CHAPTER THIRTEEN

Creation of monoclonal antibody expressing CHO cell lines grown with sodium butyrate and characterization of resulting antibody glycosylation

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Contents

Methods in Enzymology, Volume 660
ISSN 0076-6879
https://doi.org/10.1016/bs.mie.2021.06.039

Abstract

Chinese hamster ovary (CHO) cells are the primary mammalian cell lines utilized to produce monoclonal antibodies (mAbs). The upsurge in biosimilar development and the proven health benefits of mAb treatments reinforces the need for innovative methods to generate robust CHO clones and enhance production, while maintaining desired product quality attributes. Among various product titer-enhancing approaches, the use of histone deacetylase inhibitors (HDACis) such as sodium butyrate (NaBu) has yielded promising results. The titer-enhancing effect of HDACi treatment has generally been observed in lower producer cell lines but those studies are typically done on individual clones. Here, we describe a cell line development (CLD) platform approach for creating clones with varying productivities. We then describe a method for selecting an optimal NaBu concentration to evaluate potential titer-enhancing capabilities in a fed-batch study. Finally, a method for purifying the mAb using protein A chromatography, followed by glycosylation analysis using mass spectrometry, is described. The proposed workflow can be applied for a robust CLD process optimization to generate robust clones, enhance product expression, and improve product quality attributes.

1. Introduction

Monoclonal antibodies (mAbs) have become an important tool in modern-day healthcare. In the last two decades, the approval of 79 mAbs by the US FDA highlights their growing importance as a therapeutic product class (Lu et al., 2020). Chinese hamster ovary (CHO) cells are the most widely used host cells for recombinant production of mAbs (Butler & Meneses-Acosta, 2012; Hong, Lakshmanan, Goudar, & Lee, 2018). The growing understanding of how various process variables and raw material attributes impact mAb productivity has led to multi-fold improvements in achievable titers in a typical CHO fed-batch process (Lee, Kildegaard, Lewis, & Lee, 2019).

Traditional cell line development (CLD) involves the isolation, cultivation, and amplification of high producing recombinant CHO cells using a dihydrofolate reductase (DHFR)-based methotrexate (MTX), or a glutamine synthetase (GS)-based methionine sulfoximine (MSX), selection system (De Leon Gatti et al., 2007; Doolan et al., 2013; Hong et al., 2018; Kim, Kim, & Lee, 2012; Rita Costa, Elisa Rodrigues, Henriques, Azeredo, & Oliveira, 2010; Walsh, 2018). While isolation and cultivation are critical steps in CLD, the screening of higher producing CHO cells is a time and labor-intensive process. Technological advancements such as efficient vector systems, media design, and automation in clone selection have facilitated streamlining the CLD process and achieving enhanced product

titers (Le et al., 2018; Nakamura & Omasa, 2015). Additional improvements in mAb productivity have been targeted using small-molecule additives such as histone deacetylase inhibitors (HDACis). Examples of commonly used HDACis are sodium butyrate (NaBu) or valproic acid (VPA). These molecules have the potential to aid in enhancing productivity by improving chromatin accessibility to the cellular transcription machinery. However, existing data from the literature show that the use of HDACis to enhance productivity is of variable success (Mimura et al., 2001; Ogawa et al., 2003; Yang et al., 2014). A possible reason for the observed inconsistencies is that the impact of the effect of HDCAis on productivity may depend on the productivity of the clone itself. For instance, multifold titer enhancements were observed upon HDACi treatment in CHO cell lines with baseline production ranges between 5 and 300 mg/L (Backliwal, 2008; Cherlet & Marc, 2000). In contrast, little to no improvement in product titers has been observed in CHO cell lines capable of titers closer to the g/L production range (Jiang & Sharfstein, 2008). However, there are no reports of the impact of HDACi treatment of sister clones, derived from the same transfected parent cell, covering a wide range of inherent productivities to study the relationship of HDACi-treatment and productivity.

While productivity is important in clone selection, another key requirement during the production of biotherapeutics is consistent product quality to ensure safety and efficacy. The N-linked glycosylation in mAbs is one such important product quality attribute that is influenced by process changes (Costa, Rodrigues, Henriques, Oliveira, & Azeredo, 2014). The mAb glycosylation can be present either in the crystallizable heavy chain fragment (Fc) or the antigen binding (Fab) arm, depending on the availability of consensus glycosylation sites. Fc glycan heterogeneity impacts the effector functions (Higel, Seidl, Sörgel, & Friess, 2016), and Fab glycosylation has been known to influence stability and half-lives of mAbs (van de Bovenkamp, Hafkenscheid, Rispens, & Rombouts, 2016). For instance, lack of core-fucosylation is strongly correlated with enhanced antibody-dependent cell cytotoxicity (ADCC) function, while the lack of galactose significantly lowers complement-dependent cytotoxicity (CDC) activity (Pereira, Chan, Lin, & Song, 2018; Peschke, Keller, Weber, Quast, & Lünemann, 2017). Similarly, high oligomannose structures (Man 9-Man 5 forms) result in poor pharmacokinetic profiles by reducing the circulation half-life of the mAb (Alessandri et al., 2012). High sialylation has been shown to enhance anti-inflammatory activity in intravenous immunoglobulin (IVIG) preparations (Schwab & Nimmerjahn, 2014). Overall, cell line

selection should be guided by the ability to produce mAbs with optimal core-fucosylation levels, galactosylation, and sialylation depending on the desired mechanism of action, while expressing minimal levels of oligomannose structures.

This chapter presents a rapid, simplified and automation-independent protocol for developing CHO cell lines with varying production capacities to express a model IgG1 mAb, by employing a high cell density, high plasmid dose and one-step amplification method. Additionally, we describe the methodology for identifying the optimal NaBu concentration for each of the developed CHO cell lines to test further titer-improvement prospects in a fed-batch culture. Finally, we also present the detailed protocol of protein-A purification of mAbs from harvest cell culture fluid (HCCF) followed by glycosylation analysis using mass spectrometry.

2. Background

2.1 Cell line development process

CLD is an important part of process development and results in the creation of specific mAb-producing cell lines (or clones). CLD is a multi-step process involving host cell line selection, transfection of the gene of interest (GOI), clonal isolation, selection, amplification, and finally, clonal expansion (Lee et al., 2019).

The most prevalent host cell for biopharmaceutical production is CHO cells. However, production host cell lines may be viewed as being inherently heterogeneous owing to distinct genotypic and/or phenotypic characteristics. Structural changes include genetic variations such as single nucleotide polymorphism (SNPs), mutations, and chromosomal rearrangements (Bandyopadhyay et al., 2019; Vcelar et al., 2018; Wurm & Hacker, 2011). The variability in specific growth rates, product glycosylation, and biosynthetic capacities are functional variations contributing to the observed clonal variations.

Once a host cell line is chosen, the next step in creating a recombinant cell line is the integration of the GOI into the host cell genome. Classically, the chromosomal integration site for a GOI is random leading to the incorporation of the transgene in diverse genomic regions of the host cell line. The random integration of the transgene can also result in copy number variations (CNVs) and differences in the transcriptional capacity leading to heterogeneities in the productivity and product quality across the cell populations (Porter, Racher, Preziosi, & Dickson, 2010).

Clonally-derived recombinant cell lines can also undergo significant changes in the expression levels of the transgene during clonal expansion. This tendency is attributed in part to the loss or recombination of transgenes. Moreover, epigenetic changes such as promotor methylation or histone acetylation can also contribute to clonal variations in productivity (Weinguny et al., 2020).

Recent developments to address these issues include targeted strategies for gene insertion guided by a knowledge-based selection of the ideal integration sites (Shin & Lee, 2020). Examples of such strategies include recombinase-mediated targeted integration and programmable nuclease-mediated targeted integration (Baumann et al., 2017). While these methods have successfully addressed some of the limitations of early CLD activities, the field is still far from the ability to completely predict and control clone phenotypes.

2.2 Glycosylation analysis using mass spectrometry

Glycosylation is an important product quality attribute that profoundly impacts the safety and efficacy of mAbs (Mimura et al., 2018). Existing evidence suggests that the choice of the host cell line and even the choice of clone during CLD significantly impacts mAb glycosylation profile (Goh & Ng, 2018). As a result, glycosylation is a significant consideration for the choice of the host cell line for the manufacture of recombinant therapeutics.

Mass spectrometry (MS) is the primary analytical tool for characterizing protein glycosylation (Dotz et al., 2015; Leymarie & Zaia, 2012). The general approach for characterizing the glycosylation of a recombinant protein begins with the release of the glycan from the protein *via* chemical or enzymatic methods (Grunow & Blanchard, 2019; Saldova & Wilkinson, 2020). The most commonly used enzyme for N-glycan release is PNGase F, which acts by cleaving the bond between the innermost N-acetylglucosamine (GlcNAc) and the asparagine side chain of the protein at the N-glycosylation site (Vilaj, Lauc, & Trbojević-Akmačić, 2020). In contrast, chemical release is usually carried out by alkaline elimination or hydrazinolysis methods and is more commonly employed to release O-linked glycans (Fukuda, 1995; Kozak et al., 2014). Site-specific glycan occupancy is assessed by releasing glycans with Endo-H which cleaves the glycosidic bond between the core GlcNAcs but preserves the first GlcNAc attached to the protein (Cao et al., 2018).

By virtue of their biochemical properties, glycans exhibit low ionization efficiency in a mass spectrometer compared to peptides and proteins (Zaia, 2010). Hence, the released glycans are derivatized at their reducing end reactive carbonyl group either by reductive amination or permethylation (Banazadeh, Veillon, Wooding, Zabet-moghaddam, & Mechref, 2017). Derivatization improves the ionization efficiency and, thereby, facilitates accurate identification using mass spectrometry. Also, the released glycans may be modified by adding a chromophore for improving the detection by high-performance liquid chromatography (HPLC) (Ruhaak et al., 2010).

The released and derivatized glycans are usually separated using HPLC before MS analysis. The commonly employed chromatographic modes include reversed-phase, hydrophilic interaction (HILIC), and porous graphitized carbon (PGC) chromatography (Qing, Yan, He, Li, & Liang, 2020; Vreeker & Wuhrer, 2017; Zhou et al., 2017). The choice of these techniques is dependent on the sample complexity, nature of derivatization, and desired separation selectivity (Gaunitz, Nagy, Pohl, & Novotny, 2017).

The MS analysis of the released glycans is accomplished either by Matrix-Assisted Laser Desorption Ionization (MALDI) or Electrospray Ionization (ESI) techniques (Grünwald-Gruber, Thader, Maresch, Dalik, & Altmann, 2017; Morelle, Faid, Chirat, & Michalski, 2009). These approaches enable glycan detection as a metal (sodium) adduct in the positive ion mode or as a deprotonated or anion-adducted species in the negative ion mode (Han & Costello, 2013). While detecting neutral glycans with MALDI is independent of the size of the molecule, the ionization efficiency using ESI decreases with an increase in the molecular weight (Wada et al., 2007). Additionally, while MALDI yields singly charged ions, ESI exhibits a multiply charged spectrum that requires deconvolution to produce a singly charged glycan spectrum (Zaia, 2010). The structural information derived from both of these techniques is limited as either of these approaches does not generate fragmentation data required for detailed structural elucidation of glycans. Advanced MS acquisition methods utilizing tandem mass spectrometry such as collision-induced dissociation (CID) or electron-dissociation methods have proven to be a powerful means to bridge this gap (Mechref, 2012; Yang et al., 2019; Zhu, Qiu, Gryniewicz-Ruzicka, Keire, & Ye, 2020).

Finally, various software tools and glycoinformatic database platforms include GlycoMod, GlyReSoft, UniCarbkB, UNIFI, simGlycan, Byonic, and GPQuest, have facilitated in streamlining glycan analysis and data interpretation (Abrahams et al., 2020).

3. Materials and equipment

1. CHO host cell line (MTX selection compatible)
2. CHO mAb encoding plasmid (MTX selection compatible; for example: Addgene plasmid #80684 with heavy chain and light chain sequences inserted)
3. Lonza Biosystems—Nucleofector II
4. Sterile 125 mL polycarbonate Erlenmeyer flasks
5. HyClone ActiPro media supplemented with 6 mM L-glutamine
6. ClonaCell-TCS medium
7. Sterilized 1 mM methotrexate (MTX) in dimethyl sulfoxide (DMSO)
8. Well plates (6, 24, 96)
9. Heracell VIOS 160i CO_2 humified incubator (37 °C, 5% CO_2, 135 rpm, 25 mm orbital diameter, 80% relative humidity)
10. Beckman Coulter Vi-CELL Cell Viability Analyzer
11. 15 and 50 mL centrifuge tubes
12. 1000, 200, 20 μL pipettes and sterile tips
13. −20 °C fridge and −80 °C freezer
14. Eppendorf Centrifuge 5804 R
15. Sterile water bath (37 °C)
16. Labgard Class II, Type A2 biological safety cabinet
17. Life technologies microscope EVOS XL
18. Drummond pipet aid (controller) XP
19. Falcon sterile serological pipet
20. 100, 500 mL beakers
21. Corning stirrer (stir plate) and stir bar
22. Ohaus Voyager Pro scientific weigh scale
23. Millex sterile filter unit
24. DB 5 mL sterile syringe
25. Sterile 10% DMSO in ActiPro media
26. NALGENE Cyro 1 °C freezing container
27. Thermo Scientific liquid nitrogen tank with monitor
28. VWR 1.5 mL microcentrifuge tubes
29. Lonza Nucleofector Solution
30. CHO cell lines
31. Sterile filtered 0.5 M stock solution of NaBu dissolved in Dulbecco's Phosphate Buffered Saline (DPBS)

32. Sterile glucose stock solution-45%
33. Cytiva Cell boost Feed 7A
34. Cytiva Cell boost Feed 7B
35. Octet RED96e biolayer interferometry (BLI) instrument with protein A sensors
36. 2950D Biochemistry Analyzer (YSI)
37. Clarified cell culture supernatant
38. Buffer A: 50 mM Phosphate, 150 mM NaCl, pH 7.5
39. Buffer B: 100 mM Glycine, pH 3.0
40. Wash Buffer: 50 mM Phosphate, 150 mM NaCl, pH 7.5
41. Clean-In-Place (CIP) Buffers: 2 M NaCl, 0.5 M NaOH
42. 1 M Tris-HCl buffer
43. Water +0.1% formic acid buffer
44. Amicon Ultra 4 mL 10 K cut-off filter unit
45. HiTrap Protein A HP Column (Cat.17040201, Cytiva); Column Specifications: Bed height: 25 mm; Bed Volume: 5 mL; Column I·D: 1.6 mm
46. UV-Spectrophotometer
47. GlycoWorks RapiFluor-MS N-Glycan Kit
48. 18.2 MΩ/cm^{-1} LC-MS grade water
49. LC-MS grade Acetonitrile
50. 15:85 (v/v) water/acetonitrile
51. 1:9:90 (v/v/v) formic acid/water/acetonitrile
52. 5% RapiGest (Cat. 186,001,861, Waters Corp)
53. Intact mAb mass check standard (Cat. 186,006,552, Waters Corp)
54. GlycoWorks RapiFluor-MS dextran calibration ladder
55. NIST Reference Material 8671 NISTmAb
56. 96-well plate extraction vacuum manifold
57. μ-HILIC elution plate (Cat No. 186002780, Waters Corp)
58. Vacuum pump
59. Vacuum manifold shims
60. Heat block/water bath
61. Centrifugal vacuum evaporator

4. Cell line development of mAb producing clones with varying productivities

Cell line development can be very time-consuming and laborious due to extensive screening and process scaling required to achieve desired

productivities and product quality. Here, we describe a traditional but reliable method for developing mAb producing CHO cell lines. The resulting pool of clones will have various productivities. This process takes approximately 3–4 months. A summary of the described CLD method is depicted in Fig. 1.

4.1 Transfection and expansion of single-cell colonies

1. Sanitize by wiping the work surface with 70% isopropanol. Turn on the airflow of the biological safety cabinet for the execution of cell culture work 15 min prior to the start
2. Thaw a vial of host cells from the −80 °C freezer, and use a pipette to transfer $\sim 10^7$ cells to a sterile 15 mL centrifuge tube (See Note 1).
3. Pipette 50 mL of ActiPro media supplemented with 6 mM L-glutamine into sterile 50 mL centrifuge tubes. Warm 50 mL centrifuge tube in a sterile water bath set at 37 °C for 3–5 min
4. Wash cell pellet with 5 mL of ActiPro media supplemented with 6 mM L-glutamine (add slowly using a pipette). Centrifuge cells at $120 \times g$ for 3 min, and use pipette to remove the supernatant from the cell pellet
5. Pipette 5 mL of fresh ActiPro media supplemented with 6 mM L-glutamine in a centrifuge tube containing cell pellet, and transfer all the contents to a sterile 125 mL shake flask. Finally, pipette 15 mL of additional ActiPro media supplemented with 6 mM L-glutamine to bring host cell culture to a seeding density of 0.4×10^6 cells/mL in the shake flask for incubation at 37 °C, 5% CO_2, 135 rpm, and 80% relative humidity (RH)
6. Maintaining a seeding density of 0.4×10^6 cells/mL for each passage, allow host cells to undergo 3 passages (approximately 3 days per passage), and measure viability and viable cell density (VCD) using ViCell to ensure at least 97% viability and representative cell-specific doubling time (See Note 2).
7. On day 3 of the 3rd passage, measure viability and VCD of host cell culture on ViCell. Using the cell count, pipette the appropriate culture volume to obtain $\sim 10^7$ host cells into a 1.5 mL microcentrifuge tube. Centrifuge at $120 \times g$ for 5 min, and then remove the supernatant using a pipette
8. Pre-warm Nucleofector Solution recommended by Lonza to room temperature. Resuspend the host cell pellet in room temperature

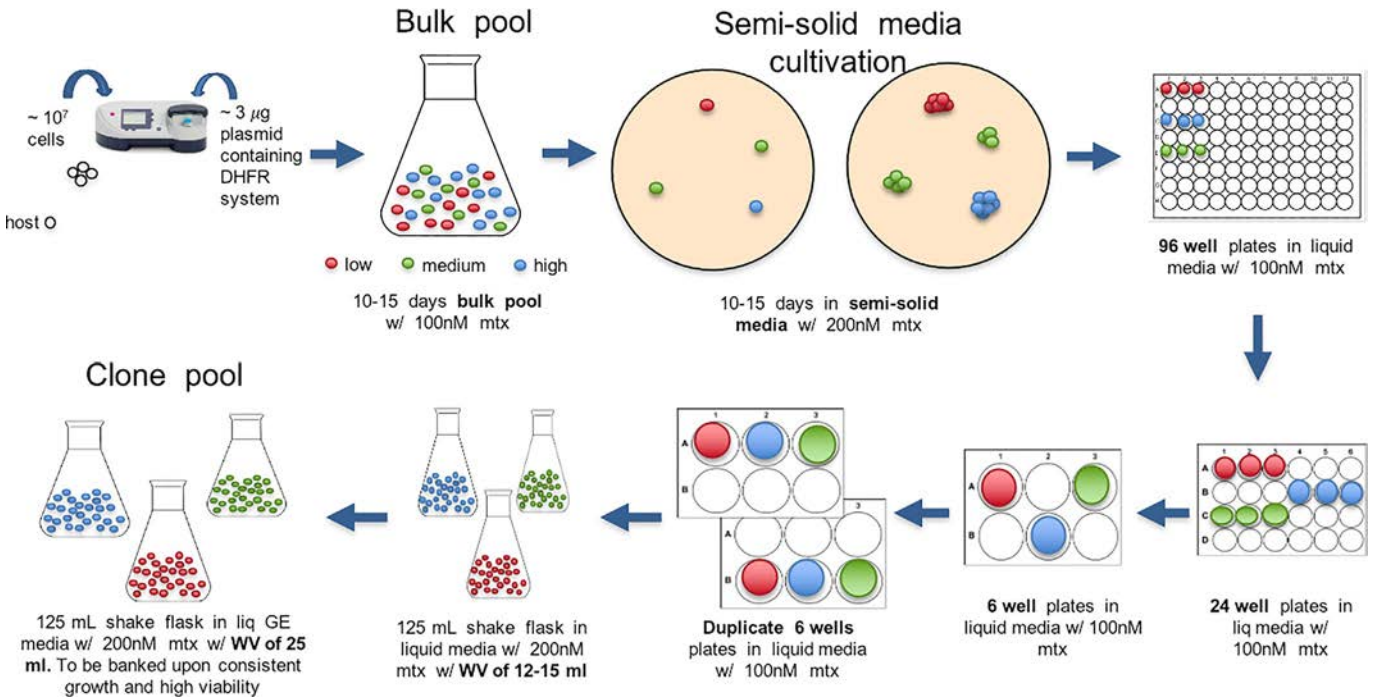

Fig. 1 Summarized workflow of cell line development used to obtain clones with varying productivities. Upon transfection of host cells with plasmid genomic material containing a DHFR sequence, a bulk pool is initiated with supplemented MTX. Next, individual cells are plated in semi-solid media before expanded colonies undergo a series of increasing-volume plate cultures in liquid media. The expanded cells are transferred to the shake flask and banked upon achieving consistent growth and high viability.

Nucleofector Solution to a final concentration of 1×10^7 cells/100 μL. Mix 100 μL of cell suspension (in Nucleofector solution) with 3 μg of mAb plasmid using a pipette

9. Transfer the sample into an Lonza certified cuvette using pipette, and cover with blue cap. Insert the cuvette into the cuvette holder of the Lonza—Nucleofector II, and select the appropriate Nucleofector program to start transfection according to the manufacturer's instructions (See Note 3).
10. Pipette 50 mL of ActiPro media supplemented with 6 mM L-glutamine into sterile 50 mL centrifuge tubes. Warm 50 mL centrifuge tube(s) for heating in a sterile water bath set at 37 °C for 3–5 min
11. Following the transfection, seed cells at 0.4×10^6 cells/mL in 125 mL shake flask using ActiPro media at a working volume of 25 mL, and maintain for 4 passages. After the first passage, supplement media with sterile filtered MTX up to a concentration of 100 nM. Tip: The MTX is added after a single passage to ensure the cells have slightly recovered from the initial cellular stress induced from transfection. Check and ensure cell culture's viability to be at least 80% before adding MTX to maintain the culture's viability
12. Cultivate single cells from bulk transfection pools using ClonaCell-TCS medium (semi-solid media) in sterile 6-well plates according to media manufacturer's instructions. Ensure that semi-solid media is well mixed and supplemented with concentrations of 6 mM glutamine and 250 nM sterile filtered MTX. Additionally, measure VCD and viability of bulk transfection pool flask using ViCell to ensure the appropriate number of cells are added from the bulk pool to aliquot 50–100 cells per well during plating. Fill each well with 3 mL of semi-solid media using a sterile 5 mL syringe for a total of 5 plates. Tip: It is recommended to use various concentrations of cells during plating as colony expansion can vary based on the cell line
13. Incubate the plates at 37 °C in a humidified incubator at 5% CO_2, 80% RH, and 0 rpm for 10–14 days. Tip: Some cells may need additional days for colony expansion
14. Inspect plates under a microscope at 20× magnification and harvest single-cell colony outgrowths using a pipette. Transfer each colony to individual wells of a 96-plate well. Each well should be filled with

0.1 mL of media supplemented with 200 nM sterile filtered MTX. After 2 days, add 0.1 mL 200 mM MTX-supplemented media to each well

15. Inspect individual wells under a scientific microscope at 20 × for 80% confluency. Transfer the contents of the respective well to a 24-well plate. Add 0.8 mL 200 nM sterile filtered MTX supplemented media to each well to bring working volume to 1 mL
16. Inspect wells under a microscope for 80% confluency. Transfer the contents of the respective well to a 6-well plate. Add 2 mL of 200 nM sterile filtered MTX supplemented media to each well to bring working volume to 3 mL
17. Inspect wells under a microscope for 80% confluency. Transfer half of the contents of each well individually to a new well of a 6-well plate. Add 1.5 mL 200 nM MTX supplemented media to all split wells to bring working volume to 3 mL
18. Inspect wells under a microscope for 80% confluency. Transfer both wells of a corresponding clone expansion to a 125 mL shake flask. Add 4 mL 200 nM MTX supplemented media to bring working volume to 10 mL
19. Measure viability and VCD of each flask after 2–3 days. Passage at a seeding density of 0.4×10^6 cells with fresh 200 nM MTX supplemented media every 3 days until viability is greater than 95% and consistent growth profiles are achieved. Once consistent growth is achieved at a working volume of 10 mL, increase the working volume of shake flasks to 25 mL and repeat passage criteria. Shake flask should be incubated in a humified incubator at 37 °C, 5% CO_2, with an agitation of 135 rpm
20. Upon completion, bank and label cells from each shake flask. Approximately 10^7 cells should be centrifuged at $120 \times g$ to remove the supernatant. Transfer cell pellet to vials with 1 mL of ActiPro media supplemented with 10% DMSO for −80 °C storage
21. Thaw one vial of each different cell line banked, and perform a batch and fed-batch study in 125 mL shake flasks to characterize growth and titer of each cell line. An example of growth (viability and VCD) and mAb production (titer) characterization data from a fed-batch study utilizing four clones after CLD is shown in Fig. 2

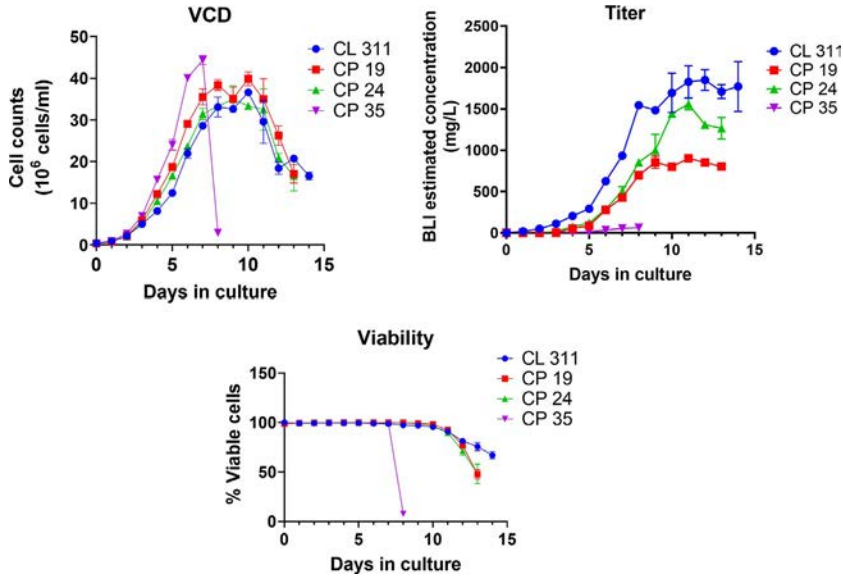

Fig. 2 Example data of VCD, mAb titer, and viability (*VIA*) stemming from the fed-batch study of clones post CLD. CL311, CP19, CP24, and CP35 are representative clones that exhibit differential mAb production after undergoing identical development conditions.

5. Sodium butyrate (NaBu) concentration optimization and treatment of developed cell lines

Many small-molecule culture additives have been utilized in CHO cell cultures for enhancing mAb production or quality. The addition of NaBu, which is a HDACi, to cell cultures has demonstrated improvement in the specific productivity of certain CHO cell lines. This effect on specific productivity stems from HDACis' ability to promote chromatin accessibility *via* a more euchromatin (less condensed) form (Jiang & Sharfstein, 2008). Chromatin accessibility is regulated by histone modifications such as acetylation, phosphorylation, and methylation. NaBu induces histone hyperacetylation leading to improved access of transcription factors to protein biosynthetic machinery by lowering the DNA binding affinity to the histones (Jiang & Sharfstein, 2008; Yang et al., 2014). While this mechanism may lead to higher productivity, its titer effects are must be balanced with cytotoxic effects, requiring the amount of NaBu added to cultures to be

optimized. Determining the optimal NaBu concentration to administer in each cell line is critical. Using a concentration too high induces apoptosis in the cell culture, leading to decreased titers due to poor culture viability; however, using a NaBu concentration too low may not induce the desired process change. Here, we describe how to determine the optimal NaBu concentration for each cell line in a batch experiment before starting a fed-batch experiment.

5.1 Determining optimal NaBu concentration for cell lines to improve titer

1. Sanitize by wiping the work surface with 70% isopropanol. Turn on the airflow of the biological safety cabinet for the execution of cell culture work 15 min prior to start
2. Thaw a vial of transfected cells from the −80 °C freezer, and use a pipette to transfer $\sim 10^7$ cells to a sterile 15 mL centrifuge tube (See Note 1).
3. Pipette 50 mL of ActiPro media supplemented with 6 mM L-glutamine into sterile 50 mL centrifuge tubes. Warm 50 mL centrifuge tube in a sterile water bath set at 37 °C for 3–5 min
4. Wash cell pellet with 5 mL of ActiPro media supplemented with 6 mM L-glutamine (add slowly using a pipette). Centrifuge cells at $120 \times g$ for 3 min, and use pipette to remove the supernatant from the cell pellet
5. Pipette 5 mL of fresh ActiPro media supplemented with 6 mM L-glutamine in a centrifuge tube containing cell pellet, and transfer all the contents to a sterile 125 mL shake flask. Finally, pipette 15 mL of additional ActiPro media supplemented with 6 mM L-glutamine to bring cell culture to a seeding density of 0.4×10^6 cells/mL in the shake flask for incubation at 37 °C, 5% CO_2, 135 rpm, and 80% RH
6. Maintaining a seeding density of 0.4×10^6 cells/mL for each passage, allow cells to undergo 3 passages (approximately 3 days per passage), and measure viability and viable cell density (VCD) using ViCell to ensure at least 97% viability and representative cell-specific doubling time
7. To begin the batch experiment, seed 10 flasks at 0.4×10^6 cells/mL for each cell line at a working volume of 25 mL. This allows for each condition (varied NaBu concentrations) to be performed in biological duplicates. Measure viability and VCD daily using Vi-CELL count, and collect 0.6 mL samples for titer analysis by BLI after the 7-day batch run
8. On day 2 of the batch culture, administer NaBu concentrations of 0, 0.5, 1, 2, and 3 mM to each flask in duplicate (Note 4)
9. Evaluate effects of NaBu on the viability and VCD and final titer of each culture to determine appropriate optimal concentration. Tip: If the NaBu concentration used resulted in a higher final titer than the

control (0 mM NaBu), it is an ideal optimal concentration. If the NaBu concentration used resulted in a final titer similar to the control or other NaBu concentrations, then defer to the higher viability and VCD as the optimal NaBu concentration for the cell line

5.2 Fed-batch study for evaluating the effect of the optimal NaBu concentration on the cell line

1. Sanitize and turn on the airflow of the biological safety cabinet for the execution of cell culture work
2. Thaw a vial of transfected cells from the −80 °C freezer, and use a pipette to transfer ~10^7 cells to a sterile 15 mL centrifuge tube (See Note 1).
3. Pipette 50 mL of ActiPro media supplemented with 6 mM L-glutamine into sterile 50 mL centrifuge tubes. Warm 50 mL centrifuge tube in a sterile water bath set at 37 °C for 3–5 min
4. Wash cell pellet with 5 mL of ActiPro media supplemented with 6 mM L-glutamine (add slowly using a pipette). Centrifuge cells at 120 × *g* for 3 min, and use pipette to remove the supernatant from the cell pellet
5. Pipette 5 mL of fresh ActiPro media supplemented with 6 mM L-glutamine in a centrifuge tube containing cell pellet and transfer all the contents to a sterile 125 mL shake flask. Finally, pipette 15 mL of additional ActiPro media supplemented with 6 mM L-glutamine to bring cell culture to a seeding density of 0.4×10^6 cells/mL in the shake flask for incubation at 37 °C, 5% CO_2, 135 rpm, and 80% RH
6. Maintaining a seeding density of 0.4×10^6 cells/mL for each passage, allow cells to undergo 3 passages (approximately 3 days per passage), and measure viability and viable cell density (VCD) using ViCell to ensure at least 97% viability and representative cell-specific doubling time
7. To begin the fed-batch experiment, seed 6 flasks at 0.4×10^6 cells/mL for each cell line at a working volume of 25 mL. This allows for the optimal NaBu and control (0 mM) concentrations to be performed in biological triplicates. Measure viability and VCD daily using Vi-CELL count, and collect daily 0.6 mL samples for titer analysis by BLI after the 14-day batch run
8. Starting from day 3, add Feed 7A and 7B at 3% and 0.3% v/v ratio to each shake flask daily. From day 5, add glucose to bring cultures to baseline glucose concentration ranging between 6 and 7 g/L. Tip: Glucose concentration can be measured by pipetting 0.2 mL samples of culture fluid into YSI sample cups for analysis by biochemistry YSI analyzer
9. On day N of the culture, where Day N equates to the day before the cell line typically achieves its maximum VCD, administer NaBu optimal concentration to treated shake flask cultures (See Note 5).

10. Evaluate the viability, VCD, and final titer of each optimal NaBu concentration treated culture compared to its respective non-treated (0 mM) control culture to determine the effects of the optimal NaBu concentration on each cell line and whether there is a correlation with titer enhancing effects and productivity

6. Protein A chromatography

Protein A is the workhorse and the primary capture step in the downstream processing of the harvested cell culture fluid (HCCF) to purify monoclonal antibodies (mAbs). The first step is to obtain the clarified cell culture supernatant from the HCCF. This is achieved by centrifugation of the HCCF at $120 \times g$ rpm for 5 min. The supernatant is collected and directly loaded onto the protein A column at neutral pH, followed by product elution at low pH. A wash step (at intermediate pH) is usually introduced between the HCCF loading and product elution step that allows removal of host cell proteins (HCPs) and other impurities. The purification step yields >99% purity owing to the high selectivity of Protein A for mAbs. Here, we describe the step-by-step procedure of purifying mAbs from the different productivity clones. The purification process described in this protocol is pressure-driven using a syringe (details described below). The whole procedure takes approximately 30 min (for 10 mL HCCF loading) in batch mode (See Note 6).

6.1 Purification process

1. Equilibrate the HiTrap Protein A HP column with 50 mM Phosphate, 150 mM NaCl, pH 7.5 (5 CV).
2. Load the cell culture harvest onto the column such that a residence time of ~4 min is maintained during loading. For a desired residence time, the flow rate is calculated using the formula:

$$flow\ rate\left(\frac{cm^3}{min}\right) = \frac{bed\ height\ (cm) \times (column\ radius\ (cm))^2 \times \pi}{residence\ time\ (\min)}$$

Flow of 1 mL/min flow rate corresponds to 24 drops/min. Thus, for 1.25 mL/min, the flow rate maintained should correspond to 30 drops/min

3. Wash the column using 50 mM Phosphate, 150 mM NaCl, pH 7.5 (2 CV)
4. Elute the mAb using 100 mM Glycine, pH 3.0 (1.5 CV)
5. Recondition the column using 2 M NaCl (2 CV) and 0.5 M NaOH (2 CV) before the next cycle of sample loading

6. Neutralize the pH of the elute using 1 M Tris buffer
7. Determine the concentration of the eluate using UV-Spectrophotometer by recording absorbance at 280 nm

7. N-glycan analysis using mass spectrometry

Glycosylation is an important quality attribute that has a pronounced impact on the safety, efficacy, and quality of mAbs. Glycan heterogeneity contributes to the varying effector function capacity of mAbs. For instance, sialylation of N-linked glycans correlates with IVIG's anti-inflammatory activity and modulates immune effector functions of human IgGs (Mimura et al., 2018). Similarly, high mannose glycans result in rapid clearance of the mAbs, thereby affecting their pharmacokinetic half-lives (Millward et al., 2008). Specific glycans such as N-glycolylneuraminic acid are associated with cases of immunogenicity (Yehuda & Padler-Karavani, 2020).

The critical determinant of glycosylation in mAbs is governed by the choice of host cell used in the cell line development. Additionally, there could be substantial clone-to-clone variations that necessitate the optimal selection of cell lines for mAb production. Here, we present a detailed protocol for purification of mAbs from the HCCF and subsequent processing, method development, and analysis of released glycans from mAbs. The sample preparation takes about 1.5 h, while data acquisition and analysis require 5–6 h.

7.1 Sample preparation

1. The protein A purified mAbs from the different productivity clones are concentrated to 2 mg/mL in an appropriate buffer that does not contain sodium dodecyl sulfate (SDS). Also, nucleophiles (*e.g.*: Tris, DTT, glycine, or histidine), if present, should be diluted such that their final concentration is below 0.1 mM
2. Prepare an intact mAb standard such as NIST Reference Material 8671 NISTmAb (diluted to 2 mg/mL) as a positive control for processing alongside the protein A chromatography-purified mAb samples
3. For deglycosylation, first, dilute 7.5 μL of the protein A purified mAb samples (15 μg) with 15.3 μL of LC-MS grade water, and denature by adding 6 μL of 5% solution of RapiGest surfactant to each of the samples followed by heating at 90 °C for 3 min. Secondly, the denatured samples are then cooled for 3 min to room temperature, followed by the addition of 1.2 μL of PNGase F and incubated at 50 °C for 5 min

4. The deglycosylated samples are then cooled to room temperature for 3 min
5. The released glycans are then labeled by adding 12 μL of Rapi-Fluor dye dissolved in anhydrous dimethylformamide (DMF). The reaction mixture is left at room temperature for 5 min
6. The labeled glycan mixtures are then diluted with 358 μL of acetonitrile (ACN) for the subsequent clean-up step
7. Set up the μ-HILIC elution plate in a vacuum manifold with shims and a waste tray
8. Condition the wells of the μ-HILIC elution plate with 200 μL of water, followed by 200 μL of 85% ACN. Ensure that the vacuum is adjusted such that the liquid takes ~30 s to pass through each of the wells of the μ-HILIC elution plate
9. Load ~400 μL of ACN-diluted, labeled glycan samples from step 6 onto the conditioned wells of the μ-HILIC elution plate
10. Wash the wells twice with 600 μL of 1:9:90 formic acid:water: Acetonitrile
11. Replace the waste tray with 600 μL sample collection tubes
12. The labeled N-glycans are eluted with 90 μL of solid-phase extraction (SPE) elution buffer into the collection tubes
13. Dilute the eluent with 310 μL of DMF/ACN sample diluent. Mix by aspiration, and pipette into high-throughput HPLC vials for fluorescence (FLR)-mass spectrometry (MS) analysis

7.2 LC-MS analysis of released N-glycans

The mass spectrometry analysis was performed on a Waters BioAccord LC-MS system composed of a Sample Manager, column compartment, thermostat, fluorescence, and UV detector. However, the analysis can be performed on any LC-MS system with these components.

1. Create a Glycan Assay (FLR with MS confirmation Method) analysis method using UNIFI software (Waters Corp.)
2. List out the separation components that are used to identify the dextran ladder components for the separation calibration. The corresponding retention times of the components will need to be updated (See Note 7)
3. To enable Glucose Unit (GU)-based library search, do not add any entries in the component list
4. Program the gradient method for the separation of glycans using 50 mM ammonium formate pH 4.4 as the mobile phase A (%A) and 100% LC-MS grade ACN as mobile phase B (%B). The gradient method used is listed in Table 1

Table 1 Listing the gradient of mobile phase B (Acetonitrile +0.1% formic acid) used for the separation of glycans using hydrophilic interaction liquid chromatography.

Time (min)	Flow Rate (mL/min)	%B
0	0.4	75
35	0.4	54
36.5	0.2	0
39.5	0.2	0
43.1	0.2	75
47.5	0.4	75
55.0	0.4	75

5. Install Waters ACQUITY UPLC Glycan BEH Amide column (2.1 × 150 mm, 1.7 mm particle size, 130 Å pore size) in the column compartment and maintain the column temperature at 60 °C during separations
6. Set the fluorescence (FLR) detector to record signal at 265/425 nm (excitation/emission) wavelength with a sampling rate of 2 Hz
7. The ESI-QTOF-MS (electrospray ionization quadrupole time-of-flight mass spectrometer) is programmed to operate in positive mode with full scan in the mass range 50–2000 m/z with a scan rate of 2 Hz. Other MS parameters included cone voltage: 45 V, Capillary voltage: 1.5 kV
8. Use Leucine enkephalin (2 ng/μL in 50% ACN/0.1% formic acid) for lock mass correction
9. Once the glycan analysis method is set up, the next task is to create an analysis for acquiring new data
10. Equilibrate the system by setting the initial separation conditions. Fill out the sample list with an appropriate number of samples
11. *Re*-suspend the dextran ladder in 100 μL LC-MS grade water and prepare 10 μL aliquots for storage at −80 °C. The reconstituted dextran ladder degrades when kept at room temperature for more than 24 h. Also, avoid more than one freeze-thaw cycle

Tip: Water is a strong solvent in HILIC analysis, so the amount injected of a sample in aqueous solutions is limited. If larger injection volumes are needed (greater than 2 μL), then dilute samples to a final concentration of 25% water, 50% ACN, and 25% DMF.

12. Include dextran ladder injections in the newly created sample list. The retention times of the dextran ladder components are used for assigning glycan structures based on library search. The glycan assignments are further validated using the mass information
13. Inspect the FLR offset with respect to the Total Ion Chromatogram (TIC) data and apply the correction to enable detector offset alignment
14. Start the data acquisition by injecting samples in triplicates

7.3 Data analysis

Set the peak processing parameters in UNIFI with the following guidelines.

1. Find 2D peaks: Glycans are identified by their FLR peak integration. Use minimum height to limit the number of peaks integrated. In addition, the "detect shoulder" option can help with poorly resolved glycans
2. Find 3D peaks: 3D peak parameters are used to find the precursor glycan and fragment isotope masses. The retention time (RT), m/z, peak shape, and intensity of each isotope above the low energy and high energy thresholds are measured. Isotopes from the same glycan or fragment across all charge states are combined into a single component at the C12 monoisotopic +H m/z. Fragments are then aligned to precursors by RT and peak shape
3. 3D isotope clustering: The 3D isotope parameters determine how close the retention time glycan isotopes and glycan fragments need to be clustered together. In general, 1/7th of the chromatographic peak width is applied during cluster creation with the intensity threshold of 1500 during high to low energy association
4. Target by Retention time: If no components have been entered, this option applies only to the separation components. A relatively large RT tolerance (±0.2–0.3 min) and maximum area can be used to identify dextran ladder components
5. Target by mass: Set the target mass tolerance to 20 ppm
6. Discovery settings: Set the search value. Only ±value matters for processing. Select the appropriate glycan library for searching the components
7. Quantitation settings: Select the calibration curve fit type as relative response (%) and quantify by area. The amount of each glycan is calculated as a percent of all identified glycans. Choose calibration curve fit type as either cubic spline or fifth-order polynomial

8. Analysis specific settings: Define the lock mass parameters for internal mass correction
9. Once the data acquisition is complete, process the samples with the above settings
10. Review the separation calibration and confirm that the correct components are identified. For example, G4 (maltotetraose) should be 1024 m/z
11. Evaluate the reproducibility of the dextran ladder injections
12. Review the FLR integration and adjust the 2D peak integration settings in step 1 as needed
13. Library Assignment of peaks: The peak retention time is converted to a GU value (Fig. 3 and Table 2). All glycans with GU values within the tolerance set in the analysis method are returned from the library search. Mass-confirmed glycans are given the highest priority. However, a single mass confirmed glycan is selected over any non-mass confirmed glycans. Similarly, if the library search returns two glycans of the same mass, then one with the lowest delta GU is selected. If two glycans of different masses are returned, then the most abundant in the spectrum is selected. For the case where no glycans are mass confirmed, the lowest delta GU is selected
14. Check the glycan identifications of the glycan performance standard (NISTmAb) and the samples for accuracy and consistency. Adjust the library GU tolerance in step 6 as needed
15. In case the desired glycans are assigned to a different peak, they must be removed from that peak to enable its manual reassignment to the desired peak. Save the manual changes after reassigning glycans

8. Summary

This chapter presents a rapid, simplified, and easy to implement (automation-free) workflow for the generation of robust CHO cell lines with varying productivity. We also have provided a step-by-step procedure for studying the impact of HDACis on product titer. Finally, we have presented analytical workflows for determining the glycosylation profile of the mAb produced using these cell lines. We believe that the proposed workflow can be applied for various CLD campaigns that will result in clones that enhance product expression, will facilitate process optimization to and impact product quality attributes.

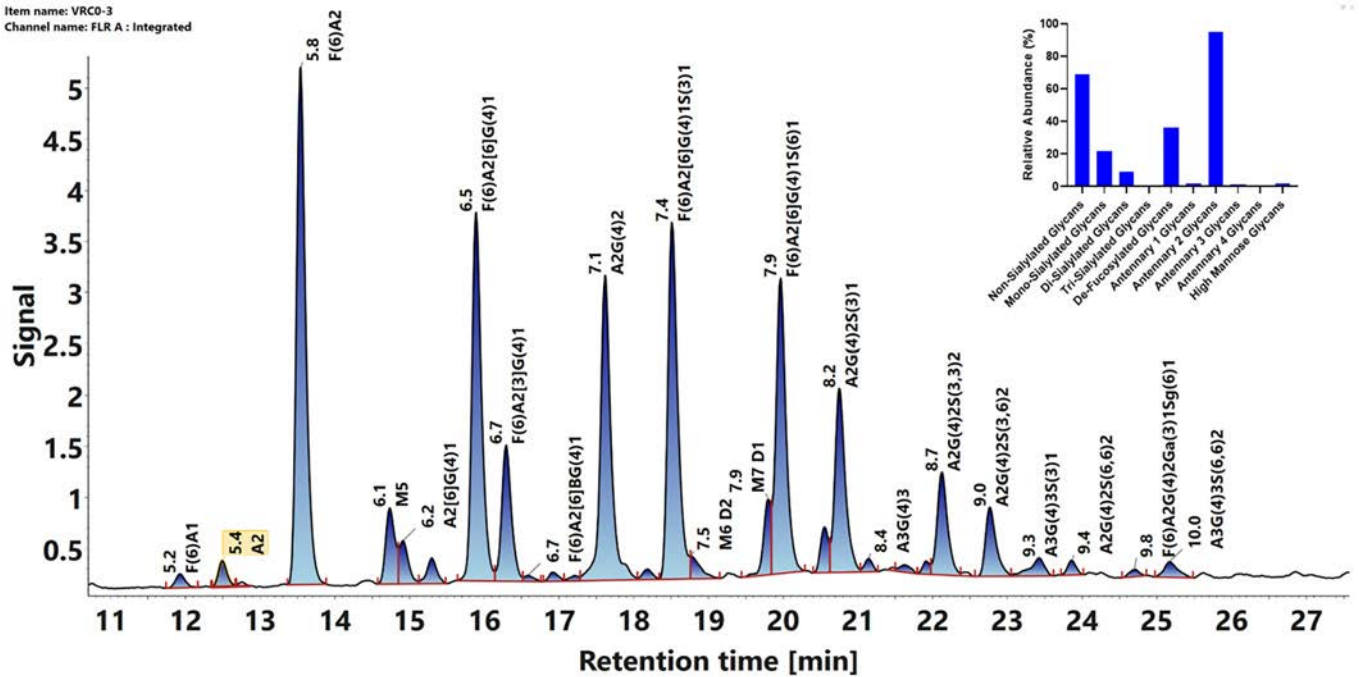

Fig. 3 Example data obtained after released glycan analysis using HILIC-FLR-MS analysis. Each peak retention time is converted into Glucose units (GU) below the corresponding peak annotation based on separation calibration. The glycan identity is then established based on library search using GU values which are finally validated with the mass values from the ToF-MS data. The cumulative percentages of various glycan groups such as mono, di, and tri-sialylated, high mannose, antennary (1–4), and de-fucosylated glycans are shown in the inset of the figure.

Table 2 A list of the mass confirmed N-glycans derived from a representative mAb sample analyzed in the study.

Component name	Observed RT (min)	% Amount	Glycan units	Expected glycan units	Expected mass (Da)	Observed mass (Da)	Charge	Response
A2	12.62	2.01	5.494	5.4619	1627.66114	1627.6656	2	70,097,021
A2[3]G(4)1	15.44	0.69	6.3646	6.29	1789.71396	1789.7191	2	24,167,661
A2G(4)2	17.78	6.59	7.1305	7.0955	1951.76678	1951.7745	2	229,496,027
A2G(4)2S(3,3)2	21.82	4.4	8.58	8.57	2533.95762	2533.9678	3	153,181,096
A3G(4)3S(3)1	23.36	0.94	9.1863	9.23	2607.99439	2608.0105	3	32,769,924
F(6)A2	13.66	25.24	5.8095	5.7879	1773.71904	1773.723	2	878,563,305
F(6)A2[3]G(4)1	16.43	6.17	6.6824	6.6633	1935.77187	1935.7786	2	214,660,159
F(6)A2[6]G(4)1	16.02	14.65	6.5507	6.5337	1935.77187	1935.7804	2	509,948,907
F(6)A2[6]G(4)1S(6)1	19.88	6.76	7.8603	7.84	2226.86728	2226.8707	2	235,229,553
F(6)A2B	14.9	1.37	6.1924	6.1175	1976.79842	1976.8144	2	47,622,306
F(6)A2BG(4)2S(6)1	22.3	0.34	8.7671	8.82	2591.99948	2592.0114	3	11,980,437
F(6)A2G(4)2	18.67	11.43	7.4346	7.4266	2097.82469	2097.8293	2	397,801,326
F(6)A2G(4)2S(3)1	20.66	5.72	8.1426	8.124	2388.92011	2388.9277	2	199,150,686
F(6)A2G(4)2S(3,3)2	22.46	3.53	8.8291	8.85	2680.01552	2680.023	3	122,713,719
F(6)A3G(4)2	19.71	1.6	7.7992	7.7585	2300.90406	2300.9272	3	55,751,428

Continued

Table 2 A list of the mass confirmed N-glycans derived from a representative mAb sample analyzed in the study.—cont'd

Component name	Observed RT (min)	% Amount	Glycan units	Expected glycan units	Expected mass (Da)	Observed mass (Da)	Charge	Response
F(6)A4G(4)4	24.9	1.35	9.8344	9.915	2828.08908	2828.1028	3	47,036,678
F(6)A4G(4)4S(3,3,3)3	28.51	0.69	11.5124	11.52	3701.37533	3701.3831	3	23,888,778
F(6)A4G(4)4S(3,3,3,3)4	29.73	0.63	12.1439	12.17	3992.47075	3992.4734	3	21,868,994
M5	14.91	2.29	6.1942	6.1692	1545.60804	1545.6114	2	79,778,922
M7 D1	20.45	1.56	8.0658	8.0285	1869.71368	1869.7217	2	54,216,136

Also included are the observed retention times, normalized abundance (% Amount), expected and observed GU values, expected and observed masses, the predominant charge state, and the response units.

9. Notes

Note 1. The following steps are based on the thaw vial containing $\sim 10^7$ host cells. If the initial thaw vial does not contain $\sim 10^7$ cells or is unknown, then a cell count will need to be performed using a Vi-CELL. Based on cell count, use a pipette to transfer $\sim 10^7$ cells to a sterile 15 mL centrifuge tube before starting step 3.

Note 2. ViCell measurement requires a 0.6 mL sample of culture fluid to be pipetted into a ViCell sample cup for analysis.

Note 3. Following the manufacturer's instructions, a program specific to the cell type should be used. In this case, the program F-137 was selected for the CHO-K1 cell type specified for this protocol (https://lonza.picturepark.com/).

Note 4. Previous literature studies have typically found that 1 mM is the optimal NaBu concentration in many CHO cell lines, so in testing other CHO cell lines, it is recommended to start with concentrations closer to that value.

Note 5. Day N should be identified based on previous fed-batch growth characterization studies during clone development. Treatment on day N ensures high VCD before NaBu addition that would minimize the cytotoxic effects of NaBu.

Note 6. Column volume (CV) is determined by the column diameter and the bed height of the resin. The amount of mobile phase used in each step is determined in terms of column volume.

Note 7. The retention times of glycans are expressed in Glucose Units (GU) by reference to the components of the dextran ladder. Each glycan structure has a GU value that is related to the number and linkage of its constituent monosaccharide units.

Acknowledgments

The authors would like to thank Dr. Hussain Dahodwala for his help during CLD experiments and critical reading of the manuscript. Additionally, we would like to thank Michael Daly from Waters Corporation for his help in troubleshooting the mass spectrometry experiments for glycan analysis at various points in this study and also the NIH-NIAID for sharing of cells. The work has been supported, in part, by the National Science Foundation (1624698, 1736123) and NIST (70NANB17H002).

Competing interests

The authors declare no competing interests.

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