FULLY AUTOMATED SAMPLE PREPARATION WITH ULTRAFAST N-GLYCOSYLATION ANALYSIS OF THERAPEUTIC ANTIBODIES

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ABSTRACT

There is a growing demand in the biopharmaceutical industry for high throughput, large scale N-glycosylation profiling of therapeutic antibodies an all phases of product development, but especially during clone selection where hundreds of samples should be analyzed in a short period of time. Our group has recently developed a magnetic bead based protocol for N-glycosylation analysis of glycoproteins to alleviate hard-to-automate centrifugation and vacuum-centrifugation steps of the currently used protocols. Glycan release, fluorophore labeling and clean-up were all optimized resulting in <4 hours magnetic bead based process with excellent yield, and high reproducibility. This paper demonstrates the next step of this work by automating all steps of the optimized magnetic bead based protocol from endoglycosidase digestion, through optimized fluorophore labeling and clean-up with high throughput sample processing in 96 well plate format using liquid handling robots. CE-LIF analysis of the fluorophore labeled glycans was also optimized for rapid (<3 min) separation to accommodate the high throughput of the automated sample preparation process.

INTRODUCTION

Generally used liquid phase based glycoanalytical methods such as capillary electrophoresis and hydrophilic interaction chromatography require time consuming sample preparation and derivatization steps including glycan release, purification and pre-concentration. In addition, current protocols include numerous centrifugation and vacuum-centrifugation steps that make full automation of the process by liquid handling robots difficult and expensive. Utilizing a novel magnetic bead based sample preparation protocol, a large number of samples can be processed within a couple hours with simple liquid handling robots requiring no centrifugation or vacuum-centrifugation steps. This new protocol has been tested on a Biomek FXP Laboratory Automation Workstation with standard immunoglobulin G samples. Ultrafast analysis (<3 min) of the resulting fluorophore labeled glycans was accomplished by capillary electrophoresis with laser induced fluorescent detection.

Figure 1. Biomek FX[P] Laboratory Automation Workstation

EXPERIMENTAL SETUP

Automated sample preparation was performed on a Biomek FX[P] Laboratory Automation Workstation (Figure 1), which was set up with 96 well plate holders, a magnetic stand, 1000 µl and 25 µl pipette tips, a quarter reservoir, along with sample and reagent vials. The quarter reservoir contained acetonitrile (Sigma Aldrich, MO) and the Agencourt CleanSeq magnetic beads (Beckman Coulter, Brea, CA). The reagent vials contained reagents for the PNGase F digestion (Prozyme, CA), 20 mM ATPS (Beckman Coulter) in 20% acetic acid and 1 M sodium-cyanoborohydrate (in THF) (Sigma Aldrich, MO). To reduce evaporation induced volume loss, a pipette box lid was used to cover the quarter reservoirs.

The glycoprotein samples were incubated in a Biomek vortex heater block. For better re-suspension, an extra plate was applied under the sample plate, in which case the magnets were positioned under the sample plate, rather that of on the side. In this way the magnet could pull down the magnetic beads to the bottom of the vials and with fast aspiration/dispensing the beads were easily re-suspendable (Figure 2).

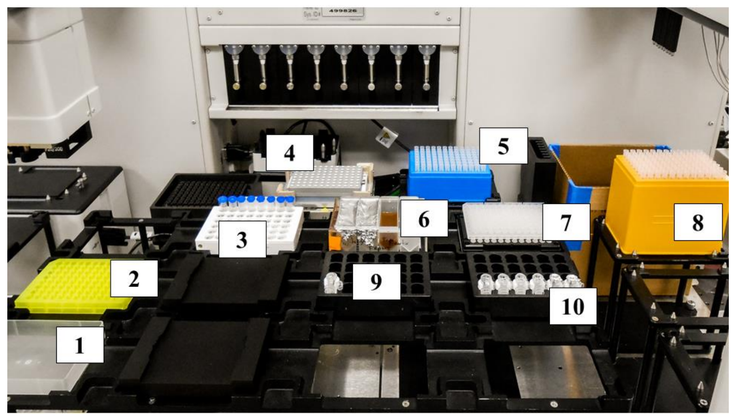

Figure 2. Experimental setup

No.	Labware	Function
1	Pipette tip box lid	Reducing the evaporation of acetonitrile
2	96 well plate (Nunc U96 0.5 ml)	Stacking plates for resuspension
3	Result sample tray	Beckman PA 800 *plus* vials
4	Shaker Peltier 96	For incubation, mixing and re-suspension
5	Pipette tip box (P20)	Small reagent amounts
6	Quarter reservoir	Acetonitrile (100% and 87.5%) and Agencourt CleanSeq magnetic beads
7	Sample plate (Thermowell 96 well PCR plate) on magnetic stand	Sample preparation and capturing magnetic beads on side of the plate vials
8	Pipette box (P1000)	Large reagent amounts
9	Sample tray (24 well tray)	Protein sample
10	Reagent tray (24 well tray)	For PNGase F digestion and labeling reagents

METHODS

The individual steps of the rapid sample preparation protocol were recently published by Varadi *et al.* [1]. The entire workflow is shown in Figure 3. The enzymatic digestion using PNGase F was performed at 50 ˚ C for 1 hour followed by glycan capture on the magnetic beads using 87.5% MeCN medium [2]. APTS labeling of the bound carbohydrates was initiated in situ on the beads at 37 ˚ C for 2 hours by the addition of sodium cyanoborohydride. The fluorophore labeled glycans were eluted from the beads by the addition of 25 µl of water and were ready for CE-LIF analysis by a PA 800 *plus* equipment with LIF detection (488 nm excitation, 520 nm emission) (Beckman Coulter, sold through AB Sciex, Brea, CA). For the separation, 20 cm effective length NCHO capillaries (Beckman Coulter) were used (30 cm total length, 50 µm ID). The applied voltage was 30 kV. The samples were pressure injected by 3 psi for 6 seconds. The entire liquid handling protocol was programmed using the Biomek Software version 4.0 (Figure 4) and the CE-LIF data acquisition and analysis by the Karat 32 software package (Beckman Coulter, sold through AB Sciex, Brea, CA).

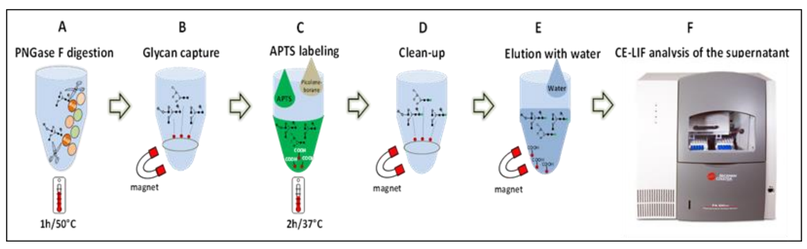

Figure 3. Magnetic bead based sample preparation flowchart for N-glycosylation analysis of therapeutic antibodies by CE-LIF

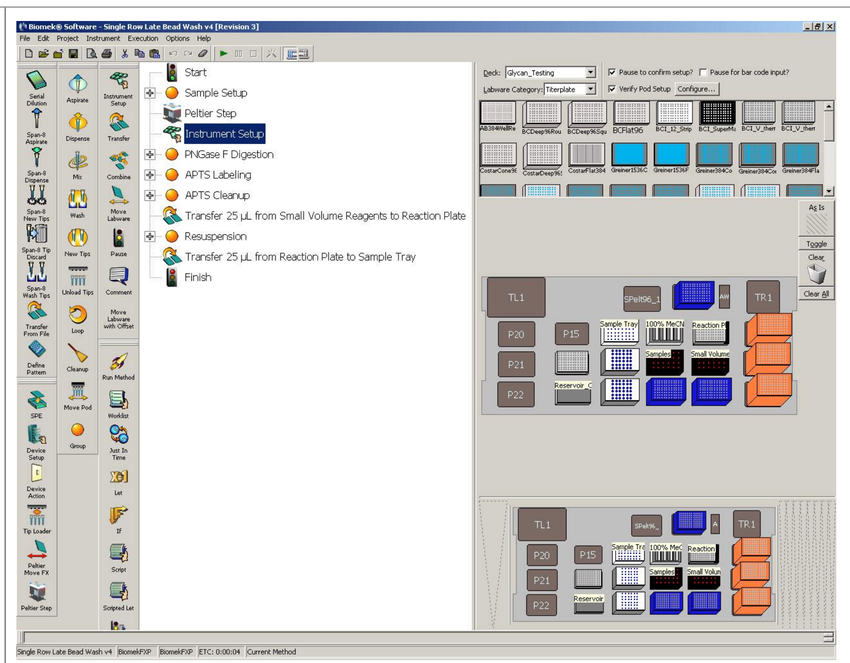

Figure 4. Biomek software

RESULTS AND DISCUSSION

Glycan release, APTS-labeling and sample clean-up were all optimized with a magnetic bead based protocol resulting in < 4 hours processing time with excellent yield and high reproducibility [1]. CE-LIF analysis of the fluorophore labeled glycans was also optimized for rapid separation to accommodate the high throughput of the automated sample preparation process. The electropherograms of APTS labeled IgG glycans from a 96 well plate are shown in Figure 5. Please note that full separation of the major IgG glycans were obtained in less than 3 minutes.

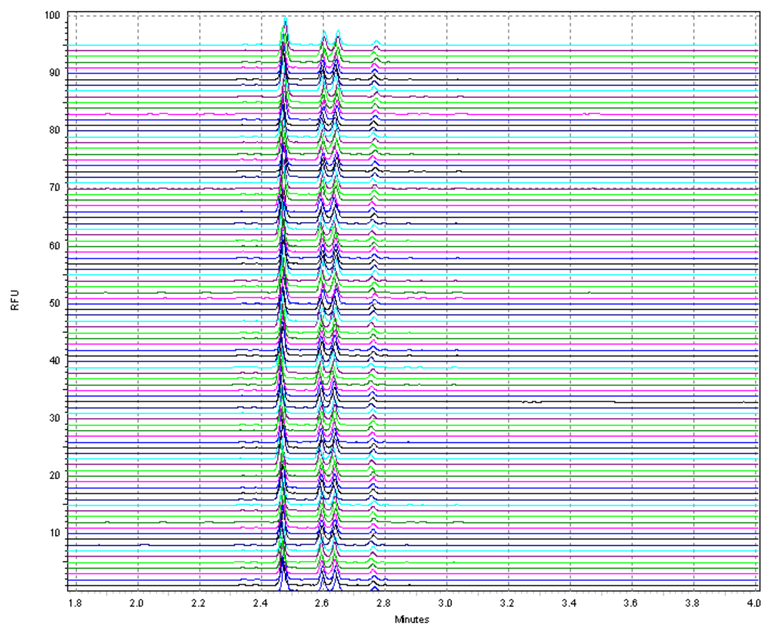

Figure 5. Ultrafast CE-LIF analysis of APTS labeled IgG glycans prepared by a liquid handling robot in a 96 well plate format utilizing the magnetic bead based protocol

Programing of the liquid handling platform was simple and the system was flexible and robust to handle large number of samples. The Laboratory Automation Workstation provided fast sample preparation, reduced flow-induced shear strain on native biological sample matrices and minimized contamination risks. Due to the large amount of deck space available in the system, the buffer preparation for the CE measurement was also done automatically. For higher accuracy liquid handling or unknown source and amount of samples conductive pipette tips can be used that capable of high precision liquid handling. In summary, the Laboratory Automation Workstation in conjunction with CE-LIF used in these experiments was capable of high throughput sample preparation for rapid glycosylation analysis of IgG molecules.

REFERENCES

[1] Varadi et al., Anal. Chem., 2014, 86 (12), pp 5682–5687
[2] Kieleczawa, Jan, ed. DNA sequencing II: optimizing preparation and cleanup. Vol. 2. 2006. p 132.

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TRADEMARKS/LICENSING