

Chemo-Enzymatic Production of *O*-Glycopeptides for the Detection of Serum Glycopeptide Antibodies

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Abstract

Protein microarray is a highly sensitive tool for antibody detection in serum. Monitoring of patients' antibody titers to specific antigens is increasingly employed in the diagnosis of several conditions, ranging from infectious diseases, allergies, autoimmune diseases, and cancer. In this protocol we present a detailed method for enzymatic generation of disease-specific *O*-glycopeptides and how to monitor the antibody response to these in serum using microarray technology.

Key words Glycopeptide microarray, GalNAc, Sialylation, Serum biomarkers, Autoantibodies, Posttranslational modification, Glycosylation

1 Introduction

Protein microarray enables simultaneous measurement of several biomarkers, such as antibodies against foreign antigens or autoantibodies [1, 2]. Antibody profiling has been shown to be useful in differential diagnosis of infectious diseases [3, 4] and allergies [5, 6]. Furthermore, elevated levels of specific autoantibodies are able to identify autoimmune disorders prior to onset of clinical symptoms [7, 8]. In recent years, the autoantibody response to tumor-associated antigens has received much interest for its potential capabilities as a biomarker for early detection of cancer [9]. Compared to the measurement of peptide and protein levels in bodily fluids, antibodies are produced in high, relatively stable titers, and have longer half-lives [10]. Besides their use as biomarkers, the identification of antibody targets could reveal specific vaccine candidates, for example viral and cancer vaccines [11–14].

The discovery of disease-specific antibodies has been approached through several different proteome-wide screening techniques, including expressed cDNA libraries (SEREX) [15], protein and peptide arrays [16, 17], both random and designed phage displays [18], and self-assembling protein arrays [19, 20].

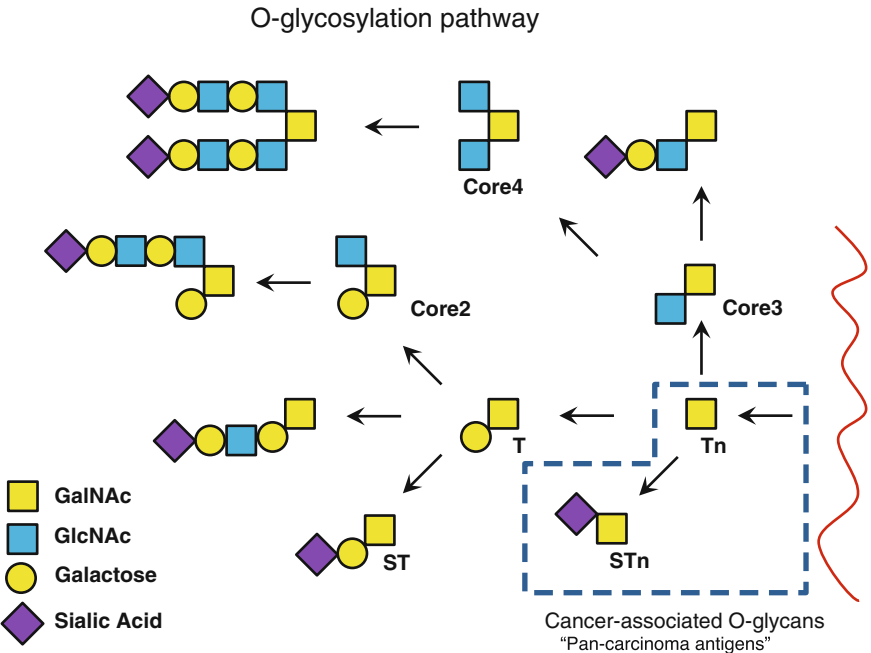


Fig. 1 The *O*-linked GalNAc glycosylation pathway. Glycosylation is initiated by the addition of GalNAc to Serine or Threonine residues in the protein core, and are elongated through subsequent enzyme reactions. Symbols for monosaccharides GalNAc, Gal, GlcNAc and Neu5Ac (sialic acid) are indicated

These methods are, however, designed to examine the proteome in the absence of posttranslational modification, which might limit the potential output of antibody screening [13].

Aberrant patterns of *O*-linked glycosylation (*see* Fig. 1) represent one of the most important cancer associated posttranslational changes [11, 21]. Changes in *O*-linked glycosylation are also associated with other pathological conditions, such as autoimmune diseases [22–25] and viral infection [26, 27]. Up regulation of unique short aberrant *O*-glyco-signatures on proteins may introduce novel glycopeptide epitopes that can elicit autoantibodies because of lack of tolerance.

By employing purified glycosyltransferases, we have previously described a method for *in vitro* *O*-glycosylation of synthetic MUC1 peptides [12, 13]. MUC1 is a heavily *O*-glycosylated mucin, with the glycans located predominately in a large 20 amino acid tandem repeat region [28]. While immunological tolerance is experienced to the tandem repeat protein core and its normal glycosylated forms [28, 29], aberrant truncation of the sugar chains results in the induction of autoantibodies to immunodominant *O*-glycopeptide epitopes. Importantly, the elicited immune response is specifically directed to combined glycopeptide epitopes, with little or no antibody specificity for the Tn carbohydrate hapten.

Through strict control of reactive enzymes we are able to recreate known disease-associated glycosylation patterns on our chosen antigens. In combination with microarray hydrogel slides, which provides remarkably low background levels [13], this offers a high-throughput method for screening patient sera for antibody reactivity.

In this protocol we are presenting a chemo-enzymatic approach using a synthetic 60-mer tandem repeat MUC1 peptide as an example to produce cancer-associated *O*-glycopeptides. The concept and methods described here could easily be transferred to other kinds of posttranslational modifications. The purpose of this chapter is to provide a detailed protocol for microarray detection of antibodies to glycosylated peptides and to offer aid with troubleshooting based on our experience and studies.

2 Materials

2.1 Generation of *O*-Glycopeptides

2.1.1 Enzymatic GalNAc-Glycosylation

1. 5× glycosylation buffer: 125 mM Cacodylate, 50 mM MnCl₂ (pH 7.4).

Dissolve 802.7 mg C₂H₆AsO₂Na·3H₂O and 296.9 mg MnCl₂·4H₂O in ~20 mL MilliQ (MQ)-H₂O. Titer the solution to pH 7.4 using 6 M HCl, and once this is achieved add MQ-H₂O until total volume reach 30 mL. Store at 4 °C, up to 2 months.

2. 100 mM UDP-GalNAc.
3. Relevant GalNAc-Transferase(s): in this protocol we use GalNAc-T1, GalNAc-T2 [30], and GalNAc-T4 [31] to glycosylate our MUC1-peptide. Several trials using different enzymes, with subsequent Mass Spectrometry (MS) analysis, might be necessary to find a GalNAc-T with a suitable low *K_m* for the specific glycosylation site(s) (see Subheading 3.1 and **Note 1**). Subsequent elongation of GalNAc (Tn)-glycans, as shown in Fig. 1, into NeuAcα2,6GalNAc-S/T (STn) and GlcNAcβ3GalNAc-S/T (Core3) structures requires ST6GalNAc-I [32] and β3GnT6 [33], respectively.
4. Heating incubator (set to 37 °C).

2.1.2 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

1. Buffer A: 0.1 % trifluoroacetic acid (TFA), CF₃CO₂H. Mix 1 L MQ-H₂O with 1 mL TFA.
2. Matrix: 10 mg/mL 2,5-dihydroxybenzoic acid (DHB) dissolved in MQ-H₂O.
3. Matrix-assisted laser desorption/ionization (MALDI) sample plate.
4. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) instrument.

2.1.3 Peptide Purification by High-Pressure Liquid Chromatography

1. HPLC grade H₂O.
2. Buffer A: 0.1 % TFA (Subheading 2.1.2, item 1).
3. Buffer B: 90 % acetonitrile (ACN), 0.08 % TFA.
4. HPLC 1100 Hewlett Packard system (Avondale, PA).
5. Zorbax 300SB-C18 column, 100 mm×4.6 mm (Agilent Technologies, Waldbronn, Germany).
6. Labconco Lyph-lock 1 L Lyophilizer (Labconco Corporation, Kansas City, MO).

2.2 Printing

1. Print buffer: 150 mM sodium phosphate (pH 8.5) with 0.005 % CHAPS and 0.03 % NaN₃
Weigh 1.17 g NaH₂PO₄·H₂O, 11.8 g Na₂HPO₄·2H₂O, and 25 mg CHAPS, and dissolve in MQ-H₂O. Add 5 mL 3 % NaN₃ and titer the solution to pH 8.5 using 6 M NaOH, and add MQ-H₂O until total volume reaches 500 mL.
2. Nexterion® Slide H MPX-48 (Schott AG, Mainz, Germany).
3. Light Microscope.
4. BioRobotics MicroGrid II spotter (Genomics Solution).
5. Stealth 3B Micro Spotting Pins (Telechem International ArrayIt Division).
6. Humidity chamber (*see* Note 2).

2.3 Scanning

1. Blocking buffer: 50 mM ethanolamine in 50 mM sodium borate (pH 8.5).
Weigh 3.05 g ethanolamine and 10.06 g Na₂B₄O₇ (alternatively 19.6 g Na₂B₄O₇·H₂O) and dissolve in MQ-H₂O. Titer the solution to pH 8.5 using 6 M HCl, and add MQ-H₂O until total volume reaches 1 L. Store in amber glass bottle, at room temperature.
2. Phosphate-buffered saline (PBS) pH 7.4.
3. PBS 0.05 % Tween (PBS-T) pH 7.4.
4. Cy3-conjugated goat anti-human IgG (Fc-specific) antibodies.
5. Microarray Slide centrifuge.
6. ProScanArray HT Microarray Scanner (Perkin-Elmer).
7. ProScanArray Express 4.0 software (Perkin-Elmer), for image analysis.

3 Methods

3.1 Enzymatic In Vitro O-Glycosylation

In this protocol, we have glycosylated a 60mer MUC1 peptide (VTSAPDTRPAPGSTAPPAHG)_{n=3} representing three tandem repeats, using purified GalNAc transferases. It is advised that you test

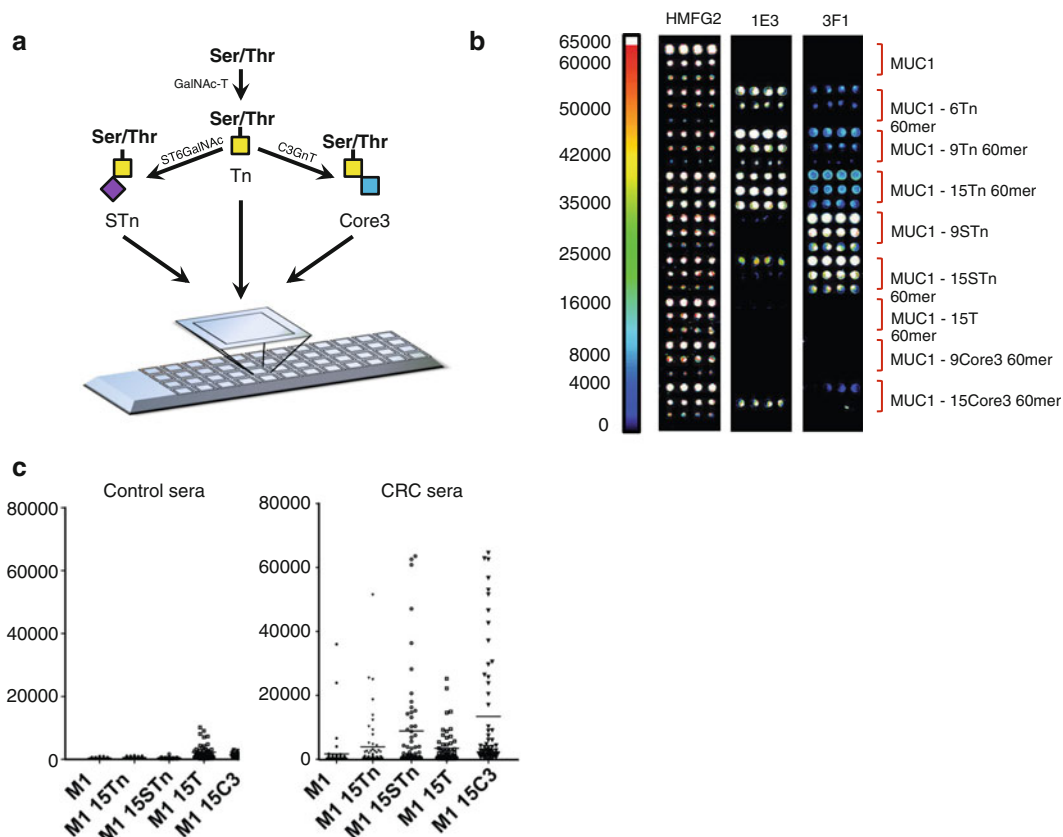


Fig. 2 MUC1 glycopeptides displayed on Micro Array. (a) GalNAc is added to Threonine and Serine residues on the peptide chain, directed by the specific activity of GalNAc-Ts, creating the Tn-antigen. The Tn structure can be elongated by subsequent enzyme reactions, e.g., into STn or Core3 structures. The Sialyl-Tn antigen is synthesized by the addition of a sialyl residue to GalNAc, whereas addition of a GlcNAc residue to GalNAc results in the formation of the Core3 structure. After purification the glycopeptide are displayed onto a micro array. (b) Each peptide is printed in quadruplicates, in three dilutions, and probed with the following antibodies (left to right): MUC1 specific mAb HMFG2; Tn specific mAb 1E3; STn specific mAb 3F1. (c) Dot-plot diagram presenting IgG autoantibody reactivity against 60mer MUC1, MUC1 Tn, MUC1 STn, and MUC1 Core3. Each dot represents one individual of colorectal cancer patients (serum at time of diagnosis, pretreatment) or healthy controls. Figure elements previously published in Pedersen et al. [12]

your reaction on a small quantity of substrate with several different kinds of GalNAc-Transferases, to determine the most suitable transferase(s) (*see Note 1*).

More complex glycan structures (e.g., Core3 or STn) can be generated through elongating preexisting GalNAc residues (Tn antigens), as illustrated in Fig. 2a. The procedures generally follow the same steps as described here, after substituting enzymes and donor sugars. In some cases adjustments to the reaction buffers are necessary (*see Notes 3 and 4*).

The quantities used in the protocol are optimized for the glycosylation of 10 µg of the aforementioned peptide (60mer MUC1).

Adjustments to the concentrations of UDP-GalNAc, substrate, and enzyme might be necessary for other targets. Likewise the suggested timeframe might need adjustments based on the enzyme's potency and affinity for other targets.

1. Mix 5 μL 5 $\times$ glycosylation buffer (Subheading 2.1.1) and 1 μL 100 mM UDP-GalNAc in an eppendorf tube.
2. Add 1 μL substrate (10 $\mu\text{g}/\mu\text{L}$).
3. Add 0.1 mU GalNAc-T1, 0.1 mU GalNAc-T2, and 0.1 mU GalNAc-T4 (*see* **Note 5**).
4. Add MQ-H₂O to a total volume of 25 μL .
5. Vortex-mix briefly and place the Eppendorf tube in the heating incubator at 37 °C, for 12–16 h.
6. Use MALDI-TOF to monitor glycosylation of peptide (Subheading 3.2).
7. STn-MUC1 and Core3-MUC1 is enzymatically synthesized based on purified GalNAc-MUC1 and is characterized and purified as described for GalNAc-MUC1 below (*see* **Notes 3 and 4**).

Note that no detergent (e.g., Triton) is added to the reaction (*see* **Note 6**).

3.2 MALDI-TOF Mass Spectrometer

1. Place a 0.5 μL droplet of DHB on the MALDI sample plate.
2. Dilute 0.5 μL of your sample in 10 μL buffer A (Subheading 2.2, **item 1**), and transfer 0.5 μL of this solution to the DHB on the sample plate.
3. Repeat **steps 1 and 2** of Subheading 3.2 for unglycosylated version of your peptide, for reference.
4. Let the matrix-sample-mix dry on the plate; then place the plate in the MALDI-TOF instrument.

Ionize the sample. Use high laser power until a peak correlating to the expected molecular weight appears, then lower laser power to minimize background noise.

After verification of the correct number of GalNAc additions by MALDI-TOF it is advisable, if possible, to confirm the position of the GalNAc in the intended site(s) [34, 35].

3.3 Peptide Purification by HPLC

Following verification of glycosylation, purify the peptide by high-performance liquid chromatography on a C18 column (Subheading 2.1.3, **item 4**), eluting with a 40 min gradient from 0 to 90 % ACN in 0.08 % TFA (buffer B, Subheading 2.1.3, **item 2**), at 0.800 mL/min flow. Lyophilize the sample and redissolve in MQ-H₂O the following day. Verify successful elution of your peptide through MALDI (Subheading 3.2).

3.4 Printing

We employed a BioRobotics MicroGrid II spotter (Genomics Solution) for printing our microarrays. When designing your own microarray, be sure that you make yourself familiar with the hardware and software by reading through the relevant chapters in the user manual. It offers detailed directions by the manufacturer on how to adjust settings to fit your needs.

We recommend printing each compound in duplicates or triplicates if space allows it on the array, and use the mean value of these in the analysis.

3.4.1 Preparing the Sample Source Plate (Biobank)

1. Dilute your peptides in Print buffer (Subheading 2.1.1). For the first run with new peptides, we recommend running a test-print with numerous dilutions, to determine the ideal concentration for each compound. Other methods of optimization might be necessary; for example, highly hydrophobic peptides might benefit from addition of dimethyl sulfoxide (DMSO) in the Print buffer, to be fully dissolved (*see* **Note 7**).
2. Seal the biobank with Parafilm to prevent evaporation and store at -20°C between runs (*see* **Notes 8** and **9**).
3. Before printing it is important to remove any air bubbles from the wells of the biobank, as they may interfere with the pins' correct acquisition of compounds when dipping into the source plate. Air bubbles are easily removed by centrifugation (e.g., $200\times g$ for 1–2 min).

3.4.2 Printing Glycopeptides

1. We store microarray slides at -20°C prior to use. Let them equilibrate at room temperature for approximately 30 min before printing; keep them in their sealed envelope for as long as possible. This will prevent condensation on the slides.
2. Fill the 6 L water reservoir. It might be necessary to refill during the print-run, depending on the number of slides, spots per slide, wash-settings and total run time.
3. Examine spotting pins in light microscope. Make sure the pins you choose for your print are clean and unbent. If needed, follow the manufacturer's recommendations for cleaning the pins. Place pins in tool, and load it onto the printer.
4. Prime the main wash station. Make sure the water flow is continuous and without air bubbles. To remove air, drain water from the 6 L water reservoir until no air bubbles are visible in the waste-tube. Prime the main wash station again, and perform the program "Regular wash cycle (without move to bath)" three consecutive times. Repeat if air bubbles persist.
5. Perform one "Regular wash cycle (with move to bath)"; make sure that the pins are lowered into the wash station accurately.

6. Load the source plate (biobank).
7. Place slides on the tray. Fill out empty slots with spare glass slides. After switching on the vacuum, make sure all slides are aligned correctly.
8. Start the run. The steps for selecting and adjusting run preferences will not be covered here. We advise that PreSpotting and Soft Touch (see user manual) are used, for the sake of consistent spot morphology. Make sure the pins are lowered correctly into the source plate wells during their route through first source visit to spotting; and that both PreSpotting and Soft Touch are working as intended.
9. After printing, place the slides in a humidity chamber for 2 h, before you either freeze the slides (-20°C) for later use or proceed directly with serum placement and scanning.

3.5 Serum Placement and Scanning

1. Block slides in blocking buffer (Subheading 2.3, item 1) for 1 h. While the slides are being blocked, thaw serum samples on ice. Vortex mix briefly before use when thawed.
2. Wash slides in PBS for 2×5 min.
3. Rinse slides in MQ- H_2O and dry through centrifugation ($200 \times g$). This ensures that the teflon-coating separating each well will be capable of upholding its function.
4. Place slides in a petri dish.
5. Dilute the sera 1:5 in PBS-T, and add $9\ \mu\text{L}$ to each well of the slide. This can also be done on slide, by placing a $7.5\ \mu\text{L}$ droplet of PBS-T in each well and then adding $1.5\ \mu\text{L}$ undiluted sera (see Note 10).

6. The addition of Tween to the diluted sera prevents nonspecific binding of antibodies.

When doing large batches, we recommend devoting one to four wells per slide to the same sera or antibodies to estimate slide-to-slide variation. This also ensures that in the case of scanner malfunction or service, scanning parameters can be calibrated using the control wells.

7. Let serum incubate on slide for 1 h, under gentle agitation (~ 50 motions per minute).
8. Wash off the sera: wash with PBS-T for 2×5 min; then a third 5-min wash cycle where PBS-T is swapped for PBS after 2.5 min.
9. While the slide(s) are washed, prepare a clean petri dish for the next steps, inside a light-blocking medium (e.g., a box or a sheet of aluminum foil).
10. Dry the edges of the slide off with a tissue, creating a dry teflon-frame around the wells.

11. Dilute Cy3-conjugated Goat anti-Human IgG (Fc-specific) antibodies 1:4,000 in MQ-H₂O.

Place 1 mL per slide inside the dried teflon frame and incubate for 1 h.

The slides should henceforth be shielded from light, by closing the box/aluminum foil around the petri dish.

12. Wash off the goat anti human antibodies: wash with PBS-T for 2× 5 min; then a third 5-min wash cycle with PBS. The slides should be shielded from light during these washes.
13. Rinse slides in MQ-H₂O, dry by centrifugation (200×*g*) and place slide in scanner-cassette.

Scan the slides using ProScanArray HT Microarray Scanner (Perkin-Elmer). We recommend scanning with 100 % laser power at several different PMT gain (parameter for the activity of the Photomultiplier tubes of the scanner; typically ranging from 70 to 90 %) to find a suitable standard for your samples, with reasonably low background levels and where the reactive spots' intensity is easily readable. Resolution of the images should not surpass 10 μm (*see Note 11*).

14. After scanning, seal slides in a container by Parafilm, and freeze at −20 °C. If needed, slides can be thawed and rescanned (e.g., at other scanning parameters).

3.6 Quantification and Data Analysis

After scanning, set up a quantification protocol as per the Scan Array Express user manual. To simplify the data analysis, we highly recommend the creation and use of a .GAL-file for the quantification template, so that the Name- and Id-tag for each printed compound is imported automatically to their corresponding spots. By following the printing specifications used in this protocol, each spot should average 150 μm in diameter. When the quantification template is correctly aligned on top of the scanned image of the MicroArray, export the result spreadsheet to excel, and convert the text to columns.

Each spot is now easily identifiable with correct name; and the Array Column and Row information enables sorting of the data after serum sample. The final data output is gained from the mean spot Relative Fluorescence Units (RFU) after subtracting the surrounding background (found in column Y in the excel spreadsheet; labeled as “Ch1 Mean – B”) (*see Note 12*).

4 Notes

1. There are over 200 glycosyltransferases in human cells, divided into 90 distinct families sharing mutual donor-sugar substrates. Of the GalNAc-Transferase (GalNAc-Ts) family, there are 20 known members, with varying affinity and specific activity for

each potential glycosylation site. Although some effort has been put into mapping glycosylation sites in different proteins for specific GalNAc-Ts, some trial and error employing different enzymes is usually needed to get the desired sites glycosylated. Also, if a peptide holds more than one potential site, it is not uncommon that several different transferases are needed to glycosylate them all.

2. Commercial humidity chambers are available; however, we made our own using a sealable plastic container with a perforated tray for slides. The lower compartment was filled with NaCl-saturated water, stabilizing the relative humidity at ~75 %, room temperature.
3. The purified Tn-bearing peptide can be enzymatically sialylated with ST6GalNAc-I (Sialyl Transferase), in the following reaction mixture: 50 mM MES (pH 6.5), 20 mM EDTA, and 2 mM DTT. The donor sugar used for this reaction is CMP-NeuAc, at 2 mM.
4. Enzymatic elongation of the purified Tn-bearing peptide can be performed by β 3Gn-T6 (Core3 Synthase), in the following reaction mixture: 50 mM Cacodylate (pH 7.4) and 10 mM MnCl_2 . The donor sugar used for this reaction is UDP-GlcNAc, at 2 mM.
5. The amounts of enzyme (0.1 mU GalNAc-T1, 0.1 mU GalNAc-T2 and 0.1 mU GalNAc-T4) was determined by scintillation counting after Dowex-1 formic acid chromatography of 25 μL reaction assays. The procedure is as described in Subheading 3.1, with UDP-GalNAc substituted for UDP-[^{14}C]GalNAc (2,000 cpm/nmol) (Amersham Biosciences).
For GalNAc-T1 and -T2, IgA-Hinge peptide (VPSTPPTPS PSTPPPTSPSK) was used for the activity assay; for GalNAc-T4, a MUC7 peptide (APPTPSATTPAPPSSSAPPETTAA) was used.
6. Avoid using detergent during enzymatic O-glycosylation with soluble glycosyltransferases when possible. It is difficult to completely remove detergent during purification, and this could later cause problems when printing. Compounds mixed with detergent will often create non-homogenous spot morphologies and may in worst case cause spots to merge, rendering slide uninformative and unusable.
7. Very high concentrations of DMSO is not recommended for printing, as it may interfere with the hydrogel surface of the microarray slides; we have however, successfully printed with up to 60 % DMSO in our Print buffer.
8. During long runs, water evaporation of the biobank will lower the volume of each sample, and increase the concentration in each well. In these cases, it is possible to refill the wells with MQ- H_2O , before a new print.

9. Precipitation issues may arise after thawing a frozen biobank. A way to solve this problem is to incubate the thawed biobank at 37 °C for 20 min before printing. A more laborious method is to manually pipette up and down repeatedly in the well of each compound, thus homogenizing the content of each well.
10. Dilution of sera is application and source dependent, and should be optimized for each experimental design. In the current protocol we use a serum dilution of 1:5.
11. As with the MicroArray printer hardware and -software, it is important that you have made yourself familiar with the scanner and its software. The manufacturer's user manual offers a great overview on how to use the scanner, and after reading the relevant chapters in the manual, it is fairly easy to adjust the default settings to those specified in this protocol.
12. Presumably due to varying content composition between different serum samples, it is not unusual to observe varying levels of background signal between subarrays of a MicroArray slide, directly corresponding to the area covered by serum. To remove this variance from the results, the background level is automatically measured by the software, and subtracted from the mean spot RFU.

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