An Integrated Strategy for N-Glycan Sample Preparation and Analysis Suitable for All Stages of Therapeutic Protein Discovery, Characterization, Manufacture and Quality Release

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Abstract

The GlykoPrep™ N-Glycan Sample Preparation (GlykoPrep) Modules have been expanded to allow the conversion of most methods and fluorescent dye combinations for a number of analytical platforms for fast, automatable sample preparation. Waters has expanded its analytical capabilities for increased sensitivity, speed and resolution of glycans: Waters® UPLC®-HILIC/FLR/Q-Tof™ MS system enables high-resolution glycan separation using the ACQUITY® UPLC BEH Glycan 1.7 µm column (Glycan Separation Technology, GST Column), and produces sensitive FLR and MS chromatographic signals in a single analysis without splitting the flow. The combination of improved options for sample preparation with selected instruments and consumables reduces time to results for new screening applications such as discovery, clone selection and cell-culture optimization that have hardly been contemplated. In addition, such applications as bioreactor monitoring and control (PAT assays) are now within reach, as a small number of samples can return results within a single shift. Standard procedures have benefitted as well, due to highly robust and reproducible UPLC technology, and the improved sensitivity and resolution of the SYNAPT G2-S MS, which has the capability of detecting glycans, even minor ones, with high mass accuracy and sensitivity.

In this poster we outline an integrated strategy for N-Glycan sample preparation and analysis that yields reliable results with different fluorescent labels and methods, which can be related across diverse applications, from screening to in-depth characterization to quality release and stability testing, taking into account the associated requirements for throughput and time to results vs. data quality. Specifically, we compare results of N-glycan analysis of human IgG (hIgG) and Enbrel® (an Fc-fusion protein) obtained when using various GlykoPrep modules coupled with Waters analytical instruments and protocols.

Introduction

Figure 1 presents an overview of an integrated glycoanalysis workflow comprising rapid sample preparation in an automatable format coupled with UPLC/FLR/Q-Tof MS-based N-Glycan analysis, which includes: 1) an optional purification module to allow direct screening of monoclonal antibody (MAb) cell-culture samples; 2) quantitative deglycosylation and separation of N-Glycans; 3) complete N-Glycan labeling with AB (aminobenzamide)-dye variants and chemistries; 4) efficient N-Glycan sample cleanup and desalting to reduce excess reagent peaks; and 5) N-glycan profiling and optional MS confirmation of N-Glycan identity with a high degree of accuracy.

GlykoPrep dramatically streamlines sample preparation, from the optional MAb purification to N-Glycan release, labeling and cleanup. Using proprietary reagents, the optimized labeling reagents provide complete derivatization with only one-hour incubation time, without significant degradation of important labile groups, such as sialic acid and core or outer-arm fucose. After labeling, the remaining free dye is efficiently removed and the sample eluted in water, enabling the potential for mass spectrometry confirmation of atypical peaks. The entire sample preparation for a large number of samples can be completed in ~3 hours when labeling with InstantAB, and ~4 hours for 2-AB using Rapid-Reduction-Amination™. It is clear that sample preparation for N-Glycan profiling is no longer the limiting factor for N-glycoanalysis.

GlykoPrep incorporates AssayMAP® high-throughput microchromatography Cartridges, which have been specifically designed for liquid handling systems, and is capable of chromatographic separations, chemical reactions and enzymatic digestions on workstations modified to perform operations with AssayMAP consumables. Alternatively, process may be performed in a 96-well array format in a microplate centrifuge, or individually in centrifuge tubes in a microfuge, all with comparable results.

Rapid analysis methods are needed to match the GlykoPrep throughput and allow the analysis of hundreds of samples overnight for screening applications. In-depth characterization throughput was also improved by semi-automating N-Glycan assignments and quantitation using the UPLC-HILIC/FLR/QToF analytical system solution.

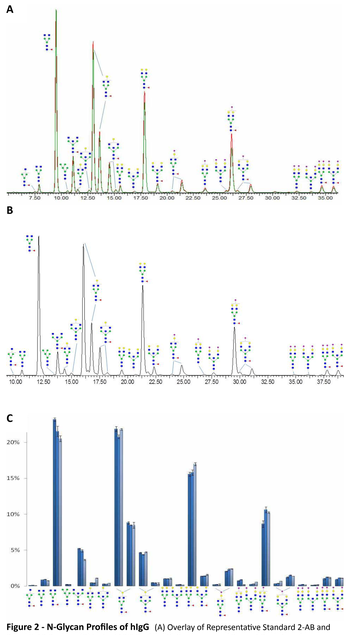

Figure 2 - N-Glycan Profiles of hIgG (A) Overlay of Representative Standard 2-AB and Rapid 2-AB Profiles; (B) InstantAB™ Profile; (C) Comparison of Percent Peak Area for Standard 2-AB, Rapid 2-AB and InstantAB

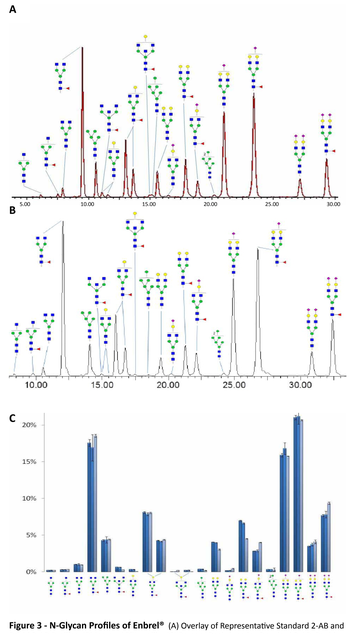

Figure 3 - N-Glycan Profiles of Enbrel® (A) Overlay of Representative Standard 2-AB and Rapid 2-AB Profiles; (B) InstantAB™ profile; (C) Percent Peak Area for Standard 2-AB, Rapid 2-AB and InstantAB

	Standard 2-AB		Rapid 2-AB		InstantAB™	
Peak Identity	% Avg	%CV	% Avg	%CV	% Avg	%CV
G0F-N	0.1	3.6	0.1	3.4	0.1	0.0
G0	0.9	1.0	0.9	3.4	0.7	2.1
G0F	23.2	1.0	21.5	3.3	20.5	1.0
Man5	0.2	7.3	0.2	14.3	0.0	0.0
G0FB	5.2	0.0	4.9	3.2	3.7	2.0
G1	0.5	2.9	0.4	3.1	1.1	1.9
G1B	0.3	2.0	0.3	1.2	0.4	1.4
G1F	21.9	1.6	20.0	1.3	21.0	0.4
G1FB	1.0	1.7	0.5	0.9	0.5	5.1
G1F1	4.6	1.7	4.4	0.7	4.7	1.4
G1FB1	0.5	0.9	0.4	15.1	0.3	1.9
G2	1.0	2.3	1.0	4.1	0.9	1.0
G2B	0.2	4.0	0.2	6.1	0.2	3.0
G2F	15.6	1.0	15.0	2.5	17.0	1.1
G2FB	1.4	2.5	1.4	2.4	1.6	4.7
G1F1S1	0.2	3.0	0.2	6.9	0.3	2.3
G1F1S1+G1FB1S1	2.1	1.7	2.3	4.6	2.4	0.6
A1	0.7	5.9	0.9	5.1	0.2	0.0
A2B	0.2	6.1	0.3	4.1	0.6	1.0
A1F	1.6	5.4	10.6	3.9	10.2	1.1
A1FB	0.3	3.0	0.4	3.3	0.7	2.2
A2FB	1.2	6.1	1.5	3.1	1.4	0.0
A2	0.2	6.0	0.2	3.7	0.2	3.7
A2B	0.1	7.3	0.1	4.3	0.2	2.7
A2F	1.0	0.9	1.3	3.7	1.2	4.5
A2FB	0.9	0.6	1.1	3.9	1.1	1.0

Table 2 - hIgG N-Glycan Profile Results (see Figure 2)

	Standard 2-AB		Rapid 2-AB		InstantAB™	
Peak Identity	% Avg	%CV	% Avg	%CV	%Avg	%CV
G0-N	0.2	3.2	0.2	19.4	0.2	2.7
G0F-N	0.3	3.1	0.3	17.2	0.3	1.7
G0	1.0	2.5	1.0	14.1	0.9	1.2
G0F	17.6	2.8	16.9	10.5	18.5	0.8
Man5	4.3	2.8	4.4	10.1	4.4	1.1
G1/G0FB	0.7	1.7	0.6	1.9	0.3	2.0
G1	0.3	1.3	0.3	1.3	0.1	0.0
G1F	8.1	1.4	7.9	2.7	8.1	1.1
G1F'	4.3	1.2	4.2	1.7	4.4	1.0
G1FB	0.0	0.7	0.0	1.3	0.2	0.0
Man6	0.2	2.3	0.2	2.4	0.0	12.4
G2	0.4	1.1	0.4	2.6	0.2	13.2
G1S1	4.1	0.8	4.0	1.1	3.5	7.7
G2F	7.2	0.6	6.8	0.8	4.5	0.6
G1FS1	2.8	1.3	2.9	4.5	4.0	0.6
Man7	0.4	1.3	0.4	1.8	0.3	1.8
A1	15.9	1.5	16.0	4.8	15.7	0.1
A1F	21.0	1.6	21.2	5.1	20.7	0.6
A2	3.5	1.7	3.7	5.6	4.1	6.4
A2F	7.7	1.7	7.8	5.7	9.4	2.1

Table 3 - Enbrel® N-Glycan Profile Results (see Figure 3)

	Standard 2-AB		Rapid 2-AB		InstantAB™	
Peak Identity	% Avg	%CV	% Avg	%CV	%Avg	%CV
G0	1.2	10.1	1.1	8.8	0.7	4.5
G0F	22.7	2.3	20.4	5.3	20.4	3.6
G0FB	5.3	3.0	4.9	4.1	3.0	2.6
G1F	21.5	2.8	20.6	2.1	21.4	1.3
G1F'	9.1	2.7	8.6	1.8	9.6	1.2
G1FB	4.8	2.7	4.7	0.5	4.9	1.6
G2	1.3	6.3	1.4	4.1	1.1	2.1
G2F	15.7	2.1	16.2	4.6	17.3	1.9
G2FB	1.8	5.7	2.0	4.4	1.9	3.5
A1	2.5	5.3	2.7	4.9	2.4	3.3
A1F	9.9	5.7	11.4	4.6	10.6	4.5
A1FB	1.6	6.3	1.8	6.7	1.6	4.2
A2F	1.3	10.0	1.5	9.3	1.4	7.0
A2FB	1.3	11.1	1.4	8.5	1.3	6.1

Table 4 - Human IgG N-Glycan Relative Abundance and Reproducibility Using the 10-Minute Screening Method (see Figure 7)

	Standard 2-AB		Rapid 2-AB		InstantAB™	
Peak Identity	% Avg	%CV	% Avg	%CV	% Avg	%CV
G0	1.0	9.8	1.0	2.7	1.0	2.7
G0F	18.1	2.6	18.8	2.2	18.8	2.2
G1F	8.4	1.4	8.1	1.3	8.1	1.3
G1F'	4.4	3.3	4.5	1.2	4.5	1.2
G2	4.8	1.4	3.2	2.2	3.2	2.1
G2F	6.7	1.1	4.9	6.5	4.9	6.5
Man5	4.3	2.5	4.7	2.3	4.7	2.3
G1F51	3.2	1.9	3.8	3.2	3.8	3.2
A1	16.2	1.4	16.0	1.7	16.0	1.7
A1F	21.3	1.4	21.1	2.3	21.1	2.3
A2	3.5	2.0	4.1	1.6	4.1	1.6
A2F	8.0	2.0	9.5	2.3	9.5	2.3

Table 5 - Enbrel® N-Glycan Relative Abundance and Reproducibility Using the 10-Minute Screening Method (see Figure 8)

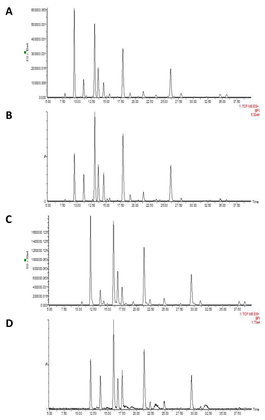

Figure 4 - FLR and MS Profiles of Rapid 2-AB and InstantAB™-labeled hIgG N-Glycans using the SYNAPT® G2-S Q-TOF MS

(A) Rapid 2-AB; (B) Rapid 2-AB Base Peak Intensity (BPI); (C) InstantAB; (D) InstantAB BPI

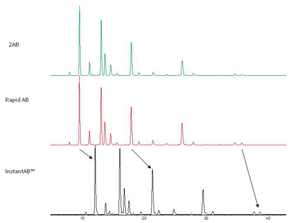

Figure 5 - Human IgG N-Glycan Profiles Using Standard 2-AB, Rapid 2-AB and InstantAB™ Labeling (FLR chromatogram) Internal Mobility Standards

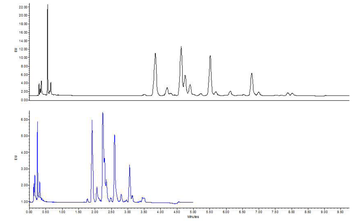

Figure 6 - Representative Profiles for 10- (above) **and 5-** (below) **minute Screening Methods (hIgG)**

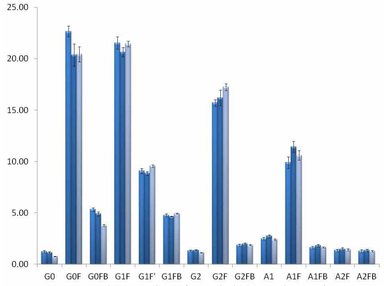

Figure 7 - Human IgG N-Glycan % Peak Area for Standard 2-AB, Rapid 2-AB and InstantAB™ Using the Rapid 10-Minute Method

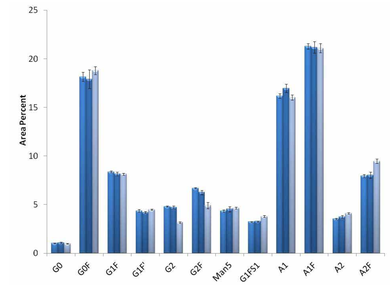

Figure 8 - Enbrel N-Glycan % Peak Area for Standard 2-AB, Rapid 2-AB and InstantAB Using the Rapid 10-Minute Method

Methods

Sample Preparation - Samples of hIgG and Enbrel were immobilized and digested with N-Glycanase® using the GlykoPrep GS96-RX Module (15- and 30-minute enzymatic digestion at 50°C). The resulting N-Glycans were labeled by one of three methods: 1) "Standard 2-AB" labeling using the Signal™ 2-AB Labeling Kit (product code GKK-404, 3 hours, 65°C); 2) "Rapid 2-AB" labeling using the GlykoPrep Rapid-Reductive-Amination™ 2-AB Labeling Module (product code GS96-AB, 1 hour, 52°C); or 3) "InstantAB" labeling using the GlykoPrep InstantAB™ Labeling Module (product code GS96-CU, ambient, within minutes). For 2-AB dye, the N-Glycans were dried before labeling; no drying is required for InstantAB. Excess dye, reagents and buffer salts were removed using the GlykoPrep Cleanup Module (product code GS96-CU). Labeling efficiency was estimated from MS results.

Analysis - All labeled N-Glycans from the two samples were analyzed by the Waters ACQUITY UPLC with 150-mm GST Column coupled to the fluorescence detector (FLR) interfaced directly with a Waters SYNAPT G2-S Mass Spectrometer (MS) operated using MassLynx™ 4.1 software. A 60-minute gradient was used to assure separation of Man5/G1 and Man6/G2. Total run time for UPLC/FLR/MS was ~1 hour/sample, or 20 samples overnight. For simple IgGs, run time maybe reduced to ~30 minutes/sample, or 40 samples overnight; additional reduction in run time or increase in throughput can be achieved with shorter, 50- or 100-mm columns.

Data reporting was semi-automated as MassLynx performs peak integration and assignment for the FLR and MS data channels. Results were pasted into Excel to calculate relative concentration and standard deviations. N-Glycans were identified once for each sample/label combination by looking at the m/z value in the raw data file. For overlapping peaks, the mass spectrum data revealed multiple ions detected, so they could be partially assigned. Reference standards may also be used to identify N-Glycans. Data was generated for 18 samples (hIgG and Enbrel: unlabeled N-Glycans, Standard 2-AB, Rapid 2-AB and InstantAB x 3 replicates of each) in ~2 hours.

All labeled N-Glycans from the two samples were also analyzed by rapid methods developed specifically for screening using the Waters ACQUITY UPLC with a 50-mm GST Column and Empower™ software. Run times of 5 and 10 minutes are demonstrated with the potential to prepare and analyze 100 - 200 samples overnight. Once appropriate N-Glycan calibration tables were established, analysis time was also reduced by automating data processing and reporting. Data was exported to Excel for rapid off-line analysis of results.

Results and Discussion

Samples - The N-Glycans for hIgG are a mixture of neutral, mono- and di-sialylated biantennary oligosaccharides, some containing bisecting GlcNAc and/or core fucose; sialylated N-Glycans and high-mannose N-Glycans were observed as minor species. Enbrel (etanercept) is a fusion protein composed of the Fc domain of IgG1 and the p75 tumor necrosis factor receptor (TNFR), and contains the hIgG CH2 and CH3 domains along with the hinge region, so that typical biantennary Fc N-glycans are present as well as highly sialylated biantennary N-Glycans from the TNFR region. Trace amounts of high mannose N-Glycans were also detected. Table 1 shows the N-Glycan notations used here and their associated structures.

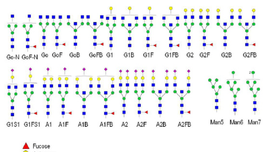

Table 1 - N-Glycan Notations and Associated Structures

Standard 2-AB, Rapid 2-AB, and InstantAB methods label the same hIgG N-Glycans. A representative chromatogram of hIgG N-Glycans labeled with Standard 2-AB and Rapid 2-AB are overlaid without normalization (Figure 2A), demonstrating that Rapid 2-AB labeling gives the same hIgG N-Glycan profile as the Standard 2-AB method; the fluorescence intensity was also equivalent. Figure 2B shows the same peaks in a chromatogram of hIgG N-Glycans labeled with InstantAB. The percent peak areas for hIgG N-Glycans are comparable but not entirely the same value (Figure 2C). The fluorescence intensity of the InstantAB chromatogram was 4x higher than either Standard 2-AB or Rapid 2-AB.

Standard 2-AB, Rapid 2-AB and InstantAB methods label the same Enbrel N-Glycans. A representative chromatogram of Enbrel N-Glycans labeled using the Standard 2-AB and Rapid 2-AB methods were overlaid without normalization (Figure 3A), demonstrating that Rapid 2-AB labeling gives the same Enbrel N-Glycan profile as the Standard 2-AB method; the fluorescence intensity was also equivalent. Figure 3B shows the same peaks in a chromatogram of Enbrel N-Glycans labeled with InstantAB. The percent peak areas for Enbrel N-Glycans are comparable but not entirely the same value (Figure 3C). The fluorescence intensity of the InstantAB chromatogram was 4x higher than either Standard 2-Ab or Rapid 2-AB. Table 6 shows the consistency of total fluorescence among the replicates for both samples.

	Standard 2-AB		Rapid 2-AB		InstantAB™	
Protein	Total area	%CV	Total area	%CV	Total area	%CV
hIgG	9.5	8	11.2	11	10.7	13
Enbrel	26.4	9.6	24.8	6.1	26.9	13

Table 6 - N-Glycan Total Peak Area Comparison for hIgG and Enbrel® with Standard 2-AB, Rapid 2-AB and InstantAB™ Using the Rapid 10-Minute Method (x 10,000)

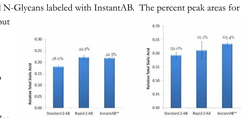

Figure 9 - Relative Total Sialic Acid of N-Glycans Labeled With Standard 2-AB, Rapid 2-AB and InstantAB™

Peak areas of mono-sialylated N-Glycans were summed; S2 N-Glycans were multiplied by 2 and added (A) hIgG (B) Enbrel®)

Comparison of UPLC and MS results. The identity of the peaks for each labeled N-Glycan was assigned based on the accurate masses measured using the Q-ToF MS detector. Figure 4 shows representative FLR and couple MS traces for hIgG N-Glycans with Rapid 2-AB (Figure 4A and B) and InstantAB (Figure 4C and D). Results for Standard 2-AB were substantially similar to Rapid 2-AB (data not shown).

The relative peak area for each N-Glycan is different in the FLR vs. MS results, probably due to the differing ionization efficiencies of the individual glycans. However, elution order is the same for all three labels, and no major extra peaks were noted. In an average of 3 replicates, the FLR signal for Rapid 2-AB (and Standard 2-AB) was ~4 times less than for InstantAB, and the MS signal was ~2.5 times higher than InstantAB (i.e. InstantAB has ~4 times more FLR signal and ~2.5 times less MS signal than for either 2-AB dye).

InstantAB shows broad peaks in the MS results between 22.5 and 25 minutes, which correspond to unlabeled N-Glycans. Labeling efficiency can be estimated here by comparing to the relative total ion counts from an unlabeled sample (data not shown). In this run, the labeling efficiency for an Enbrel sample was 83%.

Chromatographic shift in InstantAB vs. 2-AB. Figure 5 shows the FLR chromatograms for Standard 2-AB, Rapid 2-AB and InstantAB in a single stack, illustrating the retention time shift of InstantAB *vs.* Standard 2-AB and Rapid 2-AB while maintaining the general peak profile. Note that the retention time shift is not linear across the run (note slope of the arrows). Precise N-Glycan identification for the different labeled N-Glycans may be determined using appropriate standards (either 2-AB or InstantAB-labeled standards) to allow comparison of results across applications.

Rapid Screening Methods. Rapid UPLC/FLR methods were developed to match the throughput of the GlykoPrep Kit. Figure 6 shows a comparison of the 10- and 5-minute methods for hIgG with InstantAB. The 10-minute method is optimized for biantennary N-Glycans, while the 5-minute method can also accommodate triantennary N-Glycans. With some peaks, the shift from the 10- to the 5-minute method causes merging of the peaks, which may be acceptable where time to results is critical.

Figures 7 and 8 show a comparison of the relative abundance of labeled N-Gyceans analyzed using the Rapid 10-minute Method for hIgG and Enbrel, each 6 replicates. Tables 4 and 5 present the results in numerical format. It is clear from the reproducibility of the results (most CVs <5%) that a single run is sufficient during a screening campaign. However, some low-abundance N-Glycans (<1%) do not show up consistently in the 10-minute method compared to longer methods, such as G0F-N, Man5, G1, G1B, G2B, A2 and A2B for hIgG, and G0F-N, G0-N and Man7 for Enbrel. These are often below the level of detection rather than merged with other peaks, due to smaller sample injection amounts.

In addition to reproducible qualitative results, GlykoPrep/UPLC Rapid Methods produced quantitative results with sufficient reproducibility to be useful for screening; Table 6 shows total fluorescence intensity for 6 replicates. For such screening applications as clone selection or cell-culture optimization, this single metric can be used as an initial selection criteria, as all results for candidates significantly below target can be discarded. A smaller set of top candidates could then be analyzed for preferred N-Glycan species by such qualitative means as relative glycan abundance. Selected candidates can move to secondary screening using in-depth characterization methods shown herein.

InstantAB labeling is a gentler chemistry than reductive amination, and has been noted to preserve sialic acid levels. Degradation of sialic acid due to labeling was estimated by adding the relative peak areas of sialylated species multiplied by the number of equivalents of sialic acid. Figure 9A and B show the values obtained for the various labels for hIgG and Enbrel. For Enbrel, the differences are significant between InstantAB vs. Standard 2-AB, where N-Glycans are labeled at a significantly higher temperature (ambient vs. 65°C). The variability among the Rapid 2-AB replicates precludes a conclusion at this time. For hIgG, the relative amount of sialic acid is only ~30%, and the trend holds between InstantAB and Standard 2-AB; Rapid 2-AB looks equivalent in this instance. These results underscore the value of InstantAB in screening applications where accurate sialic acid quantification is important.

Depending on the application, an optimal balance exists among acceptable throughput, time to results and data quality. We present here an integrated approach to allow the user to choose, and still relate their results reliably across methods.

Conclusions

1. Signal 2-AB Labeling (Standard 2-AB) and GlykoPrep Rapid-Reductive-Amination 2-AB Labeling (Rapid 2-AB) yield comparable N-Glycan profiles with similar fluorescence intensity. Rapid 2-AB on the GlykoPrep Platform can replace manual methods and streamline work flows.
2. GlykoPrep InstantAB Labeling of N-Glycans gives the same elution order and relative concentration as Standard 2-AB and Rapid 2-AB, allowing InstantAB results to be easily related to historical results. InstantAB labeling in GlykoPrep N-Glycan Sample Preparation delivers the fastest time to results.
3. InstantAB's 4 times higher fluorescence intensity and 2.5x times lower ionization efficiency in positive ion mode increases its suitability for LC-only analysis, and reduces its applicability to coupled with MS via ESI, except for confirmatory purposes. In addition, labeling efficiency of >80% confirms its usefulness for N-glycoanalysis where time to results is critical.
4. InstantAB labeling's gentler conditions retain sialylated N-Glycans, of particular interest in screening applications.
5. GlykoPrep N-Glycan Sample Preparation with either Rapid 2-AB or InstantAB, followed by analysis using Waters instruments and consumables in different configurations, expands options for users to choose their desired throughput, data quality and time to results based on the specific application.

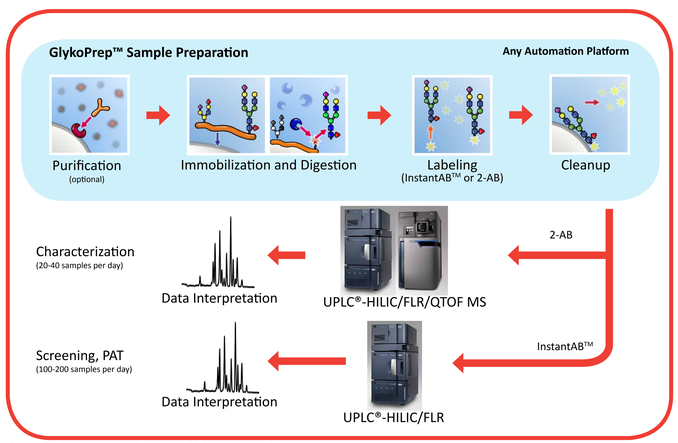

Figure 1: Overview of an Integrated N-Glycoanalysis Workflow

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