Andrew Alliance

APPLICATION NOTE

Automated Preparation Of Labeled N-Glycans With A Vision-Guided Pipetting Robot For High Sensitivity Lc-Ms Analysis

INTRODUCTION

The GlycoWorks N-glycan analysis kit with RapiFluor-MS (RFMS) ***(Figure 1)*** *labeling can provide significant time savings over reductive amination labeling methods such as 2-AB and provides sensitive MS detection. Recently, automation of this kit using an automated pipetting robot promises further gains in productivity in any laboratory setting. As shown here, automation of the GlycoWorks with RFMS kit offers an easy, reliable and robust solution to released N-glycan analysis.*

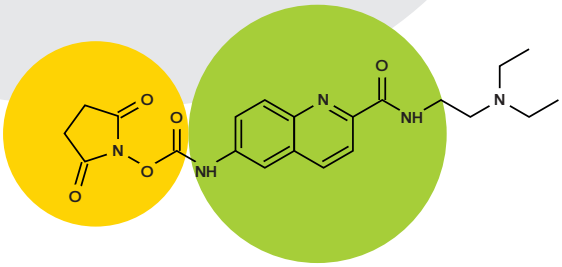


Figure 1: Structure of RFMS.

Using an Andrew Alliance pipetting robot ***(Figure 2)****, the N-glycan labeling protocol was automated. A current limitation of this robotic platform is the inability to move reaction tubes or plates while the RFMS protocol requires the different temperatures for protein denaturation and de-N-glycosylation with PNGase F. Therefore, a custom built component allowing for automation of the heating and cooling steps was constructed. As a result, optimization of these heating and cooling steps for denaturation and de-N-glycosylation was required. Several different protein samples each with unique characteristics were used to establish these optimal temperature ranges. Total area counts and relative areas of specific N-glycan peaks were used. This optimization and measures of the reliability of the automated protocol will be presented.*

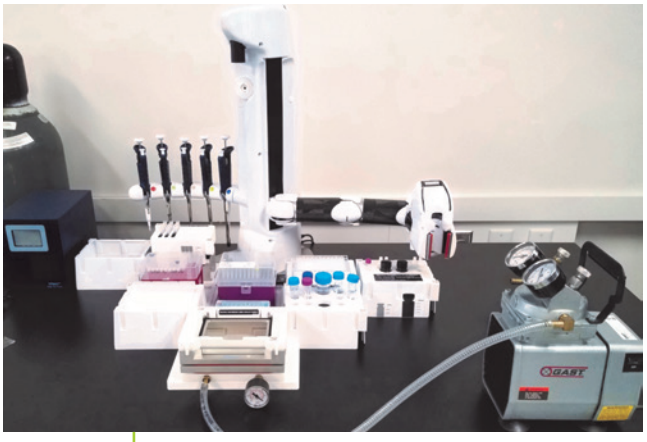


Figure 2: The workbench requirements of this automation protocol. Unique features include a heating/cooling feature, SPE cleanup component, released N-glycan kit component and vacuum manifold. Each block of the automation kit is modular allowing for adaptation to any protocol.

METHODS

Protocol optimization

Each step in the GlycoWorks with RFMS kit was automated using the intuitive Andrew Lab software **(Figure 3)**. Steps were optimized for maximum time efficiency.

Figure 3: The top panel shows the GlycoWorks with RFMS workflow. The bottom panel shows the Andrew Lab protocol development soft-ware.

Comparison to Manual Procedure

Manually and robot prepared samples were weighed following each step liquid transfer to evaluate the accuracy and reproducibility of the robot. Sample concentration normalization, which can be automated quite readily, was not included as part of this study this process.

Temperature Optimization

Denaturation and de-N-glycosylation temperatures were systematically analyzed for the most robust N-glycan release ranges:

- Denaturation temperatures between 60°C and 90°C were tested

Gradient				
Time (min)	**Flow Rate (mL/min)**	**%A**	**%B**	**Curve**
0	0.4	25	75	6
35	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55	0.4	25	75	6

Column: ACQUITY UPLC Glycan BEH Amide, 130 Å, 1.7 µm, 2.1 x 150 mm
Column Temperature: 60°C
Sample Temperature: 10°C
Mobile Phase A: 50 nM Ammonium Formate @ pH 4.4 in LC-MS-grade Water
Mobile Phase B: 100% LC-MS-grade Acetonitrile
FLR Conditions: EX 265 nm, EM 425 nm, Sampling @ 2 Hz

RESULTS & DISCUSSION

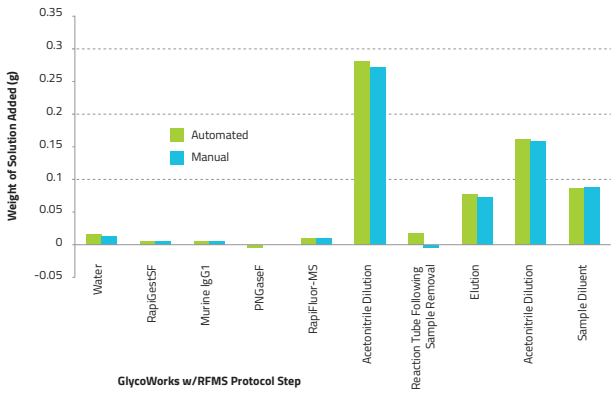


Figure 4: Reproducibility test comparing the robot's ability to perform the protocol versus a manual user performing the same proto-col side-by-side.

A main area of concern was the liquid transfer capability of the automated protocol versus the manual protocol **(Figure 4)**. Gravimetric results demonstrated that the automated protocol is comparable in performance to the manual protocol. The one deviation in procedure made was in the transfer of the fully labeled and quenched sample from the reaction tubes onto the SPE plate. A manual user can manipulate the reaction tube in order to ensure complete transfer of sample while the robot cannot. Thus, an additional step was added to the automated protocol to improve quantitative transfer where acetonitrile is added to the reaction tube to dilute the ~10 µL of remaining sample and subsequently added to the SPE plate.

For temperature optimization tests, each protein chosen provided unique characteristics and challenges for analysis **(Figure 5)**. RNase B contains high mannose structures, fetuin consists of highly sialylated glycans, cetuximab is a complex mAb with a unique N-glycan site on the Fab domain and Murine IgG1 is a control mAb with common N-glycosylation. For each individual protein, several labeled N-glycans were chosen to monitor their percent area counts in relation to one another **(Figure 5)**. Any change in these relative areas would indicate incomplete denaturation and/or de-glycosylation for that specific temperature test.

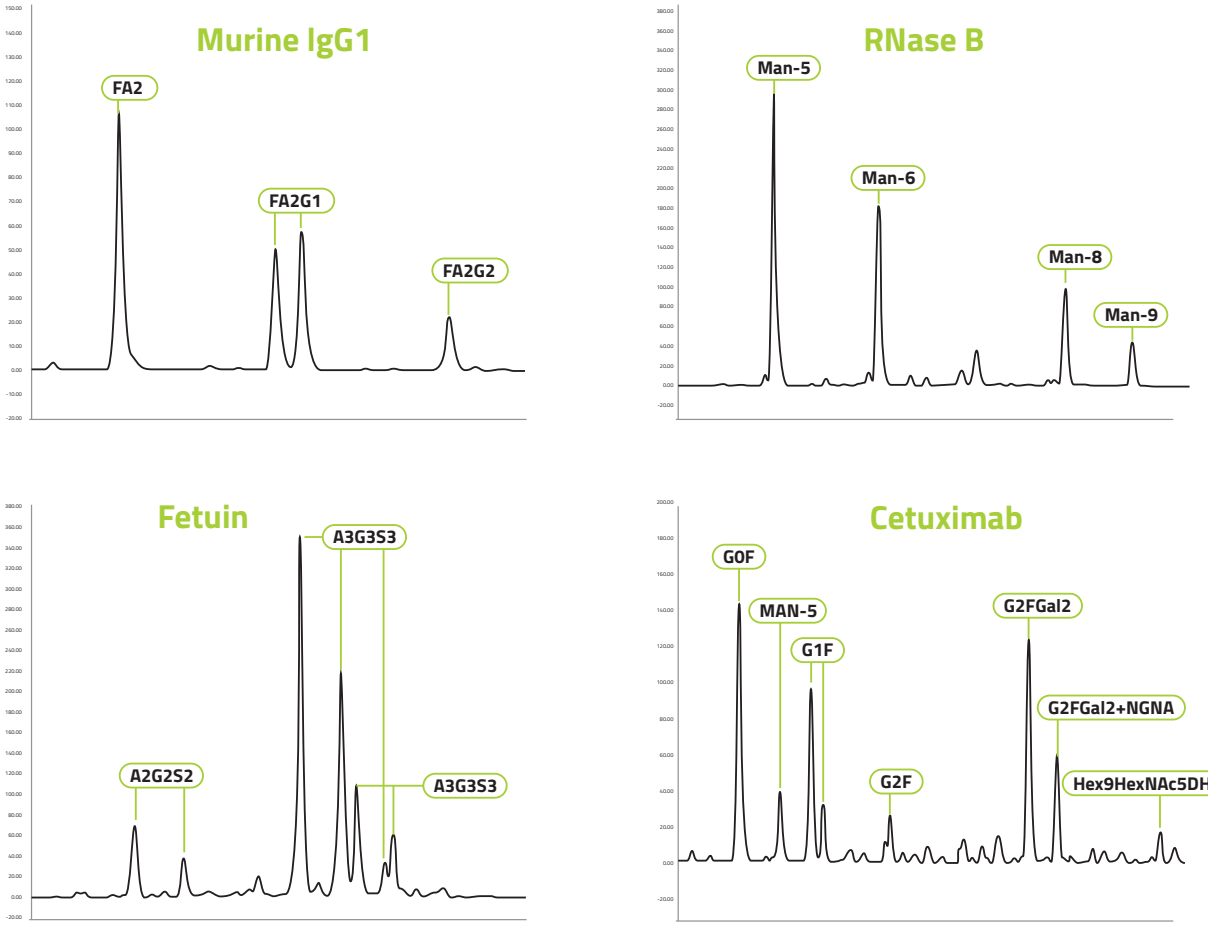
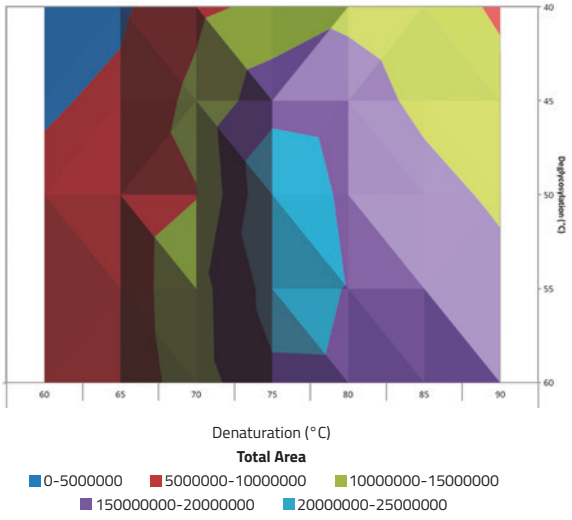


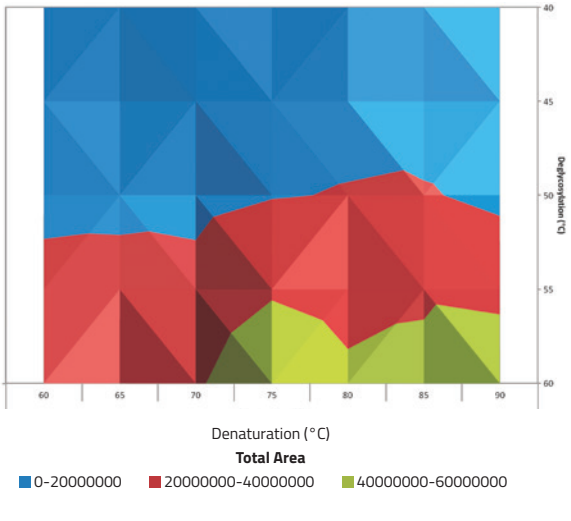
Figure 5: Representative chromatograms for each of the 4 mAbs tested with monitored N-glycans labeled.

Looking at response surfaces to visualize the highest total recovery of labeled N-glycans over all temperature tests **(Figure 6)**, the optimal temperature ranges for denaturation and de-glycosylation (PNGase F has an active temperature near 50°C[1]) are 75°C-80°C and 55°C-60°C respectively. These temperatures vary from the manual protocol due to the gradual temperature changes of the Peltier block. Another critical factor is to minimize the time between N-glycan release and labeling due to the spontaneous conversion of the N-glycosylamine to a reducing-end glycan under these conditions. Overall, the relative areas were very stable over most temperature tests indicating robust denaturation and de-glycosylation **(Figure 7.)** Deviation in all cases was low, indicating that once the optimal temperature ranges are found minor variations in temperature will not impact the quality of the results.

Optimal Denaturation and Deglycosylation Temperatures for Glycan Analysis of Murine IgG1 Using the Automated Protocol



Optimal Denaturation and Deglycosylation Temperatures for Glycan Analysis of RNase B Using the Automated Protocol



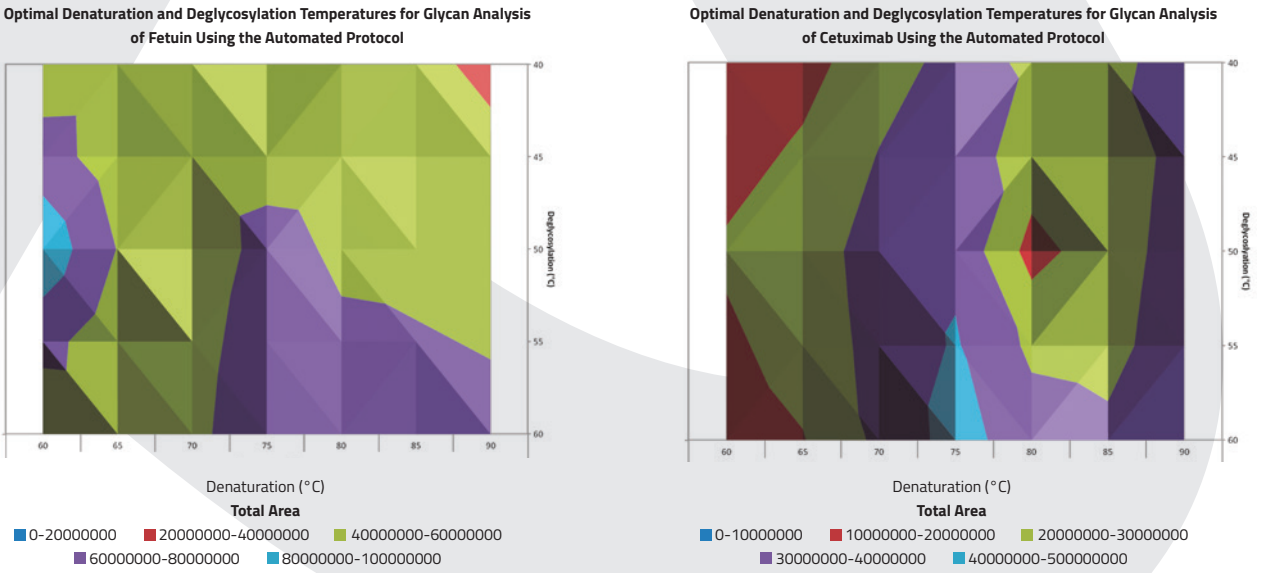


Figure 6: Surface plots showing the optimal denaturation and de-glycosylation temperatures for each mAb tested. The optimal range is from 75°C-80°C for denaturation and from 50°C-60°C for de-glycosylation.

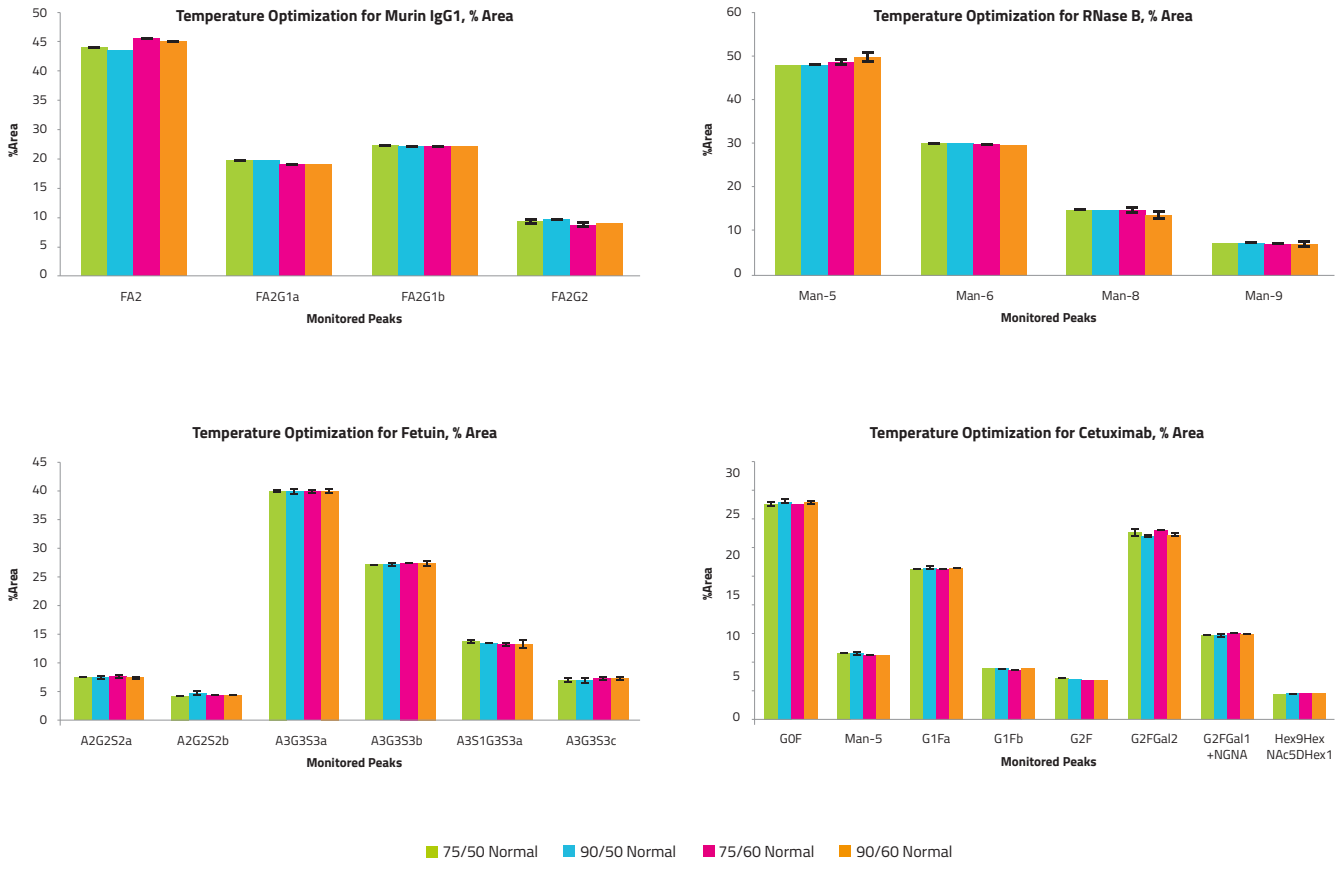


Figure 7: % areas of each glycan used to monitor relative area during tempera-ture optimization of the procedure. Legend is the format denaturation temperature (°C)/de-glycosylation temperature (°C).

CONCLUSION

- The Andrew Alliance automated pipetting robot can successfully and reproducibly perform the GlycoWorks with RapiFluor-MS N-glycan rapid labeling procedure
- Relative areas of released N-glycans remain constant at the optimal denaturation and de-glycosylation temperatures
- The optimal denaturation temperature range is from 75°C-80°C which takes between 20-24 minutes to heat and cool to room temp
- The optimal de-glycosylation temperature range is from 55°C-60°C which takes between 5-12 minutes to heat and cool to room temp
- Automating the protocol increases the preparation time approximately 2-fold but frees the analyst from tedious pipetting, effectively allowing them to perform two jobs at once

FUTURE DIRECTIONS

Adaptation and optimize of this automated protocol is ongoing with the intent to offer alternative version for QC laboratories, and further remove the need for user interventions throughout the protocol. Included in these improvements are:

1. A quality control protocol is in development and aims to eliminate small volume pipetting steps. This will reduce sample preparation time by reducing the number of times the robot needs to adjust the pipetting volumes during the protocol.
2. Automated vacuum pump control, allowing the robot to turn on the pump during SPE prep, sample cleanup and elution steps. Currently these steps require a user to be present.
3. For remaining steps that require user actions, email alerts will soon be available to notify the user that they are needed.

REFERENCES

[1] New England BioLabs.
www.neb.com/products/p0710-rapid-pngase-f#tabselect0. Accessed 3 June 2017

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