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Targeting Desired N-Linked Glycosylation Profiles Through The Use Of Glycosylation Enhancing Feeds and High Throughput and High Resolution N-Glycan Analysis By Multi Capillary Electrophoresis

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ABSTRACT

Glycosylation is a key product quality attribute for many biotherapeutic proteins expressed in CHO cells. N-linked glycans may display macro- and micro-heterogeneity; the degree of this variation can depend on several factors, including cell line, media/feeds, and process. As a consequence, it has often been challenging to achieve and maintain preferred glycosylation profiles from cell culture development through bioreactor scale-up. In order to address these challenges, we have developed a new feed technology in conjunction with a unique fed-batch process that together has been shown not only to maximize protein titers but also to modulate glycan profiles.

Precise targeting of desired N-glycan profiles necessitated the need for an analysis method that is rapid, simple, high throughput and preserves the integrity of N-glycan structure.

Current N-glycan analysis methods consist of sample prep steps that are laborious and tedious. The labeling reaction typically requires vacuum drying of purified glycans and the use of toxic reducing chemicals such as sodium cyanoborohydride.

Here we report development of an integrated N-glycan analysis platform that can analyze 96 samples in 8hrs, consist of simple magnetic bead based sample prep, multi capillary CE instrument that can analyze 8 or 24 samples in parallel and glycan specific software with novel features. Our glycan analysis workflow in conjunction with unique fed-batch process can address the needs of biopharmaceutical companies interested in precise targeting of desired glycan profiles in their biomolecules.

INTRODUCTION

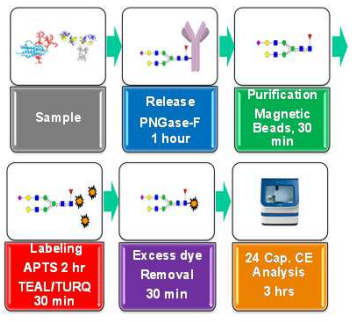
The glycosylation profile of a recombinant protein product is one of the most important attributes when defining product quality. Producing a protein with desired characteristics requires the ability to modify and target specific glycosylation profiles.

Using novel and proprietary technology, we have developed both a feed and a unique feeding process that will maximize growth and titer while being able to modulate glycan profiles that can be analyzed with a newly developed high throughput separation method to quantitate complex glycans.

This new feed can be added as a stand alone process that can result in a significant shift from G0F to G1F and G2F (maximum galactosylation). Using a unique fed-batch process, the feed can also be used with a standard feed to dial in a targeted glycosylation profile. Through process development testing, we have created a process where a transition point is used to switch from a standard feed to a glycan modulating feed. The timing of the transition point will determine the specificity of the glycan profile. A transition point early in culture will result in a greater shift from G0F to G1F and G2F. A transition midway or late in culture will result in a greater proportion of G0F compared to G1F and G2F.

Current glycan analysis methods involve laborious multistep sample preparation that involves vacuum drying of purified glycans and the use of toxic reducing chemicals such as sodium cyanoborohydride and takes anywhere from a day to multiple days for 96 samples, followed by single channel LC or CE separation. Here we report an integrated glycan solution that can generate data from 96 samples in 7-9 hrs.; consisting of an easy magnetic bead based sample prep, 24 capillary array CE instrument and a glycan specific software for analysis.

Figure 1. GlycanAssure™ glycan analysis workflow



Glycan analysis workflow – 3 hours of hands on time, 7-9 hours to process and analyze 96 samples with no vacuum centrifugation.

MATERIALS AND METHODS

All materials were from Thermo Fisher Scientific unless otherwise indicated

Cell culture: CHO DG44 derived recombinant cells expressing an IgG molecule were grown in CD CHO, CD OptiCHO™ and Dynamis™ media supplemented with 4mM L-glutamine and 1:100 Anti-Clumping Agent. Culture conditions were maintained at 37°C, 8.0% CO_2, 125 rpm. Cell densities and viabilities were measured using a Vi-CELL® counter (Beckman Coulter). Metabolites (glucose, ammonia, lactate) and IgG were measured using a Cedex® BioHT Analyzer (Roche).

Glycan Modulation Experiment 1: 250mL flasks with 60mL starting volume inoculated at $0.3x10^5$ viable cells/mL in CD CHO medium. 3X EfficientFeed™ B+ AGT™ Supplement (EFB+) and/or 3X GlycanTune™ B+ Total Feed (GTB+) were supplemented at 1.3% on days 4 through 15 (15% total). Glycan modulation conditions involved transitioning from EFB+ to GTB+ on culture days 4, 5, 7, 9, 11, 13 and 15. Glucose was supplemented as required to maintain a concentration above 3g/L.

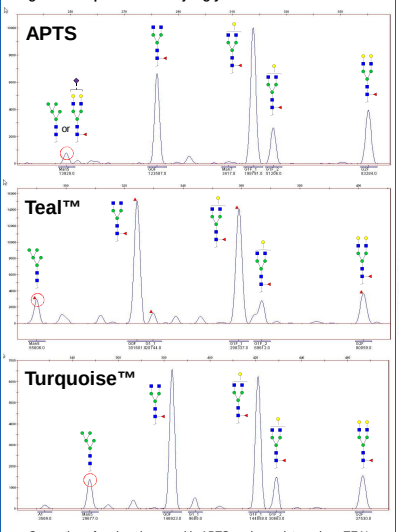
Glycan Modulation Experiment 2: 250mL flasks with 60mL starting volume inoculated at $0.3x10^5$ viable cells/mL in CD OptiCHO™ medium. 3X EfficientFeed™ A+ AGT™ Supplement (EFA+) and/or 3X GlycanTune™ A+ Total Feed (GTA+) were supplemented at 1.3% on days 4 through 15 (15% total). Glycan modulation conditions involved transitioning from EFA+ to GTA+ on culture days 4, 5, 7, 9, 11, 13 and 15. Glucose was supplemented as required to maintain a concentration above 3g/L.

Glycan Modulation Experiment 3: 250mL flasks with 60mL starting volume inoculated at $0.3x10^5$ viable cells/mL in Dynamis™ medium. 2X EfficientFeed™ C+ AGT™ Supplement (EFC+) and/or 2X GlycanTune™ C+ Total Feed (GTC+) were supplemented at 1.7% on days 4 through 15 (20% total). Glycan modulation conditions involved transitioning from EFC+ to GTC+ on culture days 4, 5, 7, 9, 11, 13 and 15. Glucose was supplemented as required to maintain a concentration above 3g/L.

Glycan Analysis: All CE separations were performed using the Applied Biosystems™ 3500xL, a system configured with a 505 nm solid state laser and laser induced fluorescence detection. 24 capillary arrays were used for separation of glycans. All other assay conditions were as described in the user guide for the Glycan Labeling and Analysis Kit (GlycanAssure user guide, Thermo Fisher Scientific, Publication Number MAN0014008).

Experimental details for this work were as follow:
- Glycan separation polymer used: POP7
- Anode Buffer (p/n 4393927); Cathode buffer (p/n 4408256)
- Capillary length: total length = 61 cm, length to detector = 50 cm
- Capillary diameter: 50 µm I.D.
- Injection conditions: 1.6 kV for 24 sec.
- Run Voltage: 19.5 kV
- Capillary oven temperature: 60° C
- APTS EX 475nm EM 501nm; TEAL™ Dye EX 466nm EM 505nm; TURQUOISE™ Dye EX 493nm EM 520nm

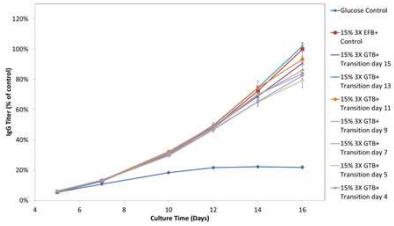
Figure 2. Separation of major glycans



Separation of major glycans with APTS and proprietary dyes TEAL and TURQUOISE. Glycans not resolved with conventional APTS (A1F/Man5) can be resolved with novel TEAL and TURQUOISE. TEAL and TURQUOISE confirm just the presence of the Man5 glycoform.

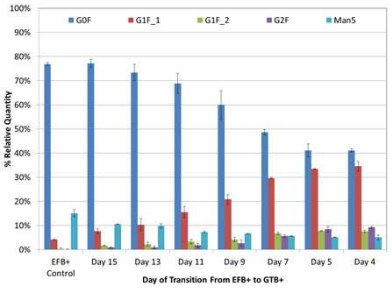
RESULTS

Figure 3. Glycan modulation experiment 1 titer data



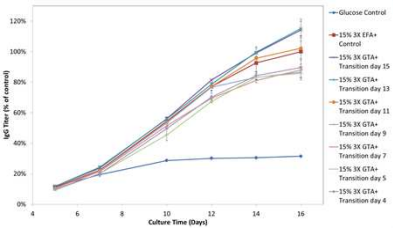
Titer comparison between feeding conditions. Titers were similar with both EFB+ and with the use of or when transitioning to GTB+. 100% titer is the titer of 15% 3X EFB+ on day 16. Titer results indicate that the use of and transition to GTB+ does not negatively affect protein production.

Figure 4. Glycan analysis from experiment 1



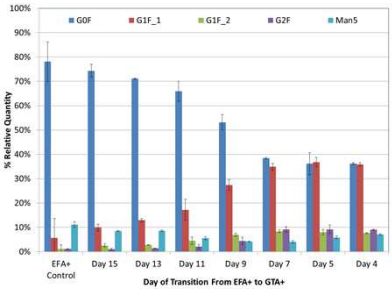
The timing of transition from EFB+ to GTB+ makes it possible to target specific glycosylation profiles. Modulating G0F from 77% down to 41%, while increasing G1F (1 and 2) and increasing G2F.

Figure 5. Glycan modulation experiment 2 titer data



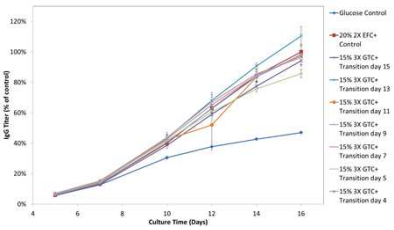
DG44 titer comparison between feeding conditions. Titers were similar with both EFA+ and with the use of or when transitioning to GTA+. 100% titer is the titer of 15% 3X EFA+ on day 16. Titer results indicate that the use of and transition to GTA+ does not negatively affect protein production.

Figure 6. Glycan analysis from experiment 2



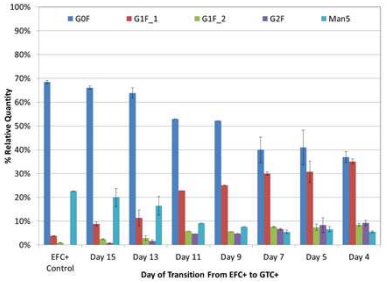
The timing of transition from EFA+ to GTA+ makes it possible to target specific glycosylation profiles. Modulating G0F from 78% down to 36%, while increasing G1F (1 and 2) and increasing G2F.

Figure 7. Glycan modulation experiment 3 titer data



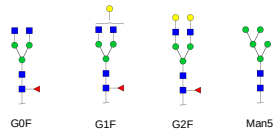
Titer comparison between feeding conditions. 100% titer is the titer of 15% 3X EFC+ on day 16. Titer results indicate that the use of and transition to GTC+ does not negatively affect protein production.

Figure 8. Glycan analysis from experiment 3



The timing of transition from EFC+ to GTC+ makes it possible to target specific glycosylation profiles. Modulating G0F from 68% down to 37%, while increasing G1F (1 and 2) and increasing G2F.

Figure 9. Major glycan structures



CONCLUSIONS

The incorporation of GlycanTune into a feed process as a stand alone feed was able to increase terminal galactosylation.

We have developed a unique process that makes it possible to target specific glycosylation profiles. Transition from standard feeding to GlycanTune allowed for precise targeting of glycan profiles. Transition to GlycanTune early in culture resulted in an increased shift from G0F to G1F and G2F. A transition late in culture resulted in increased G0F and decreased G1F and G2F.

GlycanTune can be highly concentrated to match EfficientFeed+ products and results in maximum growth and titers.

We have developed two novel fluorescent dyes to label glycans combined with a high throughput separation method to quantitate complex glycan species associated with therapeutic glycoproteins.

The new high throughput method offers a streamlined workflow. Sample preparation workflow that includes new dyes, software and automatable purification capable of parallel processing of 96 samples and analysis on Applies Biosystems 3500xL CE system in less than 9 hours.

TEAL and Turquoise fluorescent dyes offer better resolution of key glycans (Man5/A1F) with resolved baselines.

TRADEMARKS/LICENSING

ThermoFisher SCIENTIFIC