Supporting Information for

Rapid Preparation of Released *N*-Glycans for HILIC Analysis Using a Labeling Reagent that Facilitates Sensitive Fluorescence and ESI-MS Detection

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Supplemental Experimental and Results

Reagents

LC/MS grade solvents (water and acetonitrile), formic acid and trifluoracetic acid were purchased from Pierce (Rockford, IL). Ammonium acetate (73594), ammonium formate (70221), anthranilamide (2-AB, 2-aminobenzamide), bovine fetuin (F3004), dimethylformamide (DMF), dimethylsulfoxide (DMSO), glu-fibrinopeptide b (F-3261), guanidine hydrochloride, hexafluoroisopropanol (HFIP), HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) (H3375), human IgG (I4506), monosodium phosphate, n-propylamine, procainamide hydrochloride and TCEP (tris(2-carboxyethyl)phosphine) (C4706) were obtained from Sigma-Aldrich (St. Louis, MO). PNGase F (Glycerol Free) Recombinant (P0709, provided with Glycoprotein Denaturing Buffer and NP-40) were acquired from New England Biolabs (Ipswich, MA). Instant AB labeling reagent (IAB) and 2-AB labeled N-glycans from pooled human IgG were obtained from Prozyme (Hayward, CA). 2-AB labeled triacetyl chitotriose was purchased from Ludger (Oxfordshire, UK). GlycoWorks Rapid PNGase F, GlycoWorks Rapid PNGase F buffer, *Rapi*Fluor-MS labeling reagent (RFMS), and *Rapi*Gest SF surfactant (RG surfactant) were obtained from Waters (Milford, MA). Cetuximab (Erbitux, ImClone Systems, Bridgewater, NJ) was obtained from Besse Medical (West Chester, OH). Immunoglobulin degrading enzyme from *S. pyogenes* (IdeS) was obtained from Genovis (Lund, Sweden). Carboxypeptidase B was purchased from Worthington (Lakewood, NJ).

Comparison of Labeled Glycans and Measurement of Response Factors (RFMS, IAB, and 2-AB)

To compare the response factors of RFMS and IAB labeled glycans, N-glycans from anti-citrinin murine IgG1 were prepared according to the conditions outlined in the previous experimental, except that crude reaction mixtures were directly analyzed by HILIC-FLR-MS in order to avoid potential biases from SPE clean-up procedures. Response factors were determined as ratios of the FA2 N-glycan chromatographic peak area to the mass of glycoprotein from which the glycan originated. Fluorescence chromatograms were used to measure optical detection response factors, and base peak intensity (BPI) chromatograms were used to measure MS response factors. Response factors for this comparison were reported in units of peak area per µg of glycoprotein, and analyses were performed in duplicate.

To compare the response factors of 2-AB labeled versus RFMS labeled glycans, equivalent quantities of labeled N-glycans from pooled human IgG were analyzed by HILIC-FLR-MS. A sample of 2-AB labeled N-glycans from pooled human IgG was obtained from Prozyme (Hayward, CA). RFMS labeled N-glycans from pooled human IgG were prepared according to the conditions outlined in the previous experimental. Column loads were calibrated using quantitative standards of 2-AB labeled

triacetyl chitotriose and RFMS derivatized propylamine (obtained in high purity as confirmed by HPLC and ^{1}H NMR) (See **Figures S15** and **S16**). Response factors were determined as ratios of the FA2 chromatographic peak area to the mole quantity of glycan. Response factors for this comparison were reported in units of peak area per pmole of glycan. Analyses were performed in duplicate.

Metrics for the relative performance of labeling reagents were subsequently calculated based on the measured response factors. Ratios of response factors were calculated and reported as percentages in order to display the percent of RFMS signal that is obtained when an alternative labeling reagent is employed. The relative performance of procainamide was calculated using the conclusions from a published comparison of N-glycans, wherein it was found that procainamide provided comparable fluorescence and up to 50 fold greater ESI-MS sensitivity when compared to 2-AB.[1]

Comparison of Labeled Glycans and Measurement of Response Factors (2-AB and Procainamide)

N-linked glycans from pooled human IgG (ProZyme, Hayward, CA) were fluorescently labeled with either 2-aminobenzamide (2-AB) or procainamide (4-amino-N-(2-diethylaminoethyl) benzamide) reagents. Fluorescent labeling was performed in solutions of 100 µL of glacial acetic acid:DMSO (3:7, v/v) mixture with 11 mg procainamide or 5 mg 2-AB, followed by addition of 6 mg of sodium cyanoborohydrate. Labeling reagent solution was added to 2 µg of human IgG N-linked glycans and the resulting mixture was heated for 4 hours at 65 °C. All samples were reconstituted in acetonitrile/water (1:1) for injection.

Labeled N-glycans were analyzed via HILIC separations combined with fluorescence and mass spectrometric detection using a UHPLC chromatograph (ACQUITY UPLC, Waters, Milford, MA. A 2.1 x 150 mm column packed with 1.7 µm amide-bonded organosilica particles (ACQUITY UPLC Glycan BEH Amide 130Å, Waters, Milford, MA) was employed along with an aqueous mobile phase comprised of 50 mM ammonium formate (pH 4.4) and another of ACN. Samples were separated at 60°C according to the chromatographic method shown in **Table S7**. Labeled N-glycans were detected using a fluorescence detector (1 Hz scan rate, Gain=1, ACQUITY UPLC FLR, Waters, Milford, MA). Eluting glycans were also detected by positive ion mode electrospray ionization mass spectrometry with a QTof mass spectrometer (Xevo QTof, Waters, Milford, MA) operating with a capillary voltage of 3.2 kV, source temperature of 100°C, desolvation temperature of 350°C, and sample cone voltage of 30 V. Based on this experiment, procainamide labeled N-glycans were found to exhibit approximately 15 times higher MS response factors than 2-AB labeled N-glycans (**Figure S6**).

HILIC-UV-ESI-Analysis of IdeS Digested Cetuximab

Cetuximab was subjected to 'middle-up' glycan characterization[2-3] using HILIC separations combined with UV and ESI-MS detection. Prior to digestion with IdeS,[4] cetuximab was treated with carboxypeptidase B to complete the partial removal of the lysine-C-terminal residues that is typical of the antibody.[5] Formulated cetuximab was mixed with carboxypeptidase B (223 u/mg) at a ratio of 100:1 (w/w), diluted into 20 mM phosphate (pH 7.1), and incubated at a concentration of 1.8 mg/mL for 2 hours at 37°C. The carboxypeptidase B treated cetuximab was then added to 100 units of IdeS and incubated for 30 minutes at 37°C. The resulting IdeS digest was denatured and reduced by the addition of 1M TCEP and solid GuHCl. The final buffer composition for the denaturation/reduction step was approximately 6 M GuHCl, 80 mM TCEP, and 10 mM phosphate (pH 7.1). IdeS-digested cetuximab (0.9 mg/mL) was incubated in this buffer at 37°C for 1 hour.

Solutions of reduced, IdeS digested cetuximab (carboxypeptidase b treated) were injected in 1.5 µL volumes onto a 2.1 x150 column packed with 1.7µm wide-pore amide bonded organosilica (300Å) stationary phase. Separations were performed at 60˚C using the mobile phases and gradient described in **Table S8**. Eluting species were detected serially via UV absorbance (214 and 280 nm, 2 Hz) followed by online ESI-MS with a QToF mass spectrometer (Waters Xevo G2 QTof, Milford, MA) operating with a capillary voltage of 3.0 kV, source temperature of 150°C, desolvation temperature of 350°C, and sample cone voltage of 45 V. Mass spectra were acquired at a rate of 2 Hz with a resolution of approximately 20,000 over a range of 500−4000 *m/z*.

Evaluation of Deglycosylation by Gel Electrophoresis

The effectiveness of the rapid deglycosylation process was evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is an effective technique for separating proteins based on their size in solution and can often be used to separate the glycosylated and de-glycosylated forms of proteins.[6-7] A diverse set of glycoproteins were deglycosylated according to the 2-step rapid deglycosylation procedure and analyzed by SDS-PAGE along with negative controls containing no PNGase F and positive controls, in which the glycoproteins were subjected to conventional multiple step deglycosylation with SDS based denaturation and PNGase F incubation for 30 minutes at 37˚C.

The following glycoproteins were assayed for deglycosylation using gel electrophoresis: 30 µg bovine fibrinogen (F8630, Sigma, St. Louis, MO), 15 µg bovine RNaseB (New England Biolabs, Ipswich, MA), 30 µg bovine fetuin (New England Biolabs, Ipswich, MA), 13.5 µg anti-citrinin murine IgG1 (Intact mAb Mass Check Standard, Waters, Milford, MA), 10 µg human lactoferrin (L0520, Sigma, St. Louis,

MO), 30 µg chicken ovalbumin (A2512, Sigma, St. Louis, MO), 174 µg bovine holo-Transferrin (T1408, Sigma, St. Louis, MO), 30 µg human α-acid glycoprotein (G9885, Sigma, St. Louis, MO), 30 µg bovine asialofetuin (A1908, Sigma, St. Louis, MO) and 180 µg human chorionic gonadotropin (C6322, Sigma, St. Louis, MO).

Glycoprotein substrates were deglycosylated under two different conditions: a) with PNGase F (P0709, New England BioLabs, Ipswich, MA) after conventional SDS denaturation; or b) with GlycoWorks Rapid PNGase F after denaturation with GlycoWorks buffer and RapiGest SF surfactant (Waters, Milford, MA). For conventional denaturation, the glycoprotein was incubated at 95°C for 2 min in the presence of 0.5% SDS and 40 mM DTT (Glycoprotein Denaturing Buffer, New England BioLabs, Ipswich, MA), in a total volume of 16 µl. After cooling, NP-40 (to 1% v/v), sodium phosphate pH 7.5 (to 50 mM), and PNGase F (PNGase F (Glycerol Free) Recombinant, 500 units) were added to the solution, increasing the total volume of the mixture to 20 µl. The reaction was incubated at 37°C for 30 minutes. Negative controls were prepared under the same conditions, without the addition of enzyme.

The rapid deglycosylation procedure was performed with GlycoWorks Rapid PNGase F, GlycoWorks Rapid PNGase F Buffer, and RapiGest SF surfactant. Glycoproteins were incubated with GlycoWorks Rapid Buffer and 1% (w/v) RapiGest SF at 80°C for 2 minutes, in a total volume of 20 µl. After cooling, 1 µl of GlycoWorks Rapid PNGase F was added and the reaction was incubated at 50°C for 5 minutes.

Following enzymatic deglycosylation, all samples were heated to 70°C for 20 minutes to inactivate the enzyme. Each sample was diluted with 30 µl of water and 25 µl 3x Blue Loading Buffer (New England Biolabs, Ipswich, MA) containing 0.14 mM DTT. The samples were heated at 95°C for 2 minutes and 7 µl of each sample was loaded on a Novex 10-20% Tris-Glycine Mini Protein Gel (Life Technologies, Grand Island, NY). The gel was run at 150 V for 1 hour, and then stained with coomassie blue (SimplyBlue SafeStain, Life Technologies, Grand Island, NY).

Figure S10A shows the results of this study, where it can be seen that for each of the tested proteins there is a significant decrease in protein apparent molecular weight after they are subjected to the rapid deglycosylation procedure. Moreover, the apparent molecular weight decreases are visually comparable to those observed for proteins deglycosylated by the control method. These results demonstrate that the fast deglycosylation approach facilitated by a unique formulation of PNGase F (GlycoWorks Rapid PNGase F, Waters, Milford, MA) and surfactant (RapiGest SF, Waters, Milford, MA) produces deglycosylation comparable to a conventional approach but in only a fraction of the time required and with conditions that are compatible with rapid labeling and downstream mass spectrometric analyses.

It is worth noting that some glycoproteins must be subjected to reducing conditions in order for complete deglycosylation to be achieved. For instance, samples of holo-transferrin and chorionic gonadotropin were found to present higher apparent molecular bands when subjected to rapid deglycosylation according to the above procedure versus the conventional SDS procedure (**Figure S10B**). Accordingly, rapid deglycosylation was repeated with the addition of 4 mM TCEP reducing agent. TCEP, being a non-nucleophilic reducing agent, was chosen over thiol reducing agents to ensure compatibility with rapid tagging labeling reactions. TCEP hydrochloride is a strong conjugate acid and was therefore titrated upon dissolution with sodium hydroxide to a neutral pH stock solution before use. In this modified rapid deglycosylation procedure, glycoproteins were incubated with GlycoWorks Rapid Buffer, 1% (w/v) RapiGest SF, and 4 mM TCEP at 80°C for 2 minutes, in a total volume of 20 µl. After cooling, 1 µl of GlycoWorks Rapid PNGase F was added and the reaction was incubated at 50°C for 5 minutes. Results for this reducing, rapid deglycosylation condition are shown in **Figure S10B**.

Evaluation of HILIC SPE Absolute and Relative Recovery

The procedure for extracting RFMS labeled glycans after derivatization was evaluated using a test mixture containing N-glycans released and labeled from a 1:1 mixture (by weight) of pooled human IgG and bovine fetuin. The test mixture was prepared according to the conditions outlined in the previous experimental. An aliquot of crude sample was saved prior to the first SPE clean-up procedure. This sample and the one obtained after SPE clean-up (1x SPE) were analyzed by HILIC –FLR (**Figure 5A** versus **5B**). The latter sample was once again processed by HILIC SPE (2x SPE) and likewise analyzed by HILIC-FLR. To assess the relative recovery of this process, relative abundances for a selection of labeled N-glycans were then measured for the samples once and twice subjected to HILIC SPE (1x SPE versus 2x SPE) (**Table S5**, **Figure 5**). Additionally, measured peak areas for the labeled FA2 glycan were used to estimate the absolute SPE recovery (**Table S5**). Based on triplicate analysis of two different SPE sorbent batches, the average recovery for the HILIC SPE clean-up was estimated to be 74%.

Measurement of the Sample Preparation Yield

The percent yield of the entire N-glycan sample preparation was assessed in order to measure the collective efficiency of combining fast deglycosylation, rapid labeling, and HILIC SPE extraction of RFMS labeled glycans. RFMS labeled N-glycans from anti-citrinin murine IgG1 (15 µg) were prepared and analyzed by HILIC-FLR-MS as outlined in previous experimental sections (**Figure S12**). Using RFMS derivatized propylamine, the RFMS labeled FA2 glycan was quantified (**Figures S12A** and **S12B**). A percent yield for the sample preparation was subsequently calculated assuming the theoretical

yield calculation outlined in **Figure S12C**. Based on duplicate analysis, it was determined that the percent yield of the entire rapid N-glycan sample preparation was approximately 73%.

Charge States and CID Fragmentation Pathways of RFMS Labeled Glycans

Because the RFMS label has high proton affinity, derivatized glycans preferentially adopt high charge states during positive ion mode electrospray ionization. The predominant charge state for a small neutral glycan, such as an FA2 glycan, is $[M+2H]^{2+}$, although it increases to $[M+3H]^{3+}$ for larger molecular weight glycans, such as an A3G3S3 glycan (**Figure S13**). Collision induced dissociation (CID) of these doubly and triply protonated RFMS labeled glycans showed similar fragmentation pathways as those previously reported for 2AB-labeled glycans. For instance, high intensity b and y-ions from glycosidic bond cleavages were predominately observed along with low-intensity, cross-ring fragments.[8] However, unlike 2-AB labeled glycans, RFMS labeled glycans readily produced high signal-to-noise MS/MS spectra from relatively low analyte quantities. An example MS/MS spectrum for an FA2 glycan derivatized with RFMS is illustrated in **Figure S14**.

Synthesis, Purification and Characterization of RFMS Derivatized Propylamine

RFMS was reacted with propylamine to produce a material to be used as a quantitative standard. RFMS reagent (75 mg) was dissolved in methylene chloride (3 mL) and added drop-wise to a stirring solution of n-propylamine (70 mg) in methylene chloride (7 mL) over 10 minutes at room temperature. The reaction was stirred for an additional 30 minutes. Solvent was removed by rotoevaporation. The recovered brown powder was washed three times in methylene chloride (10 mL) and then dried for 48 hours under vacuum.

The dried material was then dissolved in 1% formic acid (v/v) and chromatographically purified using a 4.6 x 150 mm column packed with 2.5 µm C18 bonded, charged-doped organosilica stationary phase (130Å) (XBridge CSH C18, Waters, Milford, MA) and a UHPLC chromatograph (ACQUITY UPLC H-Class Bio, Waters, Milford, MA). Crude RFMS derivatized propylamine (1 mg) was separated at 30˚C using the mobile phases and gradient described in **Table S9**. Fractions collected from 20 gradients were pooled and dried by centrifugal evaporation to yield a yellow waxy solid. ^{1}H and ^{13}C NMR confirmed the identity of the material as the TFA salt of RFMS derivatized propylamine (**Figure S15**).

The purified RFMS derivatized propylamine (4µg) was also assayed for impurities using the reversed phase liquid chromatography (RPLC) separation by which it was purified. Eluting species were detected by UV absorbance (210 nm, 2 Hz) and fluorescence (Excitation 265 nm / Emission 425 nm, 2

Hz) followed by online ESI-MS with a QTof mass spectrometer (Waters Xevo G2 QTof, Milford, MA) operating with a capillary voltage of 3.0 kV, source temperature of 150°C, desolvation temperature of 600°C, and sample cone voltage of 40 V. Mass spectra were acquired at a rate of 1 Hz with a resolution of approximately 20,000 over a range of 100−2000 *m*/*z*. Based on these analyses, the purified RFMS derivatized propylamine was determined to be approximately 99% pure (**Figure S16**).

^{1}H NMR Analysis of IAB and RFMS Labeling Reagents

IAB labeling reagent (InstantAB, Prozyme, Hayward, CA) was characterized using a Bruker 300M NMR spectrometer (Billerica, MA). The ^{1}H NMR spectrum observed for IAB labeling reagent was found to be consistent with an NHS-carbamate derivative of 4-aminobenzamide. 1H NMR: (300MHz,) δ 8.78(s, 1H), 7.83(d, 2H, J=8.8Hz), 7.53(d, 2H, J=8.7Hz), 6.71(s, 1H), 5.94(s, 1H), 2.80(s, 4H. Use of this reagent, 4-aminobenzamidyl-N-hydroxysuccinimidyl carbamate (4-ABSC), for labeling of N-glycans has been previously disclosed.[9]

RFMS labeling reagent (RapiFluor-MS, Waters, Milford, MA) was likewise characterized using a Bruker 300M NMR spectrometer (Billerica, MA). The ^{1}H NMR spectrum observed for RFMS labeling reagent confirms our proposed structure and indicates RFMS is purified as a 1:1 complex with n-hydroxysuccinimide (NHS). 1H NMR: (300MHz, DMSO-d6) δ 8.79(t, 1H, J=5.7Hz), 8.47(d, 1H, J=8.6Hz), 8.16(d, 1H, J=1.7Hz), 8.10(d, 1H, J=8.5Hz), 8.09(d, 1H, J=9.1Hz), 7.86(dd, 1H, J=9.2, 1.9Hz), 3.40(q, 2H, J=6.5Hz), 2.85(s, 4H), 2.57(s, 4H), 2.50-2.63(m, 6H), 0.98(t, 6H, J=7.0Hz)

Table S1. Chromatographic Gradient for HILIC-Fluorescence-ESI-MS Analysis of Labeled N-Glycans with a 2.1 x 50 mm Column

Mobile Phase A: 50 mM ammonium formate, pH 4.4
Mobile Phase B: ACN

Time	Flow Rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
11.7	0.4	46	54	6
12.2	0.2	100	0	6
13.2	0.2	100	0	6
14.4	0.2	25	75	6
15.9	0.4	25	75	6
18.3	0.4	25	75	6

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