Parallel Glyco-SPOT Synthesis of Glycopeptide Libraries

Akul Y. Mehta, Ravi Kumar H. Veeraiah, Sucharita Dutta, Christoffer K. Goth, Melinda S. Hanes, Chao Gao, Kathrin Stavenhagen, Robert Kardish, Yasuyuki Matsumoto, Jamie Heimburg-Molinaro, Michael Boyce, Nicola L. B. Pohl, Richard D. Cummings

Supplementary Information

Contents

Supplementary Figures

Figure S1

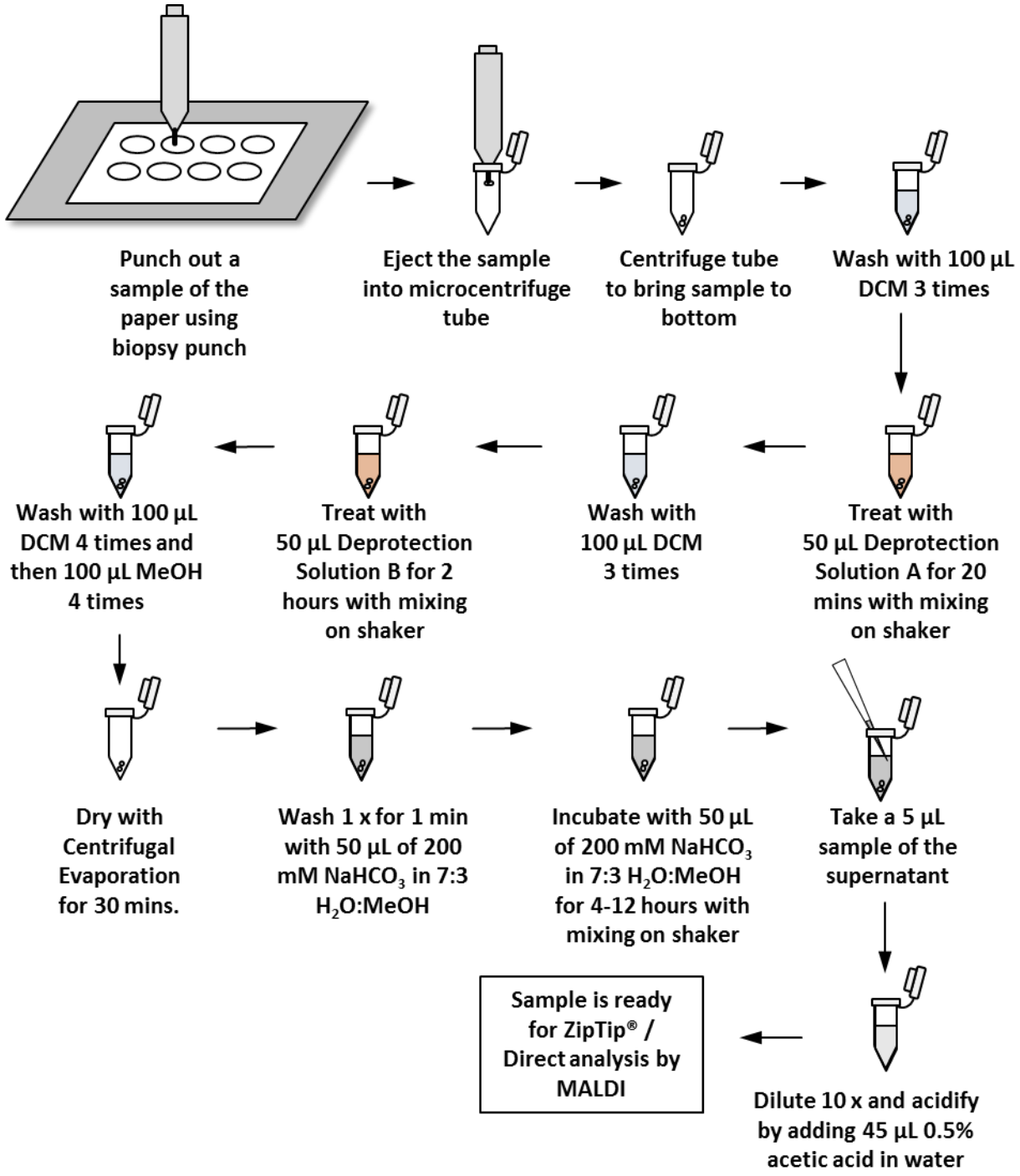


Figure S1. Related to Figure 2. Schematic diagram of the biopsy procedure to test the Glyco-SPOT synthesis. A sample of the paper can be taken any time of the synthesis and be processed to globally deprotect the side chains and then release the peptide for analysis with MALDI or other method.

Figure S2

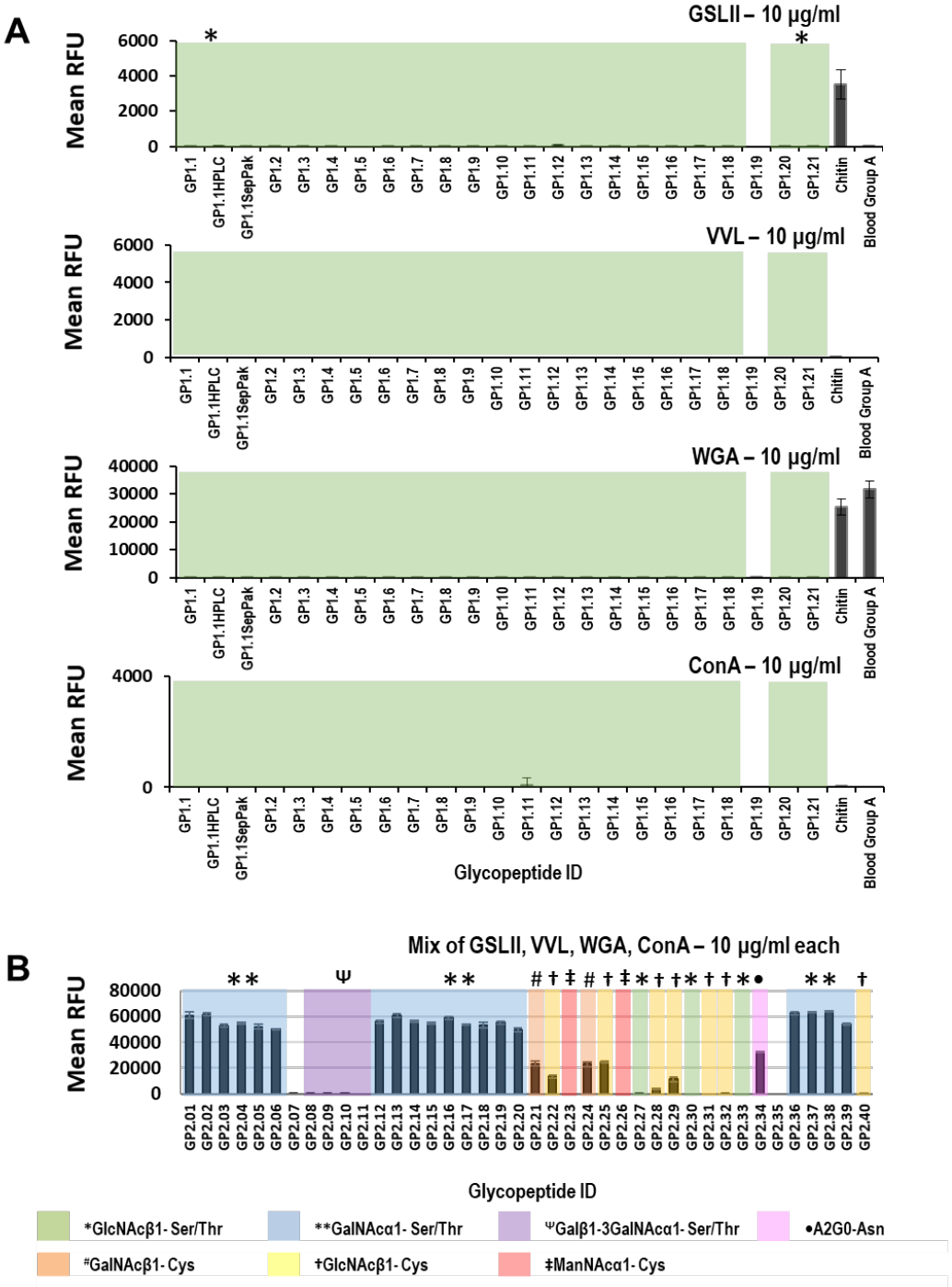


Figure S2. Related to Figure 4. Lectin binding tests performed on (A) GP1 array and (B) GP2 array. For the GP1 array individual lectins were incubated on the array at 10 µg/ml while for the GP2 array a mixture of the same lectins was utilized at 10 µg/ml concentrations. The lectins showed little to no binding to the GlcNAcβ1-Ser/Thr/Cys, however they show significant binding to other glycoamino acid containing peptides.

Figure S3

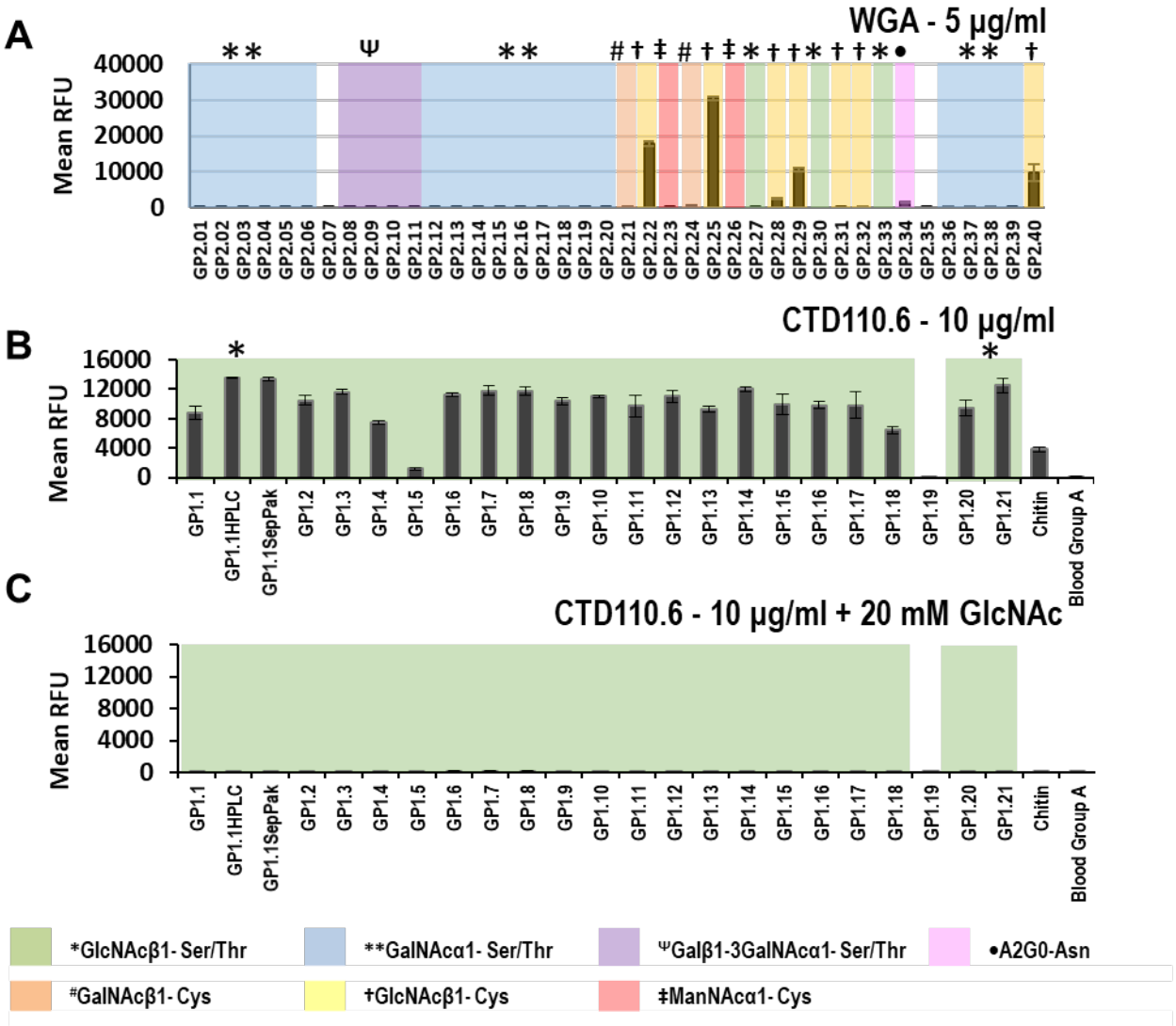


Figure S3. Related to Figure 4. (A) WGA binding at 5 µg/ml concentration on GP2 array. The data demonstrates how binding of WGA to the GlcNAc containing glycans on the microarray is very sequence dependant. For example, GP2.25 which is the 14-3-3 protein peptide with GlcNAcβ1-Cys binds strongly, while the same sequence having the GlcNAcβ1-Ser in the same position in GP2.27 shows no binding. Another observation is that GP2.28 (GlcNAcβ1-Cys) with an Asp12 shows no binding and its corresponding GlcNAcβ1-Ser congener in GP2.30 shows no binding; however, when Asp12 is mutated to Ala as in the case of GP2.29 there is slight binding by WGA. (B, C) Hapten competition on the GP1 array for CTD110.6 antibody. (B) Binding of CTD110.6 antibody without any hapten. (C) Binding of the antibody in the presence of 20 mM GlcNAc hapten. The hapten is able to completely prevent the binding of the antibody to the array, suggesting that the antibody binding to the array is primarily GlcNAc driven.

Figure S4

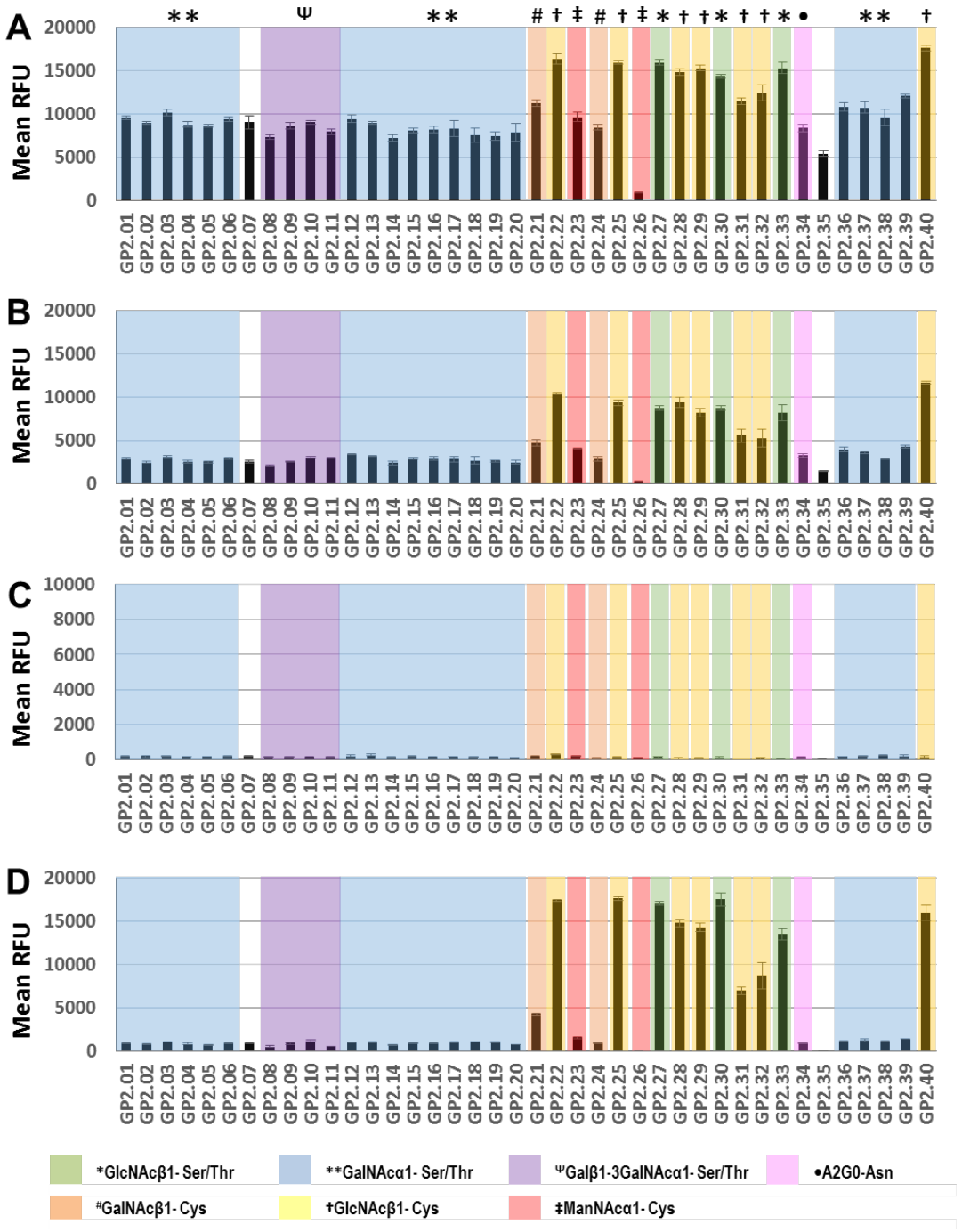


Figure S4. Related to Figure 4. Non-specific binding of CTD110.6 antibody on GP2 array when glycopeptides are printed at 100 µM concentration. (A, B) Binding of antibody when incubated at 10 µg/ml or 1 µg/ml respectively. (C) Hapten competition with 20 mM GlcNAc as hapten thereby preventing binding on the array completely. (D) Reduction of non-specific binding of the antibody by utilizing GalNAc as the hapten suggesting potential use of hapten to bring out the specificity of the antibody under high concentration conditions.

Figure S5

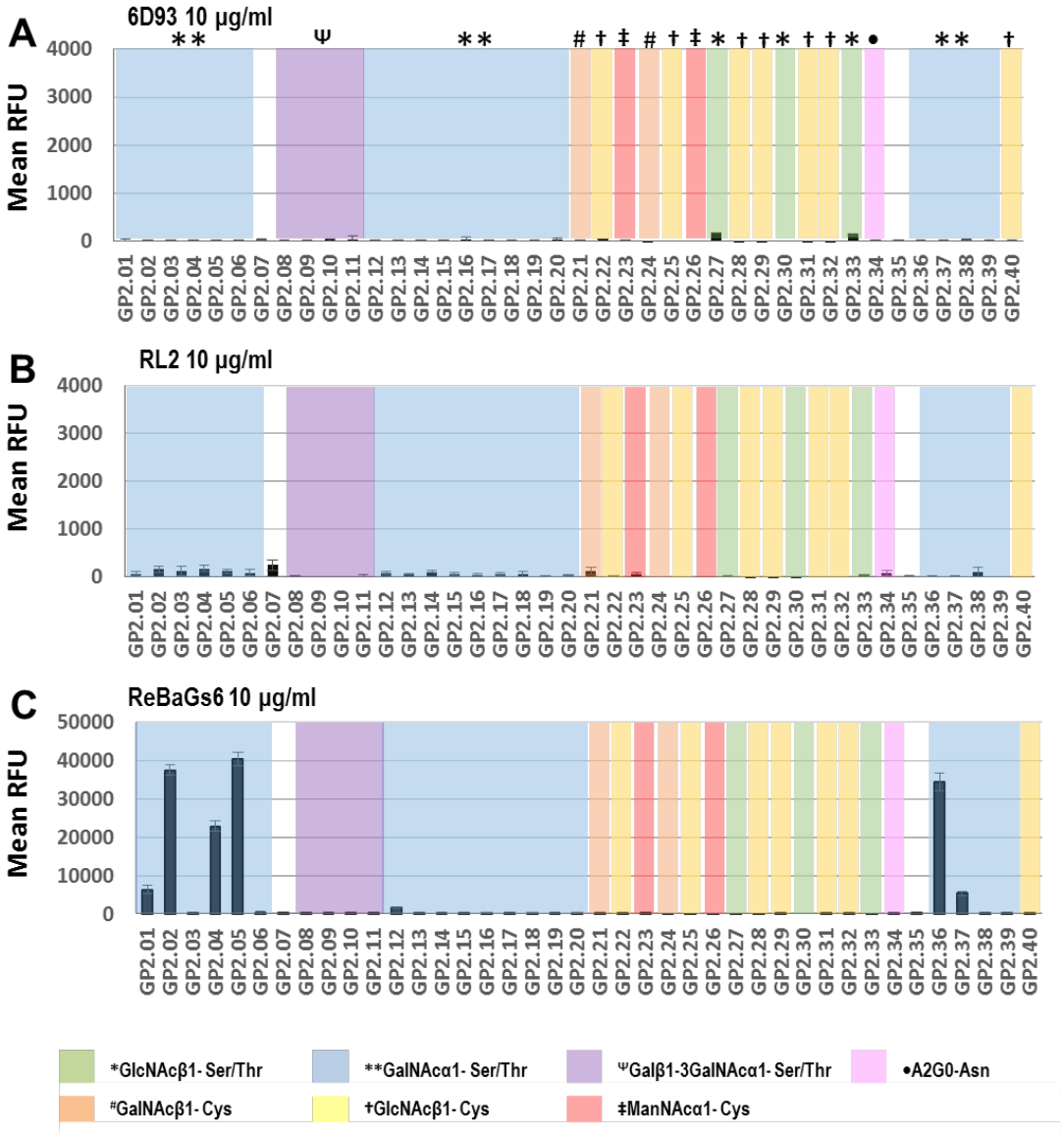


Figure S5. Related to Figure 4. Testing of other antibodies on the GP2 array. (A, B) Binding of 6D93 and RL2 antibody (believed to bind GlcNAcβ1-Ser/Thr), however both showed no significant binding on our arrays. This could indicate inactive antibodies and in fact demonstrates possible utility of such microarrays for quality control testing of such antibodies. (C) Binding of recombinant BaGs6 (ReBaGs6) antibody on the GP2 array. The result shows that the ReBaGs6 antibody binds significantly higher to peptides with GalNAcα1-Ser (GP2.02, GP2.04, GP2.05, GP2.36) over GalNAcα1-Thr (GP2.01, GP2.03, GP2.06 and GP2.37). Such specificity has never been demonstrated before for this antibody.

Figure S6

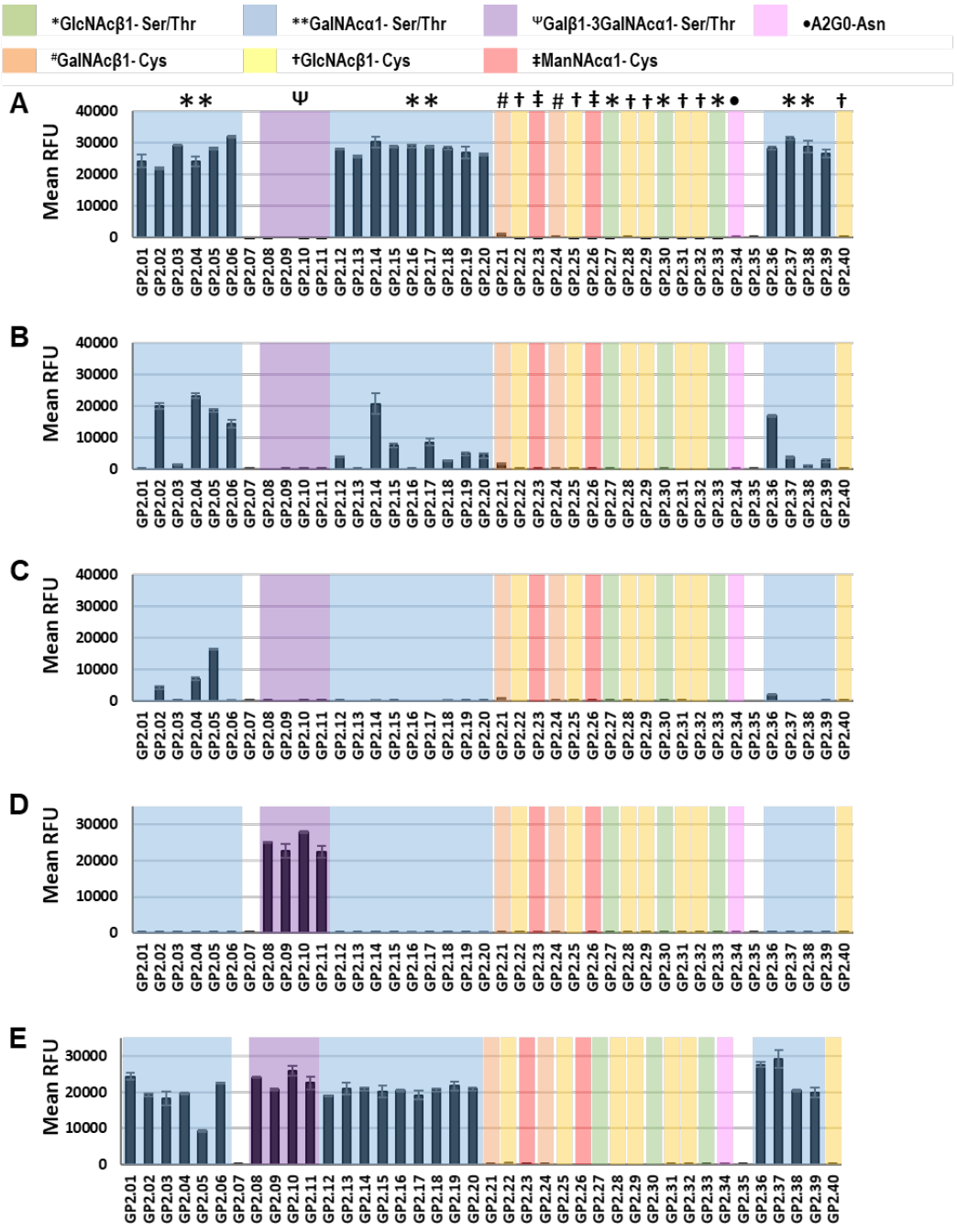


Figure S6. Related to Figure 5. On array glycosylation. (A) Biotinylated-VVL 5 µg/ml and Streptavidin-Cy5 1µg/ml (B) ST6GalNAc1 5 µg/ml with CMP-Sialic Acid (2.2 mM) was incubated for 18h at 37°C followed by staining as done in Panel A. (C) ST6GalNAc1 100 µg/ml with CMP-Sialic Acid (2.2 mM) was incubated for 18h at 37°C followed by lectin staining as done in Panel A. (D) Biotinylated-PNA 5 µg/ml and Streptavidin-Cy5 1 µg/ml (E) T-synthase (50 µg/ml) with Cosmc 1 µg/ml and UDP-Galactose (2.5 mM) was incubated for 18h at 37°C followed by lectin staining as done in Panel D.

Figure S7

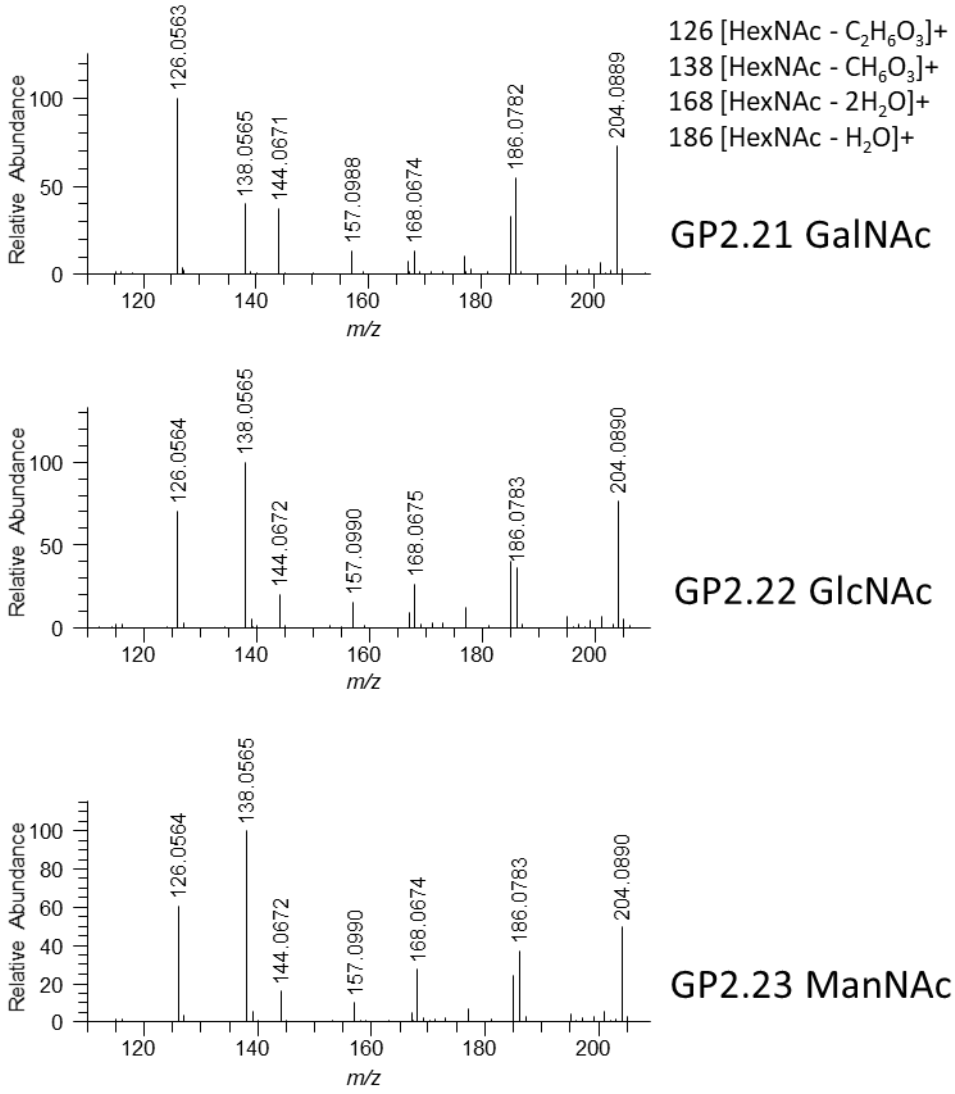


Figure S7. Related to Figure 7. Oxonium ion spectra of GP2.21-23. The HCD spectra are shown in the mass range of m/z 110 – m/z 210 using normalized collision energy of 25% (+/- 5%).

Supplementary Tables

Table S1. Related to Figure 2. GP1 library of glycopeptides. Amino acids are color coded to highlight differences. Special amino acids are highlighted in the key below. GP1.1- prototypical sequence. GP1.19- the only peptide in this library without a GlcNAc. GP1.20- a random sequence. GP1.21- the reverse sequence of the prototypical sequence. Special amino acid GlcNAcβ1-Ser is highlighted in green as shown in key below.

Pep. No.	N-Cap	Residues																					C-term
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
GP1.1	Ac	K	T	S	T	T	A	T	P	P	V	S	Q	A	S	S	T	T	T	S	T	W	A	OH
GP1.2	Ac	K	T	S	T	T	A	T	P	A	V	S	Q	A	S	S	T	T	T	S	T	W	A	OH
GP1.3	Ac	K	T	S	T	T	A	T	P	L	V	S	Q	A	S	S	T	T	T	S	T	W	A	OH
GP1.4	Ac	K	T	S	T	T	A	T	P	P	T	S	Q	A	S	S	T	T	T	S	T	W	A	OH
GP1.5	Ac	K	T	S	T	T	A	T	P	P	E	S	Q	A	S	S	T	T	T	S	T	W	A	OH
GP1.6	Ac	K	T	S	T	T	A	T	P	P	V	S	A	A	S	S	T	T	T	S	T	W	A	OH
GP1.7	Ac	K	T	S	T	T	A	T	P	P	V	S	T	A	S	S	T	T	T	S	T	W	A	OH
GP1.8	Ac	K	T	S	T	T	A	T	P	P	V	S	D	A	S	S	T	T	T	S	T	W	A	OH
GP1.9	Ac	K	T	S	T	T	A	T	P	P	V	S	Q	S	S	S	T	T	T	S	T	W	A	OH
GP1.10	Ac	K	T	S	T	T	A	T	P	P	V	S	Q	L	S	S	T	T	T	S	T	W	A	OH
GP1.11	Ac	K	T	S	T	T	R	T	P	P	V	S	Q	A	S	S	T	T	T	S	T	W	A	OH
GP1.12	Ac	K	T	S	T	T	A	T	R	P	V	S	Q	A	S	S	T	T	T	S	T	W	A	OH
GP1.13	Ac	K	T	S	T	T	A	T	P	Y	V	S	Q	A	S	S	T	T	T	S	T	W	A	OH
GP1.14	Ac	K	T	S	T	T	A	T	A	P	V	S	Q	A	S	S	T	T	T	S	T	W	A	OH
GP1.15	Ac	K	T	S	T	T	A	T	P	P	V	S	Q	P	S	S	T	T	T	S	T	W	A	OH
GP1.16	Ac	K	T	S	T	T	A	T	P	P	V	S	Q	A	P	S	T	T	T	S	T	W	A	OH
GP1.17	Ac	K	T	S	T	T	A	T	R	S	V	S	Q	P	S	S	T	T	T	S	T	W	A	OH
GP1.18	Ac	K	T	S	T	T	A	R	P	P	V	S	Q	P	S	S	T	T	T	S	T	W	A	OH
GP1.19	Ac	K	T	S	T	T	A	T	P	P	V	S	Q	A	S	S	T	T	T	S	T	W	A	OH
GP1.20	Ac	K	P	S	T	T	A	S	P	T	T	S	Q	A	T	S	S	T	T	V	T	W	A	OH
GP1.21	Ac	K	T	S	T	T	T	S	S	A	Q	S	V	P	P	T	A	T	T	S	T	W	A	OH

Key of Special Residues	
S	GlcNAc β1- Serine

Table S2. Related to Figure 2. GP2 library of glycopeptides. Amino acids are color coded to highlight differences. Special amino acids are highlighted in the key alongside.

Pep. No.	*Peptide Description*	N-term	Residues													C-term
			1	2	3	4	5	6	7	8	9	10	11	12	13	
GP2.1	IgA peptide mono-GalNAc - 01	Fl	K	V	P	S	T	P	P	T	P	S	P	S	A	OH
GP2.2	IgA peptide mono-GalNAc - 02	Fl	K	V	P	S	S	P	P	T	P	S	P	S	A	OH
GP2.3	IgA peptide mono-GalNAc - 03	Fl	K	V	P	S	T	P	P	T	P	S	P	S	A	OH
GP2.4	IgA peptide mono-GalNAc - 04	Fl	K	V	P	S	T	P	P	S	P	S	P	S	A	OH
GP2.5	IgA peptide mono-GalNAc - 05	Fl	K	V	P	S	T	P	P	T	P	S	P	S	A	OH
GP2.6	IgA peptide mono-GalNAc - 06	Fl	K	V	P	S	T	P	P	T	P	T	P	S	A	OH
GP2.7	IgA peptide mono-GalNAc - 07	Fl	K	V	P	S	T	P	P	T	P	S	P	S	A	OH
GP2.8	IgA peptide GalGalNAc -01	Fl	K	V	P	S	T	P	P	T	P	S	P	S	A	OH
GP2.9	IgA peptide GalGalNAc -02	Fl	K	V	P	S	T	P	P	T	P	S	P	S	A	OH
GP2.10	IgA peptide GalGalNAc -03	Fl	K	V	P	S	T	P	P	T	P	S	P	S	A	OH
GP2.11	IgA peptide GalGalNAc -04	Fl	K	V	P	S	T	P	P	T	P	S	P	S	A	OH
GP2.12	IgA P→A 01	Fl	K	V	P	S	T	A	P	T	P	S	P	S	A	OH
GP2.13	IgA P→A 02	Fl	K	V	P	S	T	P	A	T	P	S	P	S	A	OH
GP2.14	IgA P→A 03	Fl	K	V	P	S	T	P	P	T	A	S	P	S	A	OH
GP2.15	IgA P → X 01	Fl	K	V	P	S	T	X	P	T	P	S	P	S	A	OH
GP2.16	IgA P → X 02	Fl	K	V	P	S	T	P	X	T	P	S	P	S	A	OH
GP2.17	IgA P → X 03	Fl	K	V	P	S	T	P	P	T	X	S	P	S	A	OH
GP2.18	IgA P → x 01	Fl	K	V	P	S	T	x	P	T	P	S	P	S	A	OH
GP2.19	IgA P → x 02	Fl	K	V	P	S	T	P	x	T	P	S	P	S	A	OH
GP2.20	IgA P → x 03	Fl	K	V	P	S	T	P	P	T	x	S	P	S	A	OH
GP2.21	S-GalNAc IgA peptide	Fl	K	V	P	S	T	P	P	C	P	S	P	S	A	OH
GP2.22	S-GlcNAc IgA Peptide	Fl	K	V	P	S	T	P	P	C	P	S	P	S	A	OH
GP2.23	S-ManNAc IgA Peptide	Fl	K	V	P	S	T	P	P	C	P	S	P	S	A	OH
GP2.24	S-GalNAc 14-3-3 Peptide	Ac	K	Y	L	A	E	V	A	C	G	D	D	R	A	OH
GP2.25	S-GlcNAc 14-3-3 Peptide	Ac	K	Y	L	A	E	V	A	C	G	D	D	R	A	OH
GP2.26	S-ManNAc 14-3-3 Peptide	Ac	K	Y	L	A	E	V	A	C	G	D	D	R	A	OH
GP2.27	O-GlcNAc 14-3-3 Peptide	Ac	K	Y	L	A	E	V	A	S	G	D	D	R	A	OH
GP2.28	S-GlcNAc PCBP1 Peptide	Ac	K	S	S	P	V	I	C	A	G	G	Q	D	A	OH
GP2.29	S-GlcNAc PCBP1 Peptide D→A	Ac	K	S	S	P	V	I	C	A	G	G	Q	A	A	OH
GP2.30	O-GlcNAc PCBP1 Peptide	Ac	K	S	S	P	V	I	S	A	G	G	Q	D	A	OH
GP2.31	S-GlcNAc Antigen KI-67	Ac	K	T	T	K	I	P	C	D	S	P	Q	S	A	OH
GP2.32	S-GlcNAc Antigen KI-67 D→A	Ac	K	T	T	K	I	P	C	A	S	P	Q	S	A	OH
GP2.33	O-GlcNAc Antigen KI-67	Ac	K	T	T	K	I	P	S	D	S	P	Q	S	A	OH
GP2.34	SGP sequence with N-Glycan	Fl	K	V	A	L	N	K	T	A						OH
GP2.35	Truncate for testing	Fl	K	P	T	P	S	P	S	A						OH
GP2.36	IgA double sugar 1	Fl	K	V	P	S	T	P	P	S	P	S	P	S	A	OH
GP2.37	IgA double sugar 2	Fl	K	V	P	S	T	P	P	T	P	S	P	S	A	OH
GP2.38	IgA double X	Fl	K	V	P	S	T	X	X	T	P	S	P	S	A	OH
GP2.39	IgA double x	Fl	K	V	P	S	T	x	x	T	P	S	P	S	A	OH
GP2.40	Repeat Peptide #21	Fl	K	V	P	S	T	P	P	C	P	S	P	S	A	OH

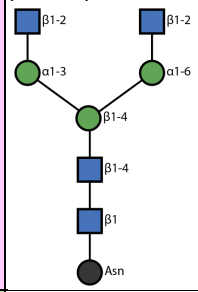
Key of Special Residues	
S	Serine
T	Threonine
S	GalNAcα1- Serine
T	GalNAcα1- Threonine
S	Galβ1-3GalNAcα1- Serine
T	Galβ1-3GalNAcα1- Threonine
X	(2S,4R)-Fluoroproline (trans)
x	(2S,4S)-fluoroproline (cis)
C	GalNAcβ1- Cysteine
C	GlcNAcβ1- Cysteine
C	ManNAcα1- Cysteine
N	N-Glycan heptasaccharide (A2G0) 
S	GlcNAcβ1- Serine
Fl	Fluorescein

Table S3. Related to STAR Methods. List of conditions tried to optimize release of glycopeptides from surface.

Base	Release	Acetyls	β-elimination with GlcNAc	TFA Adduct	Tryptophan Oxidation
0.5% Triethylamine in water 4hrs/20hrs	4hr = partial 24 = Yes	Complete	Yes	No	Yes
0.1% Triethylamine in water 20hrs	Yes	Complete	No	No	Yes
0.5% Triethylamine + 0.5% dimethylglycine pH 10.1 20hrs	Yes	Complete	No	No	Yes
0.5% Triethylamine + 0.15% Acetic Acid pH 9.8 20hrs	Yes	No	No	No	Yes
0.5% Triethylamine + 0.5% dimethylglycine + 0.25% β-mercaptoethanol in water 20hrs	Yes	Partial	No	No	Yes
0.5% Triethylamine + 0.5% β-mercaptoethanol in water 20hrs	Yes	Partial	Yes (little)	No	Yes
5mM NaOMe 4hrs/24 hrs	No	-	-	-	-
10mM NaOMe 4hrs	Yes (little)	Partial	Yes (little)	Yes	No
20mM NaOMe 4hrs	Yes	Partial	Yes	Yes	No
20mM NaOMe elution every 1 hr	Yes	No	Yes	Yes (Little)	No
Gaseous Ammonia (tried various time points)	Yes	-	Yes (complete)	No	No
Aqueous Ammonia	Yes	-	Yes (complete)	No	No
Methanolic Ammonia	Yes	-	Yes (complete)	No	No
Aqueous Hydrazine solution (5% concentration)	Yes	Partial	Yes	No	No
1mg/ml LiOH in 3:1 MeOH:H_2O 2hrs	Yes	Complete	Yes	No	No
100mM NaHCO3 in water 24-72hrs	Yes (slow sometimes partial requiring multiple releases)	Slow (sometimes partial requiring multiple treatments)	No	No	No
100mM NaHCO3 in 10% methanol:water	Yes	Partial	No	No	No
200mM NaHCO3 in 1:2 methanol:water	Yes	Complete	No	No	No

Table S4. Related to STAR Methods. Yields Obtained.

Compound	Mol. Wt.	Amount in nmoles	Amount in µg	Method Used
GP1.1	2456.6	98.7	243	A205 Scopes
GP1.2	2430.5	98.7	240	A205 Scopes
GP1.3	2472.6	97.7	242	A205 Scopes
GP1.4	2458.5	113.2	278	A205 Scopes
GP1.5	2486.6	107.2	267	A205 Scopes
GP1.6	2399.5	111.1	267	A205 Scopes
GP1.7	2429.5	64.5	157	A205 Scopes
GP1.8	2443.5	93.4	228	A205 Scopes
GP1.9	2472.6	122.0	302	A205 Scopes
GP1.10	2498.6	79.0	198	A205 Scopes
GP1.11	2541.7	100.3	255	A205 Scopes
GP1.12	2515.6	127.2	320	A205 Scopes
GP1.13	2522.6	110.0	278	A205 Scopes
GP1.14	2430.5	104.9	255	A205 Scopes
GP1.15	2482.6	109.8	273	A205 Scopes
GP1.16	2466.6	65.9	163	A205 Scopes
GP1.17	2531.6	78.0	198	A205 Scopes
GP1.18	2537.7	88.7	225	A205 Scopes
GP1.19	2253.4	66.6	150	A205 Scopes
GP1.20	2456.6	33.5	82	A205 Scopes
GP1.21	2456.6	34.8	86	A205 Scopes
GP2.01	1825.8	58.2	106	Fluorescein A453
GP2.02	1811.8	93.4	169	Fluorescein A453
GP2.03	1825.8	81.8	149	Fluorescein A453
GP2.04	1811.8	110.3	200	Fluorescein A453
GP2.05	1825.8	127.4	233	Fluorescein A453
GP2.06	1839.8	88.8	163	Fluorescein A453
GP2.07	1622.7	154.4	250	Fluorescein A453
GP2.08	1987.8	147.4	293	Fluorescein A453
GP2.09	1987.8	107.9	214	Fluorescein A453
GP2.10	1987.8	83.0	165	Fluorescein A453
GP2.11	1987.8	97.2	193	Fluorescein A453
GP2.12	1799.8	74.3	134	Fluorescein A453
GP2.13	1799.8	79.7	143	Fluorescein A453
GP2.14	1799.8	143.0	257	Fluorescein A453
GP2.15	1843.8	88.1	162	Fluorescein A453
GP2.16	1843.8	81.0	149	Fluorescein A453
GP2.17	1843.8	78.0	144	Fluorescein A453
GP2.18	1843.8	94.7	175	Fluorescein A453
GP2.19	1843.8	94.6	174	Fluorescein A453
GP2.20	1843.8	108.6	200	Fluorescein A453
GP2.21	1827.8	97.6	178	Fluorescein A453
GP2.22	1827.8	35.2	64	Fluorescein A453
GP2.23	1827.8	84.1	154	Fluorescein A453
GP2.24	1654.8	50.3	83	BCA
GP2.25	1654.8	43.3	72	BCA
GP2.26	1654.8	40.3	67	BCA
GP2.27	1638.8	41.8	68	BCA
GP2.28	1476.7	47.1	70	BCA
GP2.29	1432.7	49.2	70	BCA
GP2.30	1460.7	49.1	72	BCA
GP2.31	1619.8	41.9	68	BCA
GP2.32	1575.8	46.8	74	BCA
GP2.33	1603.8	41.6	67	BCA
GP2.34	2500.0	67.5	169	Fluorescein A453
GP2.35	1141.5	84.8	97	Fluorescein A453
GP2.36	2014.9	63.0	127	Fluorescein A453
GP2.37	2028.9	56.0	114	Fluorescein A453
GP2.38	1861.8	43.8	82	Fluorescein A453
GP2.39	1861.8	42.6	79	Fluorescein A453
GP2.40	1827.8	20.0	37	Fluorescein A453

Table S5. Related to Figure 2. Comparison of GlycoSPOT synthesis vs Other Solid Phase Peptide Synthesis Techniques.

Parameter	Glyco-SPOT Synthesis	Classical Resin Synthesis	Microwave Resin Synthesis	96-well Resin Synthesis	Notes
Ability to Synthesize Libraries	**YES**	NO	NO	Not used at sub-micromole levels. Less than 3 reports for glycopeptides.*	SPOT Synthesis and 96-well Resin Synthesis has been used to synthesize libraries of non-glycosylated peptides.
Automation	Can possibly be adapted in the future to machines (e.g. MultiPep RSi Automated Peptide Synthesizer with SPOT module from Intavis.)	Yes	Yes	Yes	Automation is key to create libraries. While currently not available, there are robotic systems which can be adapted to automated Glyco-SPOT Synthesis and our work in this paper should give impetus to develop such platforms.
Number of Peptides Synthesized in Parallel	Up to 96/membrane if performed manually (limited by individual sanity). Up to 600/membrane if adapted to machine.	1	1 at a time but can add batch sequences as usually there is a single reaction vessel.	96/plate	Significantly larger number of peptides can be synthesized in parallel if automated.
Cycle time (usually)	3-4 hours	3-4 hours	5-20 mins	2-3 hours	Cycle time is comparable to other methods where heat is not involved. Note- heat is detrimental to certain FPGA coupling. However, cycle time is significantly slower than microwave method.
Usual scale (μmoles)	0.02-0.20 μmoles (based on the two libraries we synthesized average is 0.084 μmoles)	5-100 μmoles	5-100 μmoles	1-10 μmoles	Scale is significantly smaller (>5-50 fold) in comparison to 96-well resin methods.
Visual Detection at Lower Scale at each step	Yes using bromophenol blue	Yes	Not usually performed as reaction is sealed	Possible but tedious	This is advantageous to troubleshoot the reaction.
Intermediate Sampling for MALDI during Synthesis	Yes using Biopsy technique described herein	Yes	Could result in significant losses due to density of peptide on resin at lower scales.	Could result in significant losses due to density of peptide on resin at lower scales.	This is advantageous to judge efficacy and completion of the reaction. This is especially useful in difficult FPGA couplings which require more time.
Amount of amino acid used (μmoles/peptide/cycle)	0.1-0.5 μmoles	5-200 μmoles	5-200 μmoles	2-20 μmoles (2X excess to scale)	Significantly lower absolute amounts FPGAs used allowing for more efficient utilization of precious FPGA resources.
TFA removal step	Not required if final purification on C18 performed with compatible salts (e.g. ammonium acetate).	TFA removal step usually required.	TFA removal step usually required.	TFA removal step usually required.	Since the TFA deprotection step is performed prior to the bicarbonate release step, any residual TFA is neutralized and washed away. Therefore, if appropriate salts are used for the purification, TFA removal does not need to be performed.

*to the best of our knowledge