----|------------|-------------|------------|
| 1       | male  | Caucasian             | 55  | Week 0     | Week 7      | Week 10    |
| 2       | male  | African American      | 47  | Week 0     | Week 9      |            |
| 3       | male  | Caucasian             | 46  | Week 0     | Week 8      |            |
| 4       | male  | African American      | 32  | Week 0     | Week 10     |            |
| 5       | male  | Caucasian             | 77  | Week 0     | Week 8      |            |
| 6       | male  | African American      | 49  | Week 0     | Week 9      |            |
| 7       | male  | African American      | 50  | Week 0     | Week 5      | Week 13    |
A) Pre-experiment Image (532 Channel)

B) Post-experiment images:

Figure S1: Images of pre- and post-experimental array wells for the multiplex assay. A) Signals in the pre-experimental images are from a 532-emitting fluorescent dye in the print buffer, which is later washed away during slide blocking. Blue boxes represent components that were missing during array printing. B) Array slides were incubated with human serum (1:50) and then all three secondary antibodies (1:500).
A) Scatter plot of IgA signals in singleplex format vs multiplex before optimization.

\[ y = 0.5895x + 209.98 \]
\[ R^2 = 0.9377 \]

B) IgA signal loss for multiplex relative to singleplex before optimization

Figure S2: Loss of IgA signal observed before optimization of the multiplex assay. A) Scatter plot of IgA signals in singleplex vs multiplex before optimization. The dashed line represents zero signal loss. The singleplex and multiplex data are highly correlated \((R^2 = 0.938)\), but the multiplex signal are significantly lower than expected. x and y-axis values are RFU. B) IgA signal loss for multiplex relative to singleplex before optimization. For the initial test, we used a smaller array with 307 components.
Figure S3: Comparison of singleplex and multiplex signals for IgA, IgG, and IgM after optimization
Figure S4: Loss of IgA signal observed for a high IgG, difficult serum sample (F58067-08). The percentage of IgA signal loss (y-axis) is shown for each array component (x-axis) in the bar graph: A) IgA signal loss for multiplex relative to singleplex before optimization. Many array components have greater than 50% IgA signal loss. B) IgA signal loss for multiplex relative to singleplex using low dye loaded secondary reagents.
Figure S5: Heat map showing changes for subjects 2-6. IgA, IgG, and IgM signals were measured on the glycan microarray at different time points for 7 subjects. Changes from one time point to another were determined. Representative data for Subjects 1 and 7 are shown in Figure 4. Data for subjects 2-6 are presented in this figure. White boxes indicate no change; pink boxes indicate an increase; blue boxes indicate decreases. The scale bar and coloring use a linear scale. Rows are labeled with the subject number.