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# Screening for a Biosimilar N-Glycan Profile by UHPLC

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## ABSTRACT

Since ensuring proper N-glycosylation of a biosimilar drug may be critical to proving its biosimilarity, a high-throughput, sensitive assay with fast turnaround was needed to quickly screen N-glycan profiles in order to rank candidates to advance to the next stage of development. ProZyme's N-Glycan Sample Preparation (GlykoPrep®) platform, coupled to fast analytical methods, was chosen to screen candidates to produce sensitive, accurate and precise results to assure a match with the N-glycan profile of the innovator molecule.

We present an overview for screening from cell line selection of a production cell line through cell-culture optimization. Results are shown for a series of cell-culture runs that targeted the desired N-glycan profile for a monoclonal antibody under various cell-culture conditions over multiple days, from shake flasks to small-scale bioreactors. The results revealed significant differences in N-glycosylation among the various conditions and over the growth time course. Using this approach, hundreds of samples per day may be quickly and reproducibly evaluated to move projects forward to completion.

## INTRODUCTION

Screening is a process of attrition where a large pool of candidates are systematically evaluated in a series of cell-culture steps, the candidates ranked, and the better candidates advanced to the next step. The goal is not to select the perfect candidate during any one screen, but to reduce the number of unacceptable candidates. For example, those with productivity too low to be economically viable or allow efficient process scale up; those that make misfolded protein; those without suitable post-translational modifications; or any other relevant criterion that can be easily and quickly evaluated in a screening environment.

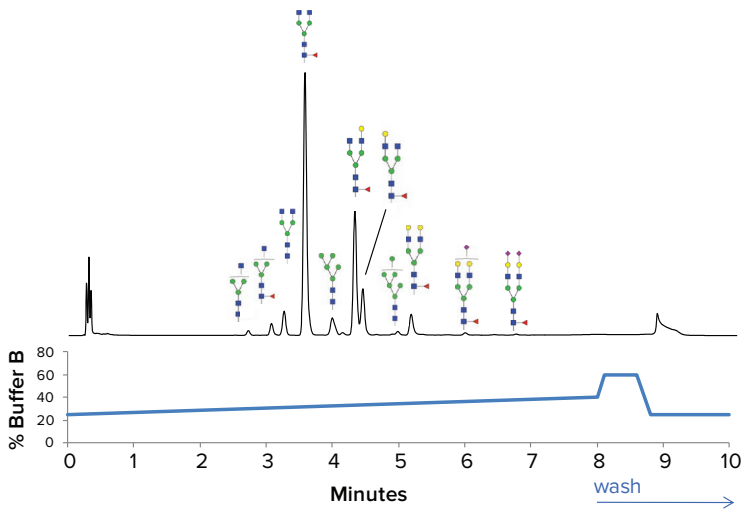



**Figure 1:** Standard N-Glycan Chromatogram. Resolution for the major biantennary N-glycan species obtained using the Rapid 10-Minute Method.

## METHODS

### N-Glycan Sample Preparation.

Up to 50 µg of protein (up to 200 µL in a single addition) was purified from each cell-culture sample using the AssayMAP Protein A (PA50) Purification Module (P50030 Kit); titer as A280 was measured on the constant volume (50 µL) of eluant of the PA50 Cartridges.

N-glycans were cleaved, labeled and excess labeling reagents removed, using GlykoPrep Rapid N-Glycan Sample Preparation with InstantAB™ (product code GK96NG-LB, includes GlykoPrep! Digestion Module with a 30-minute deglycosylation time, followed by labeling using the InstantAB™ Labeling Module and the Cleanup Module to! remove excess labeling reagents). The eluant of! the CU Cartridge contains fluorescently labeled N-glycans in 50 µL of water.

### UHPLC Methods.

Separations were performed on an Amide column (2.1 x 100 mm, 1.7 µm) using a rapid 10-minute method. A 5-minute method is also available for the earlier screening assays. Column temperature was set at 60°C, and injection volume was 1 µL of InstantAB-labeled

N-glycans (aqueous sample as eluted from the! CU Cartridge).

Figure 1 shows the N-glycan profile of a monoclonal antibody using the rapid 10-minute method.

### Exo-enzyme Treatments for Confirmation of N-Glycan Peak ID.

Exo-enzyme digests were performed to confirm the N-glycan peak identities and define the UHPLC Component Table to integrate and identify peaks in the chromatograms based on elution times. The UHPLC was able to automatically integrate and identify all peaks defined in the table.

### Shake-flask Results.

Each day during the run, each shake flasks was gently spun and the medium removed for analysis. Fresh medium was then added and growth continued.

**Bioreactor Results.**

Bioreactor results represent cumulative results over the course of the run.

**Titer.**

Titer is estimated by the A280 results after elution from the PA50 Cartridge, read on a absorbance plate reader. Titer was normalized across all of the rounds and is noted at various points on the graphs as the percent of the highest titer obtained.

**Total Fluorescence.**

Total fluorescence can be estimated after elution from the CU Cartridge, read on a fluorescence plate reader (280nm excitation, 330nm emission). Specific fluorescence can be calculated by dividing by the absorbance (A280) of the sample after purification.

The ability to rank the candidates according to selected criteria is the key, and is highly dependent on assays that are:

- high-throughput, so that lots of samples can be evaluated
- fast turnaround, because parking large numbers of samples waiting for results is inefficient
- sensitive, because only small amounts are available for testing
- linear with a wide range, so ranking is based on clear differentiation
- repeatable, to be able to discern the duds from the stars (much more important than reproducible, as generally one stage is not compared to another)
- robust, so that no real winners are lost in the process

Figure 2 shows an overview of the process to develop a production strain. The process starts with a strain capable of producing high levels of protein, but also capable of a wide variety of clonal variation for the characteristics of interest. This assures the ability to vary and control processing conditions to produce protein with the desired characteristics, in this case, a targeted N-glycan profile that matches that of the innovator molecule and its critical functional properties.

A large number of clonal cells expressing the gene of interest are produced and deposited in the wells of 96-well plates, with a dilution calculated to assure clonality in each well. The cells are grown for a week or more to allow colonies to grow up from a single cell. The assay emphasis in the early stages is on throughput and speed to results. Because the cells at the first stage are adherent and thus may not be as predictive of their performance under standard suspended growth, only growth and viability are assessed in the first screen. Titer is ranked across the wells, and empty wells or impaired growth are easily identified and excluded.

Very early in the process, N-glycans are also evaluated to assure the cells are able to make fully processed N-glycans (in antibodies, G0, G1 and G2 species). No real precision is required at this stage, only the ability to rank for content of fully processed species vs. high-mannose or hybrid species.

A predetermined number (usually based on the laboratory capacity at the next stage) of the highest-ranking candidates are advanced into tubes, where cells are grown for the first time in suspended culture, which is more predictive of their actual performance in real-world processes. The larger volumes produced here allow for evaluation of more complex criteria, such as aggregation and detailed N-glycan profiling. The number of candidates are continually reduced as the screening moves to shake flasks and eventually to small-scale bioreactors, where more assays, including functional assays are performed. Some cell-culture optimization is also employed before selection of the production cell line in order to assure the ability to meet the desired characteristics.

All results of screening and evaluation are detailed in development reports to support the submission package for regulatory approval. The information is summarized in Section 3 (Quality) of Common Technical Dossier (CTD).

Here we show the detailed results of the latter stages of cell-culture optimization for a monoclonal antibody. Two specific N-glycan species were chosen to illustrate the variability obtained when conditions were varied.

Significant clonal variation was found in the glycan distribution, with good response to process changes to further modify the distribution. Successive screens revealed responses to various conditions that were ultimately combined to produce a final process, whose characteristics met the functional requirements of the innovator molecule.

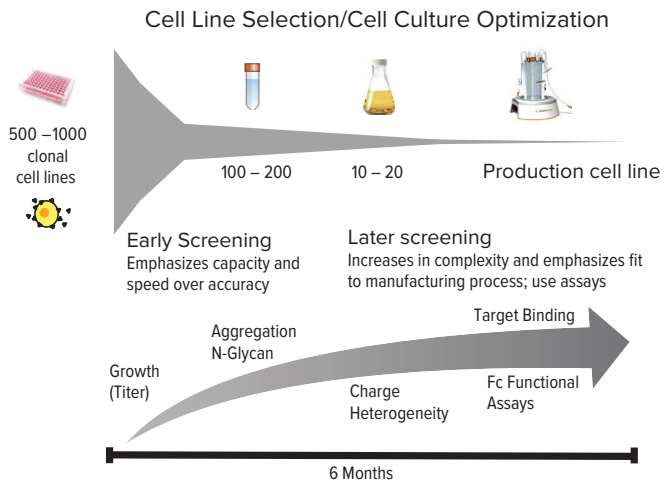



**Figure 2:** Screening Overview for Cell Line Selection and Cell Culture Optimization. *Courtesy of Mark Melville and Joe Siemiatkoski, Epirus Biopharmaceuticals, Inc*

## RESULTS AND DISCUSSION

### Sample Preparation

Figure 3 shows the sample preparation workflow using ProZyme's GlykoPrep™ N-Glycan Sample Preparation Platform, which streamlines processing of N-glycan samples. Since purification is part of the GlykoPrep workflow, estimates of titer (A280) are related from cell-culture to the N-glycan profile. Specific fluorescence was found to closely agree with the total fluorescence from the UHPLC (data not shown). Specific fluorescence can serve as an early indicator of total N-glycans in the samples, although it is a measure of total moles of N-glycans, and does not distinguish between fully pro-cessed glycans and high-mannose species.

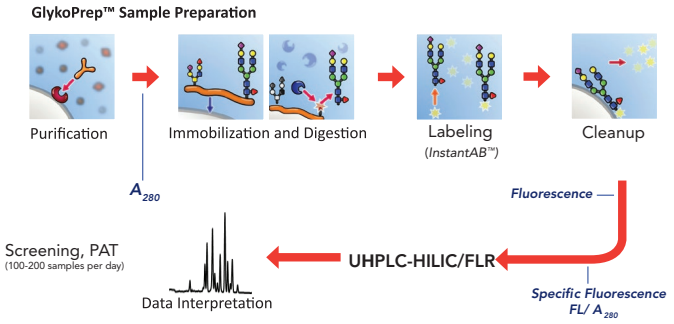



**Figure 3:** GlykoPrep Workflow.

### Reproducibility

Samples were initially run in duplicate, but minimal differences were observed, and replicates are not necessary for screening when using GlykoPrep.

### Analytical Methods

Rapid UHPLC methods were developed to match the throughput requirements of GlykoPrep, so that analysis does not lag behind sample preparation. A full plate can be analyzed overnight as 1 µL of aqueous sample is injected directly into the UHPLC every 10 min-utes (8-minute run, 2-minute wash).

Since GlykoPrep sample preparation yields a constant volume (50 µL) of IAB-labeled N-glycans for each sample, Figure 2 shows the signal and resolution for ~20 nanograms of N-glycans of a standard monoclonal antibody when starting from 50 µg of sample.

### Differences in N-Glycan Profiles, Round 1 (Shake Flasks)

N-Glycans were examined for cell cultures grown in two media (Medium A and Medium B) in order to select candidates to advance. Low titers had been an initial concern, and because Medium A gave rise to high productivity, it had been chosen as a likely base for manipulation of conditions going forward. However, the first screen revealed that several N-glycans came within the target range only in Medium B. Figure 4 shows two examples. Man5 falls into its target range beginning at around 7 days in culture, but only in Medium B. In Medium B, G2F is also within or just below its target range between 4 and 8 days in culture. Condition g (Medium B) was chosen to advance.

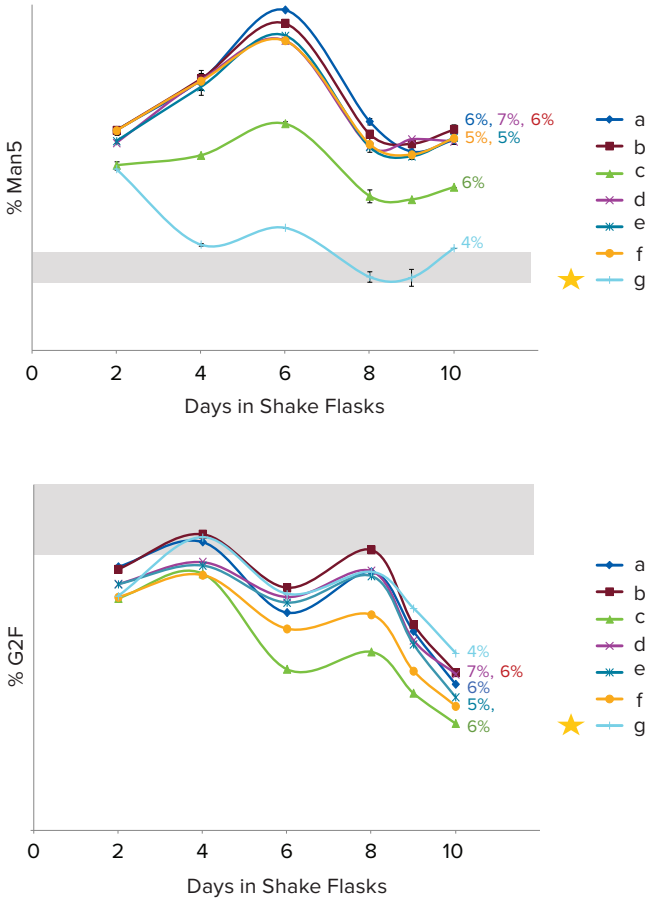


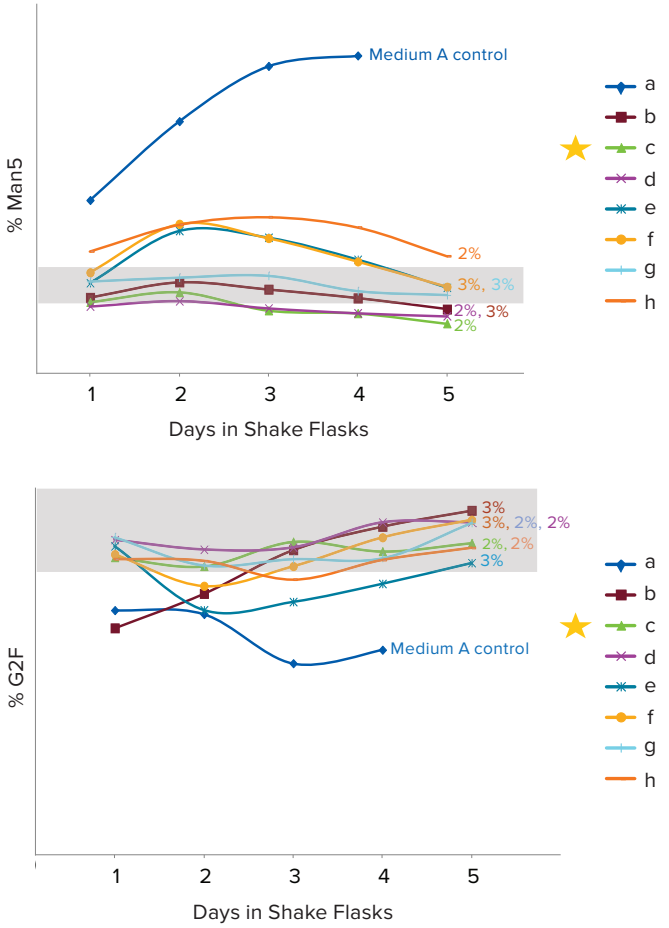

**Figure 4:** Round 1: Media, N-Glycans Man5 and G2F. Over a period of 10 days, candidates were produced in shake flasks under various cell-culture conditions in Medium A (a-f) or in Medium B (g). The target range for each N-Glycan is shown as a gray band; titer is normalized across all rounds and is noted as % at various points on the graph. Condition g (Medium B) was chosen to advance.

### Differences in N-Glycan Profiles, Round 2 (Shake Flasks)

Because Medium B gave the best results for N-glycan profile overall, it was chosen as the base medium for a second round of screening in shake flasks, varying additives to the medium over a period of 5 days. The results of this screen are shown for Glycans Man5 and G2F in Figure 5. Medium A (a) was run as a negative control. Additives to Medium B (b-h) did not have much impact on the levels of Man5 and G2F, but there were some differences in titer. With condition c additives, Man5 remains at or just below its target range while G2F remains within its target range. Condition c was chosen to advance.

### Differences in N-Glycan Profiles, Round 3 (2-L Bioreactors)

Additive c was chosen for a third round of screening, based on acceptable titer and a relatively constant N-glycan profile. In round 3, conditions that can be held constant in 2-L bioreactors were varied and Figure 6 shows the results. In Medium B with additive c, bioreactor condition a was chosen because it gave acceptable titer and fairly stable glycan levels: Man5 started off within its target range on day 7, although it crept up somewhat over the next 6 days in culture; G2F started off within its target range on day 7, fell beneath the target over the next day in culture, then leveled off. Condition a was chosen to advance.



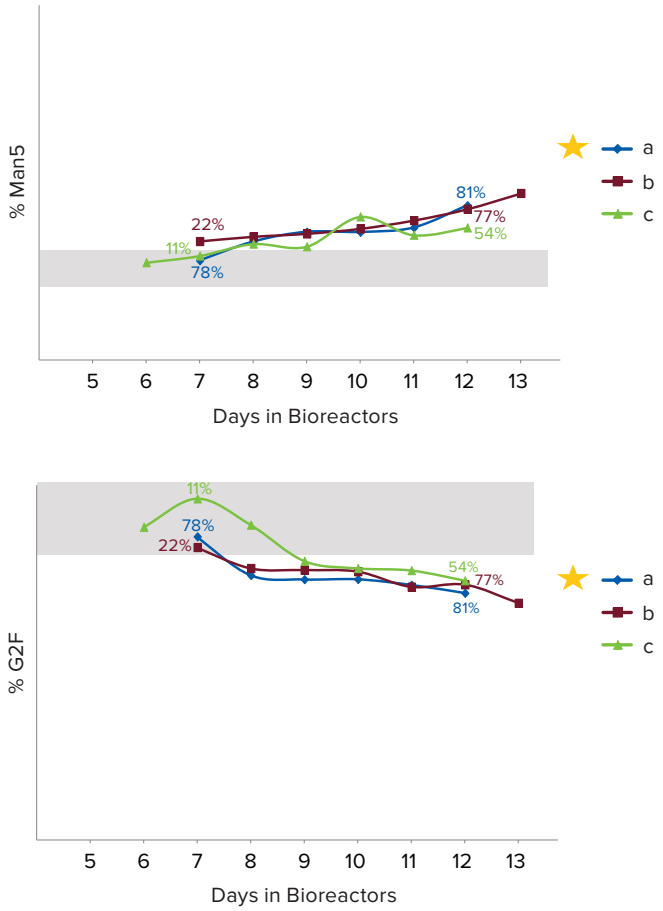




**Figure 5:** Round 2: Additives, N-Glycans Man5 and G2F. Over a period of 6 days, candidates were produced in shake flasks in Medium A (Control, a) or in Medium B with a variety of additives (b-h). The target range for each N-Glycan is shown as a gray band; titer is normalized across all rounds and is noted as % at various points on the graph. Additive c was chosen to advance.

**Figure 6:** Round 3: Bioreactor Conditions, N-Glycans Man5 and G2F. Between 7 and 13 days in culture, candidates were produced in 2-L bioreactors in Medium B with additive c, while varying conditions that can be held constant in a bioreactor (a, b and c). The target range for each N-Glycan is shown as a gray band; titer is normalized across all rounds and is noted as % at various points on the graph. Condition a was chosen to advance.

### Differences in N-Glycan Profiles, Round 4 (2-L Bioreactors)

Condition a from round 3 was chosen for a fourth round of screening, based on its relatively constant N-glycan profiles. In round 4, a variety of feeding regimes were explored to optimize titer. Figure 7 shows the results. Under feeding regime a, both Man5 and G2F stayed closest to their target ranges and remained fairly stable. Condition a was chosen to advance.

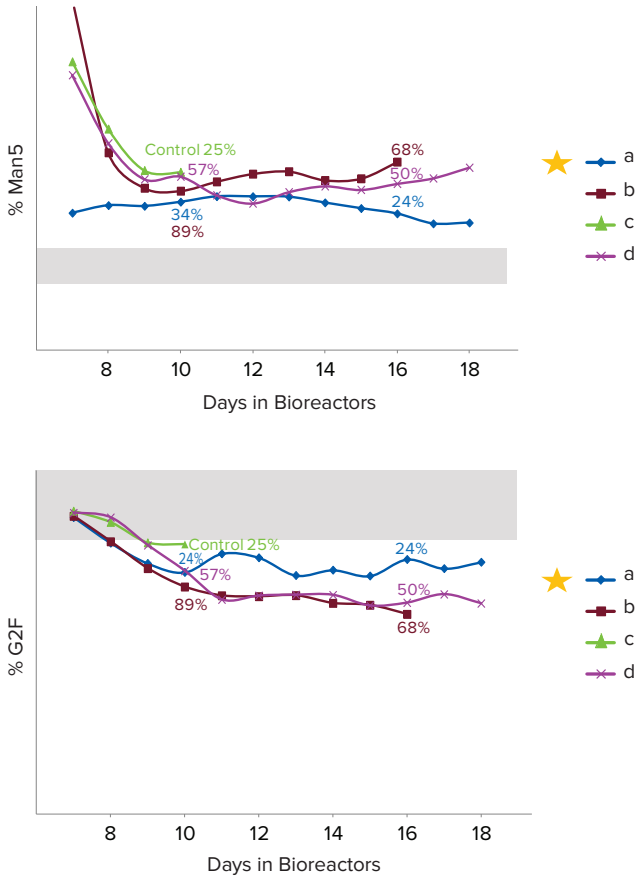



**Figure 7:** Round 4: Feeding Regimes, N-Glycans Man5 and G2F. Between 8 and 18 days in cell culture, candidates were produced in 2-L bioreactors in Medium B with additive c, under bioreactor condition a, while varying feeding regimes (a, b, c, d). The target range for each N-Glycan is shown as a gray band; titer is normalized across all rounds and is noted as % at various points on the graph. Feeding regime a was chosen to advance.

### Capabilities and Suitability as a Screening Assay

GlykoPrep paired with InstantDye chemistries (InstantAB or InstantPC®), can prepare up to 200 cell-culture samples in approximately 4 hours, ready for analysis. Since the workflow includes purification as the first step, a plate reader may be used to determine titer, so N-glycan analysis and titer be evaluated together. In early screening, comparing titre and specific fluorescence can help reduce the number of candidates to be analyzed by UHPLC.

GlykoPrep samples show a linear response from approximately 10 µg of sample up to the maximum loading of 50 µg, with sample concentrations as low as 0.05 mg/ml (up to 200 µL of sample in a single addition), although in some cases, minor species (<1% relative abundance) were not detected. Samples as low as 0.02 mg/ml have been processed successfully with additional handling (3 additions of 200 µL ea) with all species accounted for.

The ability to evaluate titre and N-glycan profiles together, throughout the development of a biosimilar monoclonal antibody, makes GlykoPrep a valuable assay in cell line selection and cell-culture optimization. To shorten time to result even further, sample prep can be preformed with Gly-X Rapid Release and Labeling with InstantPC or other dyes.

## CONCLUSIONS

1. GlykoPrep sample preparation, coupled with fast UHPLC methods, is able to meet the throughput, turnaround and sensitivity! requirements for cell line selection and cell-culture optimization.
2. Screening for N-glycan profiles, along with titer early in the! process, assures the selection of strains capable of delivering! targeted N-glycan profiles.
3. The N-glycan profile can change rapidly during cell culture,! suggesting the utility of bioreactor monitoring to better choose! time of harvest to match the N-glycan target range of the! innovator molecule.


### ACKNOWLEDGMENTS

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