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REPORT



# Process development for production of non-originator NISTmAb from CHO and NS0 cell lines

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**ABSTRACT**

Cell lines that produce non-originator versions of the National Institute of Standards and Technology (NIST) monoclonal antibody reference material 8671 (NISTmAb) are invaluable to the biopharmaceutical industry because, unlike typical commercial cell lines, they can be used on a collaborative and non-competitive basis for bioprocess development. NIST has generated NS0 clones, NISTCHO research-grade test material 10197 and reference material 8675 NISTCHO to fill this need. We set out to optimize seed train procedures, media and feeding strategies, and stirred tank and rocking bioreactor processes to facilitate our studies on the effects of cell substrate and bioreactor process parameters on non-originator NISTmAb quality attributes. For two NS0 clones and NISTCHO, we improved the baseline methods for seed train culture and demonstrated the critical roles of agitation and gassing strategies for stirred-tank bioreactor operations. For NISTCHO we also tested fed-batch and perfusion processes in rocking bioreactors, identifying several critical process parameters and in-process controls. In this work, for the NIST NS0–59 and NS0–66 clones, we demonstrated that shake flask geometry was critical for culturing a highly viable seed train with a high growth rate and exhibited impacts of feeds, agitation, and gassing during initial bioreactor process development. We identified agitation rates and gassing strategy as critical process parameters for NISTCHO stirred-tank bioreactor operations and established processes for fed-batch and perfusion rocking bioreactor operations. We anticipate this work to benefit the growing number of researchers employing non-originator NISTmAb-expressing cell lines to support precompetitive innovation in biomanufacturing.

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## Introduction

Monoclonal antibodies (mAbs) have dominated the biopharmaceutical market for over a decade[1] and, in 2024, accounted for >75%[2] of the U.S. Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) biologics approvals. As of December 2024, Chinese hamster ovary (CHO) and NS0 murine myeloma cells, which are the two most common mammalian cell lines in biomanufacturing,[3–6] are used to manufacture approximately 80% and 10%,[2] respectively, of U.S.-licensed mAb drug products.[2] The National Institute of Standards and Technology (NIST) developed and released the NIST monoclonal antibody reference material (RM) 8671 (NISTmAb) in 2016 to provide open access material for the precompetitive space in biomanufacturing and therapeutic protein characterization.[7–10] In 2018, NIST reported results for three NS0 NISTmAb-expressing clones, NS0–59, NS0–60, and NS0–66, and showed that the expression systems were functional and expressed a product that resembled NISTmAb.[11] In 2023, NIST released NIST research-grade test material (RGTM) 10197, also known as NISTCHO, to evaluate its potential as a reference material. NIST provides RGTMs to stakeholders prior to qualification as reference materials so that the community can collaborate with NIST in characterizing the material and related methods. NISTCHO is a CHO-K1 cell line expressing non-originator NISTmAb that can be used by recipients on a noncompetitive basis for bioprocess development. When in culture, cell suspensions propagated from RGTM 10197 are expected to produce a non-originator, humanized, IgG1κ monoclonal antibody, cNISTmAb, having the same primary amino acid sequence as the NISTmAb monoclonal antibody. NISTCHO was generated using the CHOZN® Platform (Sigma-Aldrich), which relies on the CHOZN® ZFN Modified $GS^{-/-}$ CHO cell line in which the endogenous glutamine synthetase (GS) is eliminated, rendering the cells auxotrophic for the essential amino acid L-glutamine.[12] GS selection technology allows for clone selection post-transfection based on incorporation of a functional GS gene along with the gene for the desired protein and eliminates the need for methionine sulfoxamine (MSX), a GS inhibitor, as a selection agent.

NIST's guidance document[13] for NISTCHO includes instructions for initial propagation and banking that refer the user to the CHOZN® Platform Technical Bulletin[12] (Sigma-Aldrich) for additional guidance on the selection of culture media, culture conditions, and scaling. Specifically, protocols

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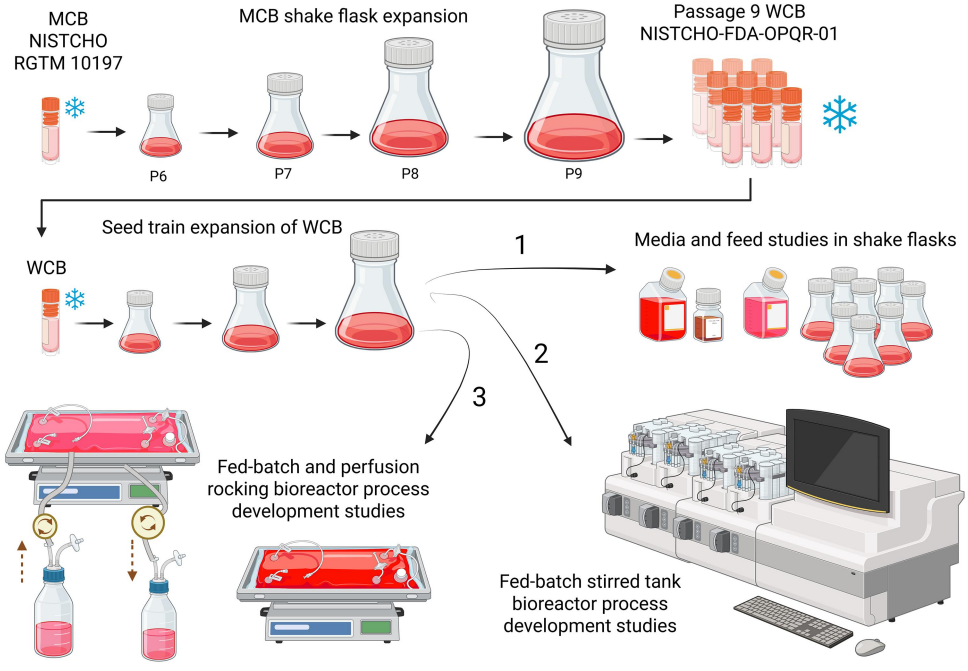
are included in the technical bulletin for stock culture maintenance and scale-up strategy of the parental cell line, which can be adapted for NISTCHO. Additionally, protocols for a rocking bioreactor 20 L wave bag method for cell expansion and benchtop scale (1 L and 5 L) bioreactor methods are presented with some suggested process parameter ranges for process development, but no guidance is included for perfusion bioreactor operations. In general, efforts in upstream process development for a cell line focus on media/feeding optimization[14,15] and identification of the ideal physical process parameters to prevent cell stress, maintain high productivity, and maintain the desired critical quality attributes of the recombinant protein drug substance.[16–18] In this study, we aimed to improve and establish additional culture methods for non-originator NISTmAb-producing cell lines. We examined critical process parameters and feeding strategies for the non-originator NISTmAb cell lines to potentially improve cell health, culture longevity, and titer. Overall, our objective was to establish a detailed process, cell growth, and cell productivity data for NISTCHO, NS0–59, and NS0–66 that we anticipated would stimulate innovation in the precompetitive space by establishing methods in stirred-tank and rocking bioreactors. Since performing our studies using RGTM 10197 NISTCHO, NIST has announced the anticipated availability of RM 8675 NISTCHO, as well as RM 8672 cNISTmAb, a CHO-expressed IgG1k monoclonal antibody based on the product produced by NISTCHO.[19]

## Materials and methods

### *Working cell bank generation for NISTCHO*

We received 1 vial of cryopreserved NISTCHO RGTM 10197 directly from NIST ($1.3 \times 10^7$ cells) that was a post-clonal isolation of six passages stored in EX-CELL® CD CHO Fusion culture medium (Sigma-Aldrich, Cat # 14365C) supplemented with seven volume percent (7% v/v) dimethylsulfoxide (DMSO). The vial was stored in the vapor phase of liquid nitrogen until the day of thaw. As instructed in the NISTCHO Test Material, Clonal CHO-K1 Cell Line Producing cNISTmAb Guidance Document,[13] we established working cell banks at culture passage 9 post-clonal isolation, primarily following the CHOZN® Platform Technical Bulletin provided by Sigma-Aldrich with some small changes to the protocol, described briefly hereafter (Scheme 1). The cell vial was thawed using an automated thawing system (BioLife Solutions ThawSTAR® AT2) and pipetted into 10 mL of prewarmed EX-CELL® CD CHO Fusion cell culture medium in a 15 mL sterile tube. The tube was centrifuged at 200 × g for 5 min to pellet the cells. The clarified medium was removed using a pipette and discarded. The cell pellet was resuspended in 30 mL of prewarmed culture media, added to a sterile 125 mL non-baffled vented-cap Erlenmeyer shake flask and placed in a INFORS HT Multitron Incubator (Infors AG) at 37°C, 5% $CO_2$, 130 rpm, and 85% humidity. The cells were checked on days 0 and 1 for cell density and viability, and split on days 4, 7, and 11, and banked on day 13 when the cells were in the mid-exponential growth phase. Two passage 9 working cell banks containing $2 \times 10^8$ cells/vial were generated and named 10197/p09/EX-CELL® CD CHO/FDA/1 (80 vials) and 10197/p09/EX-CELL® CD CHO/FDA/2 (40 vials). Testing for total cell number/vial and viability at thaw for each working cell bank (WCB) showed high similarity between banks, and therefore the WCBs were used interchangeably in our studies.




**Scheme 1.** Working cell bank generation and bioreactor process development for NISTCHO RGTM 10197. Created in BioRender. Berilla, E. (2025) https://BioRender.com/djsjkbg.

### Working cell bank generation for NIST NS0–59 and NS0–66 clones

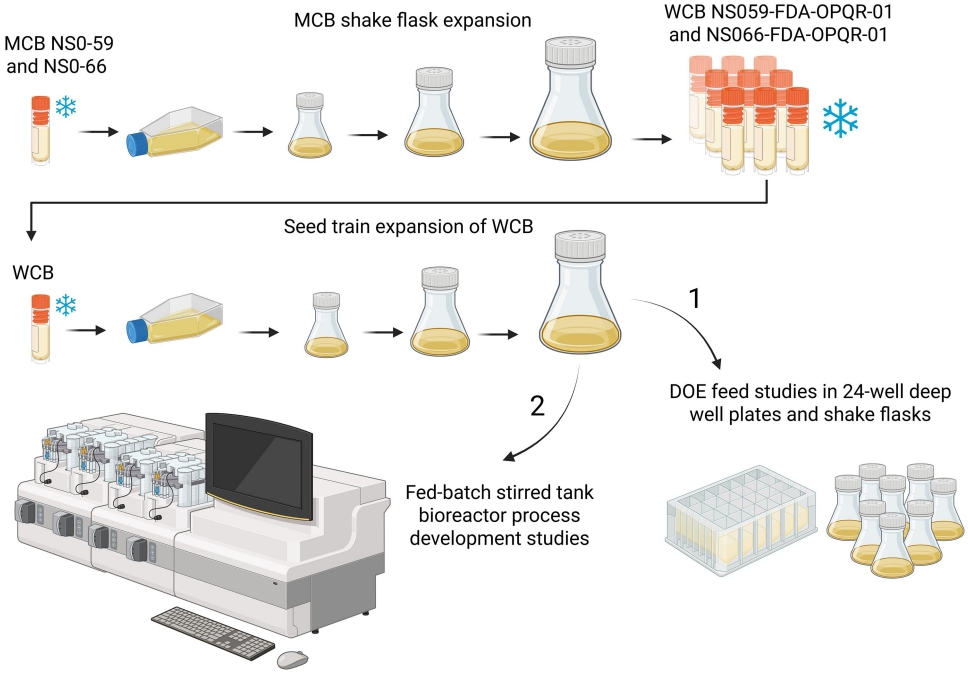
We received 1 vial each of cryopreserved NS0–59 and NS0–66 directly from NIST from which we generated our initial WCBs (Scheme 2). A vial of NS0–60 was also cryopreserved, but not used in this study due to additional media supplement requirements. The cell vials were thawed using an automated thawing system and pipetted into 10 mL of prewarmed Chemically Defined (CD) Hybridoma Medium (Gibco, Cat # 11279–023) in a 50 mL sterile tube. The tube was centrifuged at 200 × g for 5 min to pellet the cells. The clarified medium was removed using a pipette and discarded. The cell pellet was resuspended in 12 mL of medium supplemented with 2 mm L-glutamine (SAFC, Cat # 59202C) and 10 mL/L of Minimum Essential Medium Non-essential amino acids solution (MEM NEAA) (100X) (Sigma-Aldrich, Cat # M7145) in a 75-mL cell culture T-flask with vented cap (Corning, Cat # 430641 U) and placed in a INFORS HT Multitron Incubator (Infors AG) at 37°C, 5% $CO_2$, and 85% humidity. When cell density reached above $1.0 \times 10^6$ cells/mL, the T-flask cultures were scaled up to a sterile 125 mL non-baffled vented-cap Erlenmeyer shake flasks, seeded at a cell density of 0.5–0.7 × $10^6$ cells/mL and placed in the incubator at 37°C, 5% $CO_2$, 100 rpm, and 85% humidity. The cells were checked on days 0 and 3 for cell density and viability, split on days 4, 5, and 7, and banked on day 10 when the cells were in the mid-exponential growth phase. These initial WCBs were small (10 vials for NS0–66 and 30 vials for NS0–59), and therefore subsequent WCBs were generated from the initial WCBs as we acquired knowledge on improving seed train expansion.

### NISTCHO fed-batch shake flask feed optimizations

Sufficient NISTCHO cells were collected from a 500 mL shake flask culture that were day 11 post-WCB thaw to seed seven 30 mL cultures at $6.7 \times 10^6$ cells/mL. The cultures were maintained in EX-CELL® Advanced CHO Fed-batch Medium (SAFC, Cat # 14366C or 24366C) and fed on days 0, 2, and 4 with a 50% glucose solution (4 g/L) and 0–15% EX-CELL® Advanced CHO Feed 1 (without glucose) (SAFC, Cat # 24368C). Each flask was sampled on days 0, 2, 3, and 6 for pH, pO2, pCO2, osmolality, viable cell density (VCD), cell viability, cell diameter, glutamine, glucose, lactate, glutamate, ammonium, sodium, potassium, calcium, and titer measurements.

### Seed train expansion of NISTCHO for bioreactor inoculation

NISTCHO WCB (10197/p09/EX-CELL® CD CHO/FDA) vials were thawed using an automated thawing system and pipetted into 10 mL of prewarmed EX-CELL® CD CHO Fusion cell culture medium in a 15 mL sterile tube. The tube was centrifuged at 200 × g for 5 min to pellet the cells. The clarified medium was removed using a pipette and discarded. The cell pellet was resuspended in 30 mL of prewarmed culture media, added to a sterile 125 mL non-baffled vented-cap Erlenmeyer shake flask and placed in a INFORS HT Multitron Incubator (Infors AG) at 37°C, 5% $CO_2$, 130 rpm, and 85% humidity. The cells were split every 3–4 days by seeding to 0.4–0.6 × $10^6$ cells/mL in prewarmed medium in the appropriate size shake flask until there were enough cells to inoculate the desired number of bioreactors. The optimized growth (OG) protocol is the same as the standard protocol (SP) except for using patented shake flasks with optimized geometries for low shear and




**Scheme 2.** Seed train and bioreactor process development for NS0–59 and NS0–66. Created in BioRender. Berilla, E. (2025) https://BioRender.com/ohselrl.

increased aeration (Thomson Instrument Company, Cat # 931110, 931112, 931114).

On the day of inoculation, cells were counted and the appropriate volume of inoculum to reach the desired initial cell density was added to each single-use modular bioreactor (Sartorius Ambr® 250 modular bioreactors, Cat # 001-2A23) that was removed and placed in the biosafety cabinet or pumped directly from the inoculum shake flask into the cell bag (Cytiva, Cat # CB002L722–33 and CB002LFDA).

### Seed train expansion of NS0–59 and NS0–66 cells for bioreactor inoculation

NS0–59 and NS0–66 WCB cell vials were thawed using an automated thawing system and pipetted into 10 mL of pre-warmed CD Hybridoma Medium (Gibco, Cat # 11279–023) in a 50 mL sterile tube. The tube was centrifuged at 200 × g for 5 min to pellet the cells. The clarified medium was removed using a pipette and discarded. The cell pellet was resuspended in 12 mL of medium supplemented with 2 mm L-glutamine and 10 mL/L of 100X MEM NEAA in a 75-mL cell culture T-flask with vented cap (Corning, Cat # 430641 U) and placed in a INFORS HT Multitron Incubator (Infors AG) at 37°C, 5% $CO_2$, and 85% humidity. When cell density reached above $1.5 \times 10^6$ cells/mL, the T-flask cultures were scaled up to a sterile 125 mL non-baffled vented-cap Erlenmeyer shake flasks (SP) or Thomson Optimum Growth® Flasks (Thomson Instrument Company 931,110) (OG protocol), seeded at a cell density of $0.5–0.7 \times 10^6$ cells/mL and placed in the incubator at 37°C, 5% $CO_2$, 100 rpm, and 85% humidity. The cells were checked on day 0 for cell density and viability and split every 2–3 days until enough cells were generated to inoculate bioreactors.

On the day of inoculation, cells were counted and the appropriate volume of inoculum to reach the desired initial cell density was added to each single-use bioreactor that was removed and placed in the biosafety cabinet (Sartorius Ambr® 250 modular bioreactors, Cat # 001-2A23).

### Bioreactor operations and in-process sampling

NISTCHO fed-batch bioreactors were charged using EX-CELL® Advanced CHO Fed-batch Medium (SAFC 14366C or 24366C) and fed every other day with a 50% glucose solution and 5–15% EX-CELL® Advanced CHO Feed 1 (without glucose). Process parameters for single-use modular bioreactors are detailed in Table 1. NISTCHO perfusion bioreactors were charged using EX-CELL® Advanced CHO Fed-batch Medium and perfused with EX-CELL® Advanced HD Perfusion Medium (SAFC, Cat # 24370C). Process parameters for the rocking fed-batch and perfusion bioreactors (Cytiva ReadyToProcess WAVE™ 25) are detailed in Table 2.

NIST NS0 fed-batch bioreactors were charged using CD Hybridoma Medium with 2-4 mm L-glutamine and 1X NEAA. In Process C, 1X MEM Amino Acids (MEM Amino Acids (50×) solution, Sigma-Aldrich M5550) were also added and fed every other day. Process parameters for the NS0 clones in single-use modular bioreactors are detailed in Table 1.

Samples were removed from shake flasks and bioreactors at least once daily and run on the BioProfile® FLEX2 automated cell culture analyzer (Nova Biomedical) to measure pH, $pO_2$, $pCO_2$, osmolality, VCD, cell viability, cell diameter, glutamine, glucose, lactate, glutamate, ammonium, sodium, potassium, and calcium.

### Titer measurement

Samples from shake flasks or bioreactors were centrifuged at 200 × g to remove cells, sterile filtered using a syringe with 0.2 µM PVDF or PES filter (Millipore, Cat # SLGVR33RS or

**Table 1.** Process parameters and outcomes for single-use modular bioreactor systems culturing NISTCHO, NS0–59, and NS0–66 cells.

| | NISTCHO | | | NIST NS0–59 and NS0–66* | | |
|---|---|---|---|---|---|---|
| Process Parameter | Process A | Process B | Process C | Process A | Process B | Process C |
| Temperature (°C) | 37 | 37 | 37 | 37 | 37 | 37 |
| pH | 7.1 | 7.1 | 7.0 | 7.1 | 7.1 | 7.1 |
| Agitation (rpm) | 600–800 | 200–400 | 300 | 100–250 | 300 | 300 |
| DO (%) | 50 | 50 | 40 | 50 | 50 | 50 |
| $CO_2$ sparge (mL/min) | 0–2.3 | 0–2.3 | 0–2 | 0–2.3 | 0–2 | 0–2 |
| $CO_2$ headspace (mL/min) | 0 | 0 | 0.100 | 0 | 0.100 | 0.100 |
| Air sparge (mL/min) | 30–150 | 30–150 | 0.05 | 30–150 | 0.05 | 0.05 |
| Air headspace (mL/min) | 0 | 0 | 2.1 | 0 | 2.1 | 2.1 |
| $O_2$ sparge (mL/min) | 0–75 | 0–75 | 0–15 | 0–75 | 0–15 | 0–15 |
| $O_2$ headspace (mL/min) | 0 | 0 | 0 | 0 | 0 | 0 |
| Total gas flow (mL/min) | 30–150 | 30–150 | 0.05–12 | 30–150 | 0.05–12 | 0.05–12 |
| Total headspace gas flow (mL/min) | 0 | 0 | 2.2 | 0 | 2.2 | 2.2 |
| Glucose feed days | 3–7 | 3–7 | 3, 5–14 | 4,6 | 2–5 | 2–5 |
| L-gln feed days | NA | NA | NA | 4,6,7 | 2–5 | 2,4 |
| CHO Feed 1 days | 3,5,7 | 3,5,7 | 3,5,7,9,10–14 | NA | NA | NA |
| 1X NEAA, 1X EAA, and 1X vitamins days | NA | NA | NA | NA | NA | 2,4,6 |
| Run durations (days) | 8 | 8 | 15 | 9 | 7 | 8 |
| Peak VCD ($x10^6$ cells/mL) | 4.3 | 6.7 | 13 | 3.0 | 3.9 | 4.6 |
| Viability at harvest (%) | 70 | 79 | 92 | 53 | 48 | 34 |
| Titer at harvest (µg/mL) | 1374 | 1562 | 1734 | 216 | 72 | 73; 127** |

NA: not applicable.
*Process A was only tested with NS0–59
**Titer for NS0–66

**Table 2.** Culture parameters for NISTCHO fed-batch and perfusion in rocking bioreactor cultures.

| | Setpoint or Description | | |
|---|---|---|---|
| Culture Parameter | GS CHOZN® Technical Bulletin | NISTCHO Fed-batch | NISTCHO Perfusion |
| Cell bag Volume | 20 L | 2 L | 2 L or 10L |
| Starting Volume | 1–10 L | 0.55–0.7 L | 0.7 L |
| Gas Mix Flow Rate | 0.1 L/minute, fixed flow | 0.3 L/minute, fixed flow | 0.3 L/minute, fixed flow |
| O2 mix range | NS | 21–50 % | 21–50 % |
| CO2 mix range | 5% | 0–15 % | 0–15 % |
| Temperature | 37 °C | 33–37 °C * | 32–36.5 °C * |
| pH | NS | 7.1 | 7.1 |
| pH Control Method | NS | $CO_2$/Base (1M NaOH) | $CO_2$/Base (1M NaOH) |
| Dissolved Oxygen | NS | 50 % air saturation | 50 % air saturation |
| Dissolved Oxygen Control Method | NS | $O_2$ (rocking speed/angle control manually) | $O_2$ (rocking speed/angle control manually) |
| Rocking Speed | 10–25 rocks per min (rpm) | 15–28 rocks per min (rpm) | 15–28 rocks per min (rpm) |
| Rocking Angle | 8 ° | 8–11 ° | 8–11 ° |
| Glucose Feed Timing Criteria | Maintain glucose >2 g/L | Maintain glucose >1 g/L | NA |
| Glucose Feed Amount | Supplement up to 6 g/L | Supplement up to 6–8 g/L | NA |
| CHO Feed 1 Timing | Every other day starting on day 3 | Every other day starting on day 3 | NA |
| CHO Feed 1 Amount | 5–15% | 5–15% | NA |
| Perfusion Start Criteria | NA | NA | Glucose ≤ 1.5 g/L |
| Perfusion Rate | NA | NA | 0.5–2 VVD |
| Cell Bleed Criteria | NA | NA | VCD ≥ 55 x $10^6$ cells/mL |
| Harvest Criteria | NA | CHO cell viability < 60 % | CHO cell viability < 60 % or 21 days |

NA: not applicable.
NS: not specified.
*All batches were run at 37°C from inoculation through the exponential growth phase. Temperature downshift was generally performed once cell entered stationary phase for fed-batch runs, and once cells reach 50 x $10^6$ cells/mL for perfusion run.

SLGPR33RS) and stored at 4°C for up to 7 days. Titer was measured using Protein A dip-and-read biolayer interferometry (BLI) sensors (Sartorius, Cat #18–5010) on the Octet® Red96e (Sartorius) and applied to a standard curve made using NISTmAb RM 8671. For samples expected to lie close to the outside of the upper analytical range of the standard curve, dilutions of the sample in culture medium were used.

### *Screening DOE for NS0 feed optimization*

Feeds were added into 24-well deep well plates (Thomson Instrument Company 931,568) for final concentrations of 4 mm L-glutamine (Corning, Cat # 25005 CI), 1X MEM NEAA, 1X MEM Amino acids, and 1X MEM Vitamins solution (Millipore Sigma, 100×, Cat # M6895-100 mL). NS0–59 and NS0–66 were resuspended in CD Hybridoma media (Gibco, Cat # 12372001) supplemented with an additional 2 g/L glucose and 4 mL were added into each well of two 24-well plates for each clone. The starting VCD and viability for clone 59 were $0.622 \times 10^6$ cells/mL and 97.2%, respectively, and for clone 66 were $0.728 \times 10^6$ cells/mL and 94.1%, respectively. Plates were loaded into a microtiter plate box (Infors HT) and incubated at 37°C, 5% $CO_2$, 85% humidity agitating at 200rpm with a 25 mm shaking throw.

A full factorial design of experiment (DOE) was performed using JMP 17 software Easy DOE Guided Mode (Supplementary Table S1). Response data were collected daily as VCD and viability, and on Day 4/the final day of the culture, titer was measured (Supplementary Tables S2 and S3). Four discrete numeric factors with 2 levels (0 and 1-2X) were used: L-glutamine, NEAA, amino acids, and vitamin solution. A default number of runs of 16 was used. The model estimated the individual factor effects and the two-factor interaction effects. For analysis, the best model was fit for each response variable using a stepwise fitting procedure using the Akaike Information Criterion to select terms. If terms were removed from the model, fit least squares was used to recalculate the parameter estimates.

## Results and discussion

### *NISTCHO seed train cell growth and viability trends improved in shake flasks with reduced shear and increased aeration*

Our MCB vial of RGTM 10197 was 97% viable at thaw and remained > 96% viable during the 13-day expansion process to generate our WCB. NISTCHO cell vial thaws from our WCB averaged 95.5 ± 2.1% viable ($N = 12$) and consistently stayed above 92% viable during expansion in shake flasks up to 4 weeks (data not shown). While improvement was not necessarily needed for the seed train cell expansion process, we found that, when using shake flasks optimized for mammalian cell growth (OG), the growth rate trend increased in the latter half of a 13- or 14-day seed train protocol as compared to standard shake flasks (SP) (Figure 1a). The increase in total cells generated from OG reached statistical significance as defined by one-tailed homoscedastic t test between SP and OG ($^*p < 0.05$) on days 13 and 14. The maximum oxygen transfer capacity ($OTR_{max}$) for a shake flask is a measure of the maximum rate at which oxygen transfers between the gas and liquid phase (cell culture media). Several parameters can affect the shake flask culture's $OTR_{max}$, including the shaking frequency, orbital diameter of the shaker, size of the shake flask, and filling volume of the shake flask. In our experiments we did not vary the shaking frequency or orbital diameter of shaking and shake flask filling volumes did not exceed the working volume upper limits (20–35% for SPs and 50–56% for OGs, depending on size). We note that seed train cultures that used lower working volumes were those with the highest

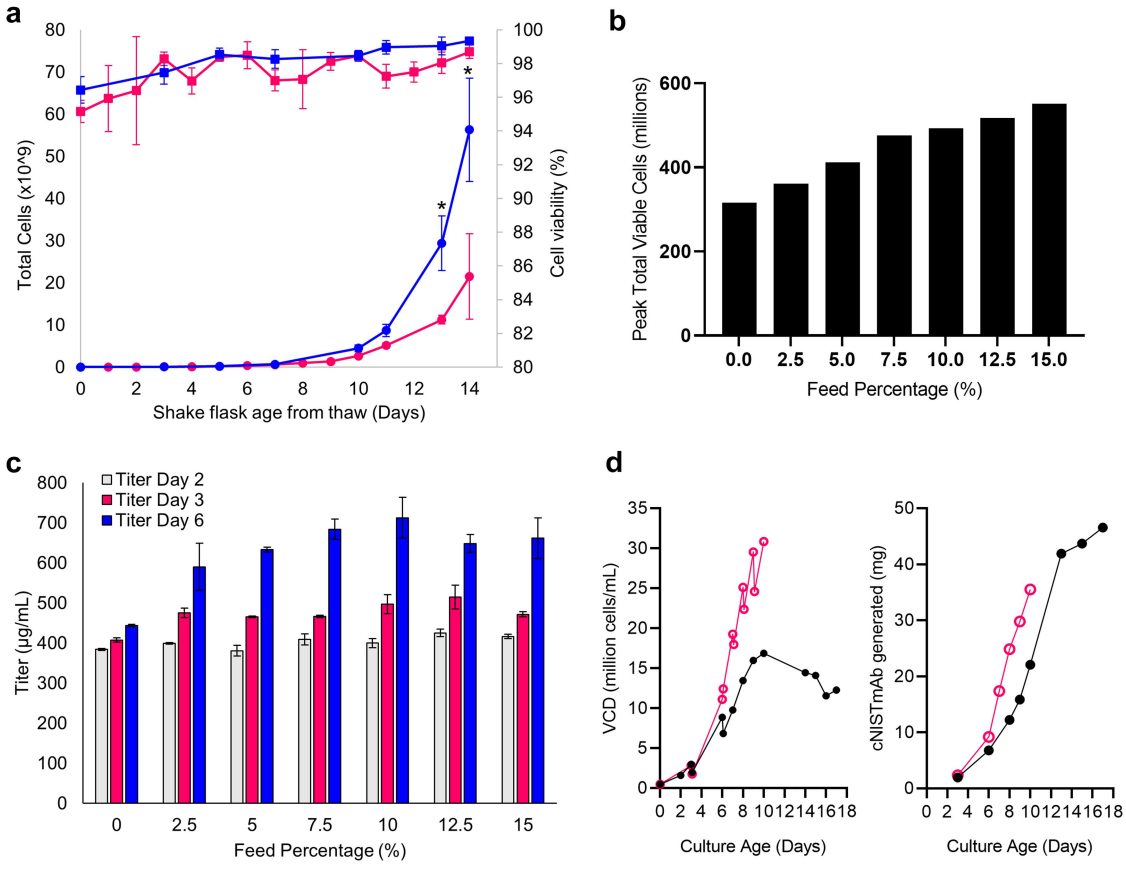



**Figure 1.** NISTCHO seed train and fed-batch development. (a) Comparison of seed train growth (circles) and viability (squares) in traditional cell culture shake flasks (SP; pink; $N = 10$) versus shake flasks with optimized aeration (OG; blue; $N = 3$) with error bars indicating ± 1 standard error of the mean. Statistical significance is defined by one-tailed homoscedastic $t$ test between SP and OG (*$p < 0.05$) (b) Peak total viable cells reached during a six-day culture and (c) volumetric titer depending on percentage of complex feed added every other day. Error bars indicate ±1 standard error of the mean of three measurements. (d) Viable cell density and total cNistmab generated in 30 mL cultures of NISTCHO in either fed batch (black) or perfusion media (pink).

growth rates, and differences in shake flask sizes and fill volumes may be the reason for the high variability within data sets. The difference in growth rate may have increased later in cell culture expansion (after day 7), as typically this is the day of the second cell split since thawing, which is when the cells are moved from a 250- or 500-mL shake flask into larger shake flask sizes (1-2 L). Aeration within shake flasks typically decreases with shake flask size,[20] which may explain why the optimized shake flasks can improve the growth of NISTCHO once the culture volumes and shake flask size are larger.

### *Evaluation of feed volume and media type on NISTCHO growth and productivity*

To assess the ideal feeding regimen for NISTCHO in fed-batch bioreactors, we ran fed-batch 30 mL shake flasks starting at high cell density to represent approximately half the expected peak VCD, and varied feeding every other day with 0, 2.5, 5, 7.5, 10, 12.5 or 15% CHO Feed 1. The most notable findings were that there were no negative effects on cell growth when feeding with the maximum 15% CHO Feed 1 and the 15% feed flask reached the highest total peak viable cells (Figure 1b). For titer, the impact of the CHO Feed was not fully apparent until day 6, at which point the titer when feeding between 5–15% was similar (Figure 1c). We used these data to inform our feeding regimen for our fed-batch stirred tank and rocking bioreactor runs, for which we used 5–15% CHO Feed 1 dependent on the VCD measured prior to feeding.

Additionally, we compared growth rate and productivity between fed-batch and perfusion media shake flasks under simulated perfusion conditions at 1 vessel volume per day (VVD) wherein we exchanged two-thirds of the media in the shake flask each day on days 3 and 6–10. The fed-batch shake flask was fed glucose daily to maintain the culture >1.5 g/L and CHO Feed 1 at 5–10% every other day starting from day 3–17. NISTCHO could grow to higher density in the perfusion media, reaching a peak VCD on day 10 that was nearly twice the VCD of the fed-batch culture (Figure 1d). The rate of mAb generation was also higher for NISTCHO under the simulated 1 VVD perfusion conditions between day 6–10, leading to higher cNISTmAb production (Figure 1d) from both increased cell density and cell-specific productivity increases of 31%.

### *Development of a fed-batch stirred tank bioreactor process for NISTCHO using a single-use modular system*

The process conditions tested for NISTCHO using a single-use modular bioreactor system are listed in Table 1. In the initial test process (Process A) using settings from a previous CHO cell line cultured in our lab, we used high agitation rates

(600–800 rpm), no headspace gassing, and high gas sparge rates (up to 150 mL/min), but those parameters led to a slow growth rate and viability drops starting on day 3 of the batch (Figure 2a). In Process B we reduced the agitation rates to 200–400 rpm, but kept the legacy gassing strategy, and peak VCD and viability were improved (Figure 2a). For Process C, we tried a gassing strategy more reliant on oxygen (instead of air) with a significantly lower maximum gas sparging rate (Table 1). We also incorporated headspace gassing with ~ 5% $CO_2$ for positive pressure, decreasing foaming and helping pH reach set point at the start of the batch. These improvements in gassing strategy led to a doubling in the peak VCD, increased batch duration (15 vs. 8 days) and maintenance of viability ≥ 92% for the 15-day batches (Figure 2a). Process C delivered slightly higher titer at batch harvest, but Process A and B batches had higher productivity with respect to batch age and higher cell-specific productivity (Figure 2b). These results highlighted the potential trade-off in optimizing cell growth and optimizing productivity.

Process C also differed in glucose feeding strategy (Figure 2c) in that glucose was fed to higher concentrations to ensure higher VCD cultures were sustained. Despite higher glucose concentrations, lactate traces resembled the previous processes (Figure 2d). After day 6, Process C batches began to consume lactate through day 10, after which lactate began rising again until the batches were harvested. Glutamine concentrations remained low, generally at or below the level of accurate measurement for the sensor, for all NISTCHO batches because NISTCHO has a functional GS and therefore does not require exogenous L-glutamine in culture (Figure 2e). Glutamate trends matched among processes through day 5, after which glutamate was consumed in Process C cultures through day 10 and then increased again until harvest as glutamate was present in the CHO Feed 1 daily feeds (Figure 2f). The apparent switch from glutamate and lactate decreasing to increasing trends may indicate that the cells were being overfed with CHO Feed 1 from day 10 onwards, representing a part of the process that could be improved through optimized feeding strategy. Ammonium concentrations remained below 3 mm for the entire batch durations for all processes (Figure 2g), which is advantageous since high levels of ammonium can decrease cell growth, cell viability, cell productivity, and impact post-translational modifications like glycosylation.[21] Osmolality was higher for Process C batches than Process B, most likely because feeds were added at higher concentrations to maintain higher cell densities and more added base was required to maintain pH (Figure 2h). Process C batches did not exceed osmolality above 400 mOsm/kg, which is within the typical range for fed-batch CHO cell cultures.

Because Process C incorporated a constant flow of $CO_2$ as part of the headspace gassing, pH was set to 7.0 in Process C instead of 7.1 as in Process A and B. A lower pH setpoint helped to avoid increasing the amount of base addition during the culture's exponential growth phase when lactate is produced in the highest amounts and drives down culture pH. Overall, pH control was more consistent with the Process C control strategy and once the batches reached a stationary phase on day 6, base additions were generally not needed and $CO_2$ sparge remained steady around 0.5 mL/min (Figure 2i). The dissolved oxygen (DO) setpoint for Process C was also set lower than Process A and B at 40%, but $O_2$ sparge rate is the parameter where Process C differs the most from Process A and B (Table 1 and Figure 2j). For Processes A and B, when DO would drop below set point, $O_2$ flow would spike up to as much as 75 mL/min, which likely caused shear stress created by the bubbles colliding with the cells and can lead to cell damage. In Process C, the maximum $O_2$ sparge flow was decreased to 15 mL/min, which decreased the potential for cell damage. Future optimizations to NISTCHO stirred tank bioreactors should take into consideration the cell's sensitivity to shear stress if using the EX-CELL® Advanced CHO Fed-batch Medium.

### *Development of a fed-batch and perfusion rocking bioreactor process for NISTCHO*

Rocking bioreactor cultures of CHO cells are mostly used in the biomanufacturing industry for scaling up prior to production stirred-tank bioreactors, but they can also be useful to compare the effects of fed-batch versus perfusion operations on cell growth, productivity, and quality attributes. We aimed to generate processes for NISTCHO in rocking bioreactors that can be used as a starting point to test questions surrounding media or feed changes, batch duration or cell age, integrating process analytical technologies and other process modifications. The process parameters used for NISTCHO fed-batch and perfusion bioreactor runs are described in Table 2. We began with developing the fed-batch process with guidance from the GS CHOZN® Technical Bulletin, but there are limited parameters described because the protocol is intended for seed culture expansion purposes. For the fed-batch process, the average peak VCD was $11.6 \times 10^6$ cells/mL, compared to $13 \times 10^6$ cells/mL in the stirred-tank Process C, and batches lasted 12 days at which point they were harvested because viability dropped to or below 60% (Figure 3a). The titer was lower for the rocking bioreactor fed-batch process with titers at 1.1–1.2 mg/mL (0.75–0.85 g total) (Figure 3b) than for the stirred tank fed-batch process with titers at 1.4–1.9 mg/mL (Figure 2b) and could likely be further optimized for increased cell health, batch duration, and cell productivity. The perfusion rocking bioreactor processes were run for up to 21 days and were maintained at ~ $50 \times 10^6$ cells/mL with only slight dips in cell viability down to 85% (Figure 3a). One perfusion batch was ended on day 21 due to fouling of the floating perfusion filter that started on day 18. Significant standard error from the mean in the VCD data was observed during exponential growth (days 6–10) due to differences in the duration of the lag phase between runs. Perfusion operations resulted in higher productivity than fed-batch, generating an average of 2.7 times as much mAb on day 12 when the fed-batch runs were harvested, and an average of more than 10 times as much mAb by day 21 (Figure 3b).

Under fed-batch conditions, glucose concentrations were more variable (1–8 g/L) whereas glucose consistently measured between 0.2–2 g/L during perfusion operation (Figure 3c), but glucose differences did not affect lactate trends, which were similar between both operation types

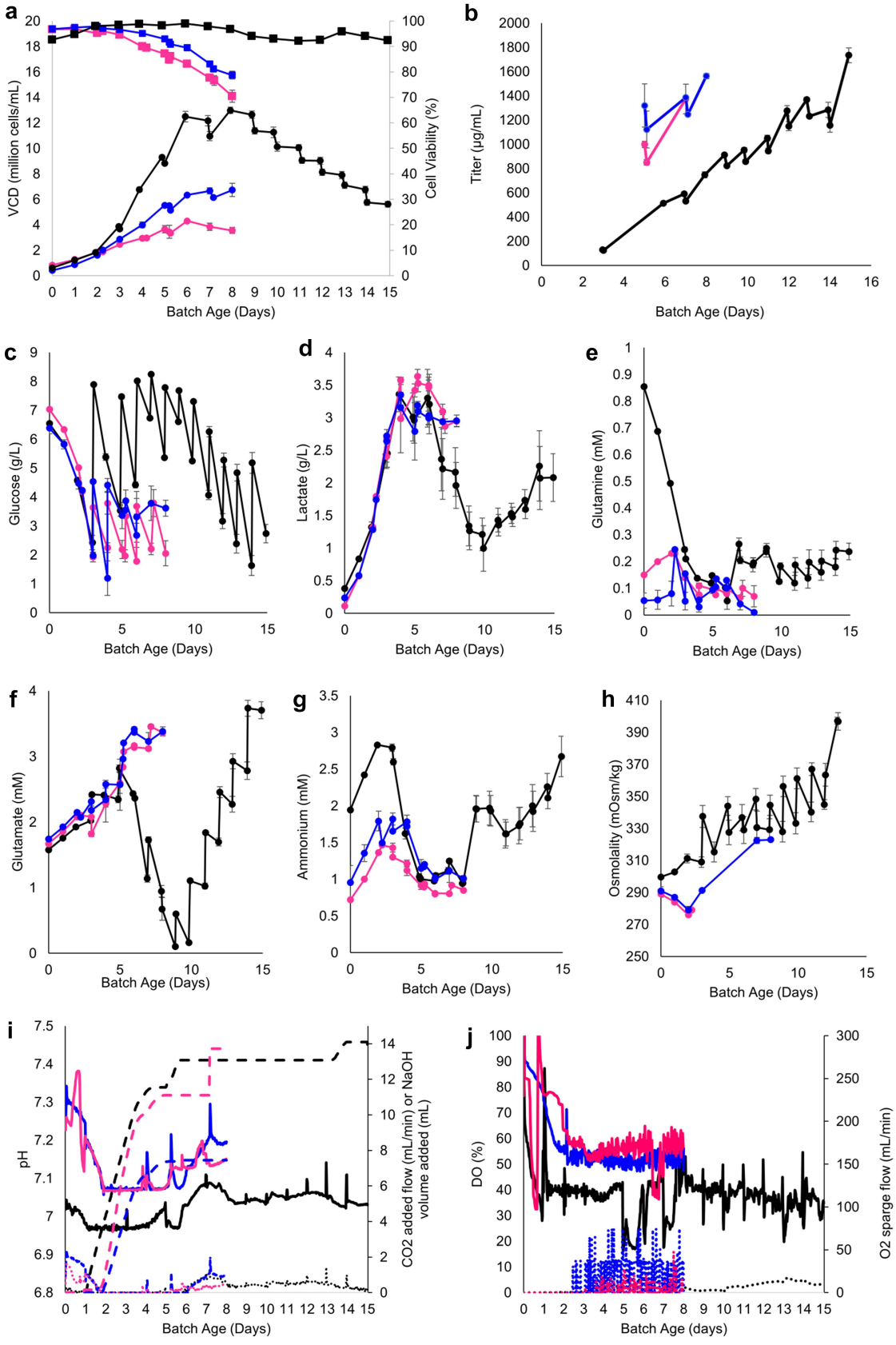



**Figure 2.** Process development of NISTCHO in single-use modular bioreactors. Process a is shown in pink, process B is in blue, and process C is in black. (a) Viable cell density (circles) and cell viability (squares) (b) volumetric titer (c) glucose (d) lactate (e) glutamine (f) glutamate (g) ammonium and (h) osmolality data from processes A, B, and C for NISTCHO that differ in agitation rates and gassing strategies. (i) pH and pH control for processes A, B, and C. Solid lines are pH, dashed lines are base addition, and dotted lines are $CO_2$ added flow. (j) DO and oxygen sparging among processes A, B, and C. Solid lines are DO and dotted lines are $O_2$ sparge flow.

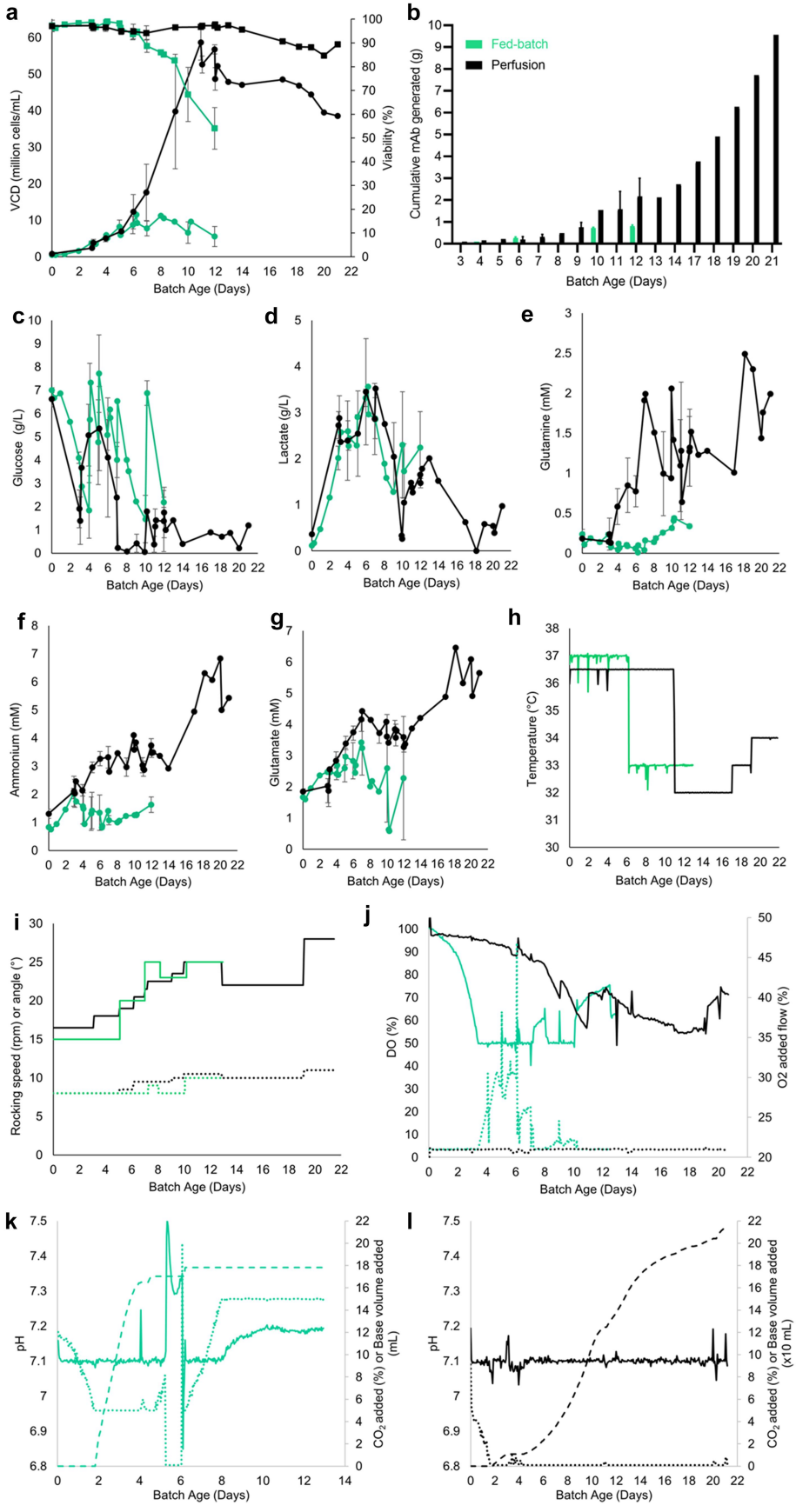



**Figure 3.** Process development of NISTCHO in fed-batch and perfusion operation in a rocking bioreactor. Fed-batch runs ($N = 2$) are shown in green and perfusion runs ($N = 2$) are shown in black. Where runs were averaged, error bars indicate ± 1 standard error of the mean. (a) Viable cell density (circles) and cell viability (squares). (b) Cumulative mAb generated during fed-batch and perfusion operations. (c) Glucose (d) lactate (e) glutamine (f) glutamate and (g) ammonium from fed-batch and perfusion bioreactor runs. (h) Representative temperature from one fed-batch and one perfusion run. (i) Rocking speed (solid lines) and angle (dotted lines) from the fed-batch and perfusion bioreactor runs. (j) Representative DO (solid lines) and oxygen percentage added overlay flow (dotted lines) from the fed-batch and perfusion bioreactor runs. (k) Representative pH (solid line), $CO_2$ overlay (dotted line), and base addition (dashed line) for a fed-batch bioreactor run. (l) Representative pH (solid line), $CO_2$ overlay (dotted line), and base addition (dashed line) for a perfusion bioreactor run.

(Figure 3d). Glutamine was very low and sometimes below the analytical range minimum (0.1 mm) for fed-batch, as expected because there is no L-glutamine in the media or feeds, but trends were unexpected for perfusion batches with glutamine measurements reaching as high as 2.5 mm despite no glutamine addition (Figure 3e). Since glutamine is not formed spontaneously, it can be assumed to be formed intracellularly. One possibility is that overexpression of glutamine synthetase within NISTCHO cells may result in the transport of excess glutamine out of the cell. Another possibility is that the glutamine in the culture media could be released from the dead cells into the culture. Ammonium (Figure 3f) and glutamate (Figure 3g) also remain low under fed-batch operations but increase over the course of the batch during perfusion, with the highest concentrations between days 17–21. Typically perfusion operations are associated with lowering the accumulation of waste products like ammonium due to the continuous removal of spent media[22] and ammonium accumulates when the cells metabolize L-glutamine from the culture media.[23,24] The mechanism by which ammonium and glutamate increase toward the end of the perfusion batches is currently undetermined, but it may be related to a shift in nitrogen metabolism and/or the start of fouling of the perfusion filter, which may cause cell stress.

For both fed-batch and perfusion processes, we incorporated temperature down-shifts to control cell density, increase batch duration, increase cell-specific productivity,[25] and increased the duration between manual cell bleeds (Figure 3h). In shake flask experiments testing temperatures to be used during the stationary phase, we found that NISTCHO could be cultured at 30°C for up to 2 weeks with only a small decrease in viability, but at 32°C NISTCHO maintained slow growth and high viability in perfusion media (data not shown). Thus, 32°C was selected as the minimum temperature to be used for perfusion operations. Increasing rocking speed and angle helped to increase aeration of the culture and was controlled manually during our processes so that cell bags could be run in dual mode. Using rocking angle and speed as part of DO control may also be a good strategy, but we did not test this because we typically operate in dual-mode, which cannot allow for incorporation of rocking parameters into feedback control loops. In general, to conserve $O_2$, rocking speed and/or angle (Figure 3i) were increased manually when increasing $O_2$ flow to the system was observed or DO approached the setpoint (Figure 3j). The pH was maintained within the desired operating ranges for the fed-batch runs except in a single batch when the $CO_2$ flow was disrupted on day 5, leading to a spike in pH that lasted through day 6 (Figure 3k). After day 8, $CO_2$ began rising to adjust the rising pH and once $CO_2$ flow reached its maximum allowable flow of 15%, the pH value remained ~ 7.2 for the remainder of the batch run. Interestingly, the perfusion batches required very little $CO_2$ and used 10 times the amount of added base in 21 days compared to the amount the fed-batch runs averaged in 12 days (Figure 3l). These differences were attributed to variations between the fed-batch and perfusion media, but also may be indicative of metabolic shifts of NISTCHO under high-density perfusion conditions.

### NIST NS0–59 and NS0–66 growth and viability improved in shake flasks with greater aeration and reduced shear stress

Successful shake flask expansion of NS0–59 and NS0–66 to generate WCBs, run feed studies, and inoculate bioreactors was a challenge for the study of the non-originator NISTmAb expressed in NS0 cells. The vials used to generate our initial WCBs of NS0–59 and NS0–66 were 82.1% and 46.7% viable at thaw, respectively, and 78–79% viable when frozen for storage as our in-house WCBs. Attempts to improve shake flask expansion included supplementing lipids, adapting to different commercial media for NS0 cells, keeping cell density lower or higher during expansion, splitting from the initial *T*-75 into multiple *T*-75s prior to moving to shake flasks, adjusting pH, changing shaking speeds, and performing daily media exchanges. However, none of these changes resulted in any consistent improvements to growth rate or viability, and cell viability was typically not high enough to inoculate the replicate bioreactors.

The shake flask expansion process was improved only when the cells were cultured in shake flasks optimized for increased aeration and reduced shear. These Optimum Growth® Flasks are patented shake flasks designed for high aeration and low shear due to a unique baffle design that has been optimized for mammalian cell lines. The improvements for both NS0–59 and NS0–66 were evident in their growth rates (Figure 4a,c) and viability (Figure 4b,d). Viability was maintained above 90% from the first measurement after the day of thaw through day 14 of culture, which allowed for inoculation of bioreactors at high cell viabilities. Reliable seed train expansion also meant that performing a DOE to test feeding regimens was feasible. While outside of the scope of this work, computational fluid dynamics modeling and efforts to measure DO and/or shear stress could be performed to strengthen the proposed rationale for culture improvements.

### NIST NS0–59 and NS0–66 growth, viability, and titer were not affected by amino acid or vitamin supplements

A preliminary screening DOE tested several feed supplements, including commercially available fatty acids, lipids, insulin, chemically defined mammalian cell feeds, soy hydrolyzate, amino acids, and vitamins (data not shown). Based on the preliminary results, a second DOE designed to estimate the individual factor effects and the two-factor interaction effects of L-glutamine, EAA mix (arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine), NEAA mix (alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, and serine), and a vitamins mix (choline, folic Acid, myo-inositol, niacinamide, D-pantothenic acid, pyridoxal-HCl, riboflavin, and thiamine) on cell growth, viability, and productivity. Despite some preliminary evidence of positive impacts of NEAA, EAA, and vitamins on the growth rate and viability of NS0–59 in shake flasks, the main factor effects of these feeds on growth, viability, and titer were minor for a 4-day process with feeds added on days 0 and 2 (Table 3). Using

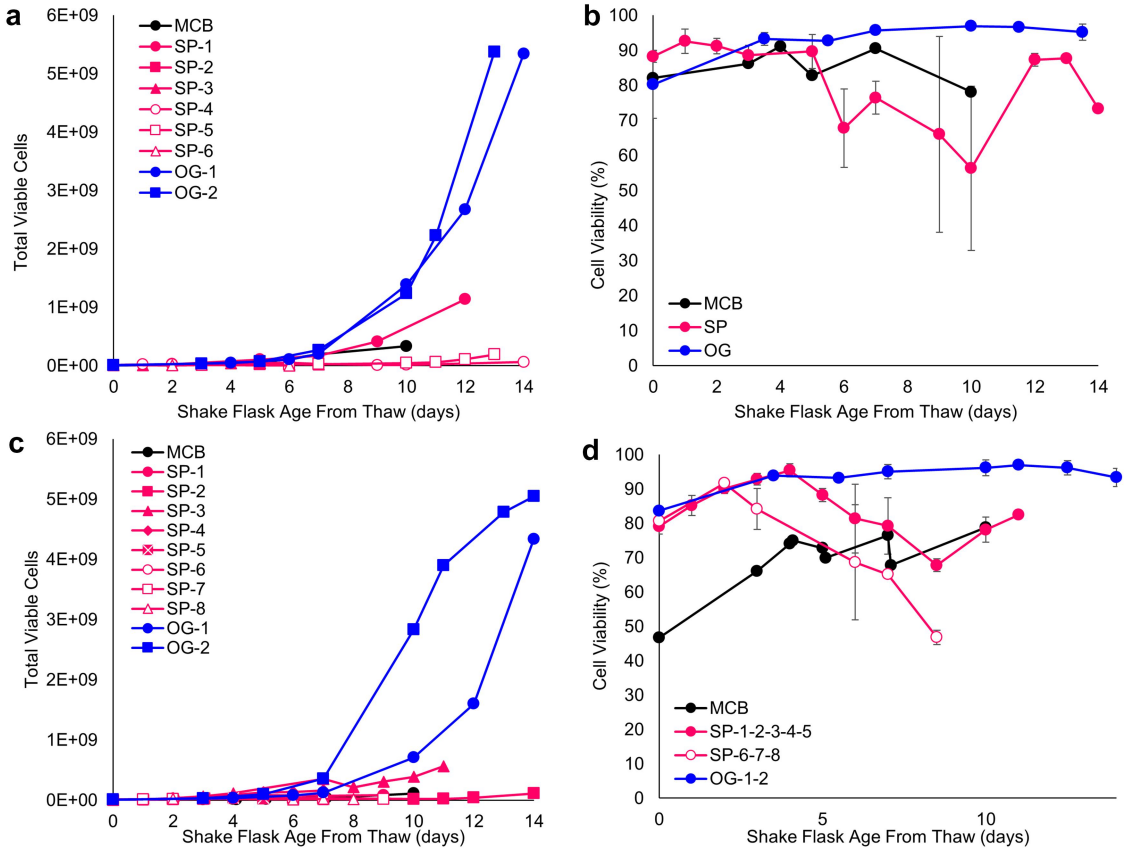



**Figure 4.** NISTNS0 clones seed train development. Seed train expansion was a major challenge with the NS0 clones using the standard protocol (SP) that was solved by using shake flasks with optimized geometry for cell culture (OG). (a) Total viable cells and (b) mean cell viability during seed train expansion for NS0 clone 59 MCB vial thaw (black line, $N = 1$), SP vial thaws (pink lines, $N = 6$), and OG vial thaws (blue lines, $N = 2$). Error bars indicate ± 1 standard error of the mean. (c) Total viable cells and (d) mean cell viability during seed train expansion for NS0 clone 66 MCB vial thaw (black line, $N = 1$), SP vial thaws (pink lines, $N = 8$), and OG vial thaws (blue lines, $N = 2$). Error bars indicate ± 1 standard error of the mean. SP-1, SP-2, SP-3, SP-4 and SP-5 were vial thaws of a different WCB than SP-6, SP-7, and SP-8, and thus for cell viability they were treated as two separate datasets.

the "best model" selection by JMP for each response variable indicated that NEAA increased NS0–59 and NS0–66 cell viability from day 2 and on, but NEAA decreased the titer. EAA and vitamins did not affect cell growth, but also decreased titer. Maintaining available L-glutamine, which is known to be required for NS0–59 and NS0–66, was the only factor that resulted in increased growth, viability, and titer. No consistently positive two-factor interaction effects were identified for either clone. However, no consistently negative effects from the NEAA, EAA or vitamins were identified other than apparent small decreases to titer. Since depletion of certain amino acids can impact mAb glycosylation, we decided to test the amino acid and vitamin supplements for our fed-batch Process C.

### *Development of a fed-batch stirred tank bioreactor process for NIST NS0–59 and NS0–66 using a single-use modular system*

NS0–66 was not used in initial fed-batch bioreactor experiments for process development because we were unable to generate enough high viability cells for inoculum from the initial seed train attempts. Thus, initial fed-batch bioreactor experiments for NS0–59 were run under Process A (Table 1). Process A runs were agitated at 100–250 rpm and gassed with high air and oxygen sparge rates (Figure 5k). While growth was slow (Figure 5a) and viability slowly decreased over the first 3 days of the batch (Figure 5b), the viability stayed above 80% for the first 7 days. Process B and C parameters are very similar, but like Process A, Process B runs also used NS0–59 cells cultured using the SP, and thus cells averaged 86% viability at inoculation. While the increased agitation (300 rpm) and decreased gas sparging rates of Process B improved the growth rate of NS0–59 and increased the peak VCD (Figure 5a), viability began to drop earlier in the batch after day 5 (Figure 5b). Process C was inoculated with cells from a WCB made from higher viability cells expanded using OG, from which a vial was thawed and expanded using OG to inoculate the bioreactors. Process C batches began with cells at an average viability of 97% (NS0–59) or 98% (NS0–66), had the highest growth rates, and peaked in VCD on day 3 (Figure 5a). Despite positive signs early in the runs, viability started dropping after day 2 and continued to drop until the batches were ended on day 8 (Figure 5b).

Process A batches resulted in higher titer than Process B and C batches, with average titers >200 µg/mL, comparable to shake flask results[11] (Figure 5c). Despite higher cell viability, faster growth rates, and higher peak VCD, Process C batches for NS0–59 averaged 73 µg/mL, highlighting that improved

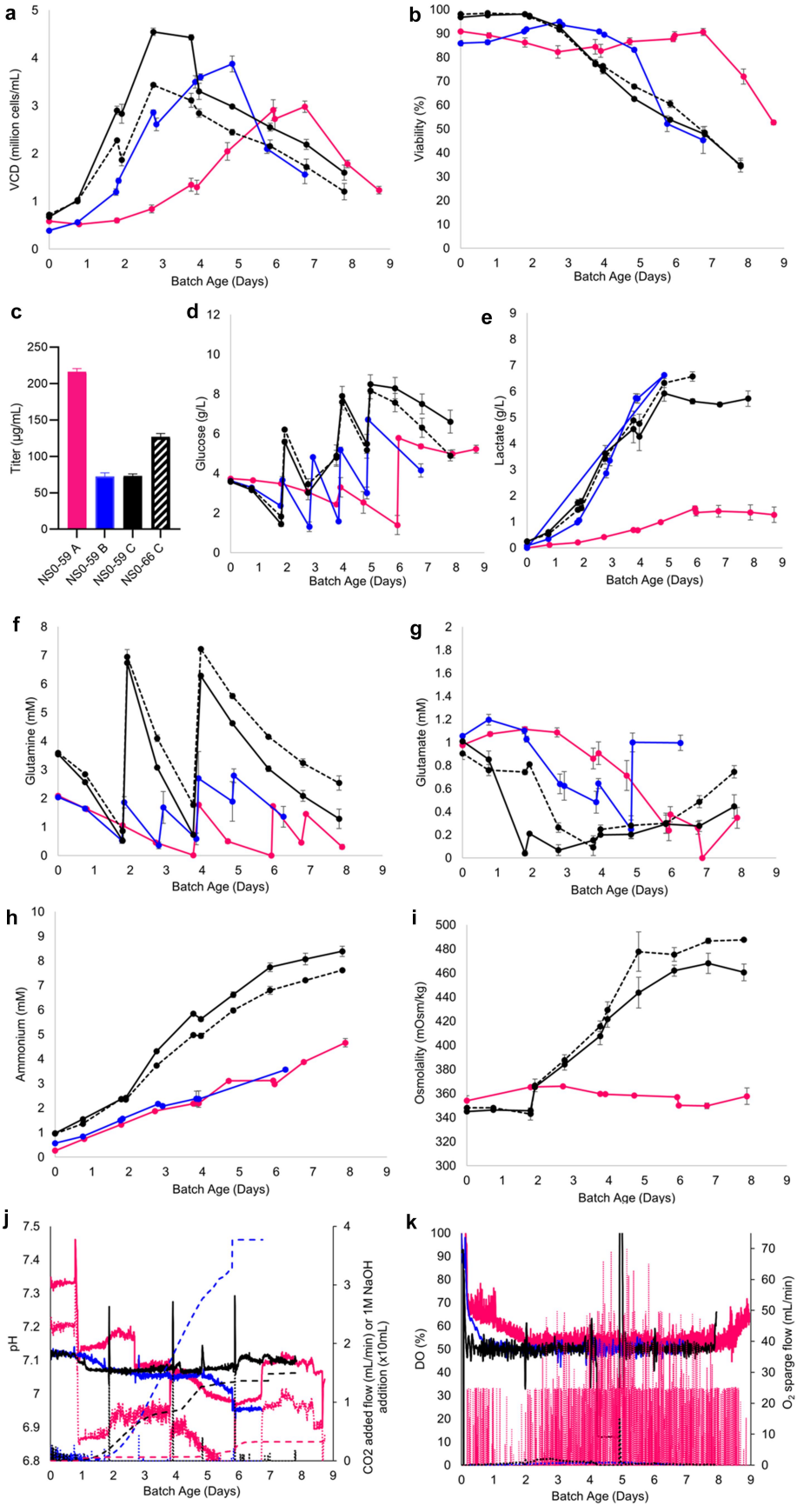



**Figure 5.** Process development of NISTNS0 clones in single-use modular bioreactors. For A-I, process a for NS0–59 is shown in pink, process B for NS0–59 is shown in blue, process C for NS0–59 is shown in black solid line or solid bar and process C for NS0–66 is shown in back dashed lines or striped bar. Error bars indicate ± 1 standard error of the mean. (a) Viable cell density (b) cell viability (c) volumetric titer (d) glucose (e) lactate (f) glutamine (g) glutamate (h) ammonium and (i) osmolality data from processes A, B, and C for NISTNS0 clone 59 and from process C for NISTNS0 clone 66. (j) Comparison of pH and pH control for NS0–59 among process a (pink), process B (blue), and process C (black). Solid lines indicate pH, dashed lines indicate base volume added (x10) and dotted lines show $CO_2$ added flow. (k) Comparison of DO and DO control for NS0–59 among process a (pink), process B (blue), and process C (black). Solid lines indicate DO and dotted lines show $O_2$ sparge flow.

**Table 3.** Individual factor and factor interaction effects on NS0–59 and NS0–66 growth and productivity.

| | | NS0 Clone 59 | | NS0 Clone 66 | |
|---|---|---|---|---|---|
| Factor or Factor Interaction | Response Variable | p value | Effect | p value | Effect |
| L-gln | Titer Day 4 | <0.001 | Increase | <0.001 | Increase |
| L-gln | VCD Day 1 | <0.001 | Increase | <0.001 | Increase |
| L-gln | VCD Day 2 | <0.001 | Increase | <0.001 | Increase |
| L-gln | VCD Day 3 | <0.001 | Increase | <0.001 | Increase |
| L-gln | VCD Day 4 | <0.001 | Increase | ND | ND |
| L-gln | Viability Day 1 | <0.001 | Increase | 0.005 | Increase |
| L-gln | Viability Day 2 | <0.001 | Increase | <0.001 | Increase |
| L-gln | Viability Day 3 | <0.001 | Increase | <0.001 | Increase |
| L-gln | Viability Day 4 | <0.001 | Increase | ND | ND |
| NEAA | Titer Day 4 | 0.009 | Decrease | 0.038 | Decrease |
| NEAA | VCD Day 1 | NI | NI | NI | NI |
| NEAA | VCD Day 2 | NI | NI | 0.041 | Increase |
| NEAA | VCD Day 3 | 0.115 | None | NI | NI |
| NEAA | VCD Day 4 | NI | NI | ND | ND |
| NEAA | Viability Day 1 | 0.152 | None | NI | NI |
| NEAA | Viability Day 2 | 0.02 | Increase | 0.039 | Increase |
| NEAA | Viability Day 3 | 0.009 | Increase | 0.023 | Increase |
| NEAA | Viability Day 4 | 0.01 | Increase | ND | ND |
| EAA | Titer Day 4 | <0.001 | Decrease | 0.001 | Decrease |
| EAA | VCD Day 1 | NI | NI | NI | NI |
| EAA | VCD Day 2 | NI | NI | NI | NI |
| EAA | VCD Day 3 | NI | NI | NI | NI |
| EAA | VCD Day 4 | NI | NI | ND | ND |
| EAA | Viability Day 1 | NI | NI | 0.158 | None |
| EAA | Viability Day 2 | NI | NI | NI | NI |
| EAA | Viability Day 3 | NI | NI | 0.072 | None |
| EAA | Viability Day 4 | 0.048 | Decrease | ND | ND |
| Vitamins | Titer Day 4 | <0.001 | Decrease | 0.01 | Decrease |
| Vitamins | VCD Day 1 | NI | NI | 0.123 | None |
| Vitamins | VCD Day 2 | 0.098 | None | NI | NI |
| Vitamins | VCD Day 3 | 0.211 | None | NI | NI |
| Vitamins | VCD Day 4 | NI | NI | ND | ND |
| Vitamins | Viability Day 1 | NI | NI | 0.103 | None |
| Vitamins | Viability Day 2 | NI | NI | NI | NI |
| Vitamins | Viability Day 3 | NI | NI | NI | NI |
| Vitamins | Viability Day 4 | 0.206 | None | ND | ND |
| L-gln*EAA | Titer Day 4 | <0.001 | Decrease | 0.004 | Decrease |
| L-gln*EAA | VCD Day 1 | 0.126 | None | NI | NI |
| L-gln*NEAA | Titer Day 4 | 0.001 | Decrease | 0.003 | Decrease |
| L-gln*NEAA | VCD Day 1 | NI | NI | 0.047 | Increase |
| L-gln*NEAA | VCD Day 3 | 0.162 | None | NI | NI |
| L-gln*NEAA | Viability Day 1 | 0.14 | None | 0.137 | None |
| L-gln*NEAA | Viability Day 2 | 0.021 | Decrease | 0.046 | Decrease |
| L-gln*NEAA | Viability Day 3 | 0.038 | Decrease | 0.008 | Decrease |
| L-gln*NEAA | Viability Day 4 | 0.009 | Decrease | ND | ND |
| L-gln*Vitamins | Titer Day 4 | <0.001 | Decrease | 0.017 | Decrease |
| L-gln*Vitamins | VCD Day 3 | 0.196 | None | NI | NI |
| L-gln*Vitamins | Viability Day 1 | 0.114 | None | NI | NI |
| L-gln*Vitamins | Viability Day 4 | 0.068 | None | ND | ND |
| NEAA*EAA | Titer Day 4 | NI | NI | 0.21 | None |
| NEAA*EAA | VCD Day 3 | 0.112 | None | NI | NI |
| NEAA*EAA | Viability Day 1 | 0.013 | Decrease | 0.077 | None |
| NEAA*EAA | Viability Day 2 | 0.081 | None | 0.169 | None |
| NEAA*EAA | Viability Day 4 | 0.076 | None | ND | ND |
| NEAA*Vitamins | Titer Day 4 | NI | NI | 0.15 | None |
| NEAA*Vitamins | VCD Day 3 | 0.059 | None | 0.054 | None |
| NEAA*Vitamins | Viability Day 1 | 0.212 | None | NI | NI |
| NEAA*Vitamins | Viability Day 2 | 0.181 | None | NI | NI |
| NEAA*Vitamins | Viability Day 3 | 0.03 | Decrease | NI | NI |
| NEAA*Vitamins | Viability Day 4 | 0.148 | None | ND | ND |
| EAA*Vitamins | Titer Day 4 | 0.246 | None | NI | NI |
| EAA*Vitamins | VCD Day 2 | 0.004 | Increase | NI | NI |
| EAA*Vitamins | Viability Day 1 | NI | NI | 0.207 | None |

ND: not determined.
NI: not included in model.

cell growth in early culture does not result in improved NISTmAb productivity. NS0–66 has higher cell specific productivity than NS0–59, but Process C batch titers were less than optimal at an average of 127 µg/mL at harvest. Altogether, the growth, viability, and titer data from the NS0–59 stirred-tank bioreactor processes suggest that the lower agitation rates and gassing strategy of Process A may be closer to the optimal process conditions for cell-specific productivity.

Due to higher growth rates early in the batch for Processes B and C, glucose was fed earlier and to higher concentrations throughout the batch (Figure 5d), which led to higher lactate production than Process A (Figure 5e). The increased lactate led to larger volumes of base added during the Process B and C batches to maintain pH (Figure 5j), which in turn added to increased osmolality (Figure 5i). Osmolality data is not shown for Process B batches due to incomplete data sets obtained from those runs. Glutamine was fed to higher concentrations in Process C to avoid culture depletion that occurred in some Process A batches (Figure 5f), which resulted in increased ammonium accumulation in the Process C batches (Figure 5h) but slightly lower or similar glutamate concentrations (Figure 5g). Because glutamate is known to be rapidly depleted in NS0 cells,[26] that only ammonium accumulates as a result of increasing glutamine metabolism was not unexpected. The increased ammonium concentrations in Process C batches could be inhibiting growth and decreasing productivity later in culture[27] or alternatively other inhibitory metabolites could play a role in the rapid decrease in viability for the NS0 cell lines.[28] While none of the processes resulted in sustained high cell viability and increased titer over measurements taken from fed-batch shake flasks, the batch data highlighted some key areas for process improvement. For example, process improvements could be aimed at improving the gassing strategy, testing increased agitation rates, avoiding accumulation of lactate and ammonium through adaptive glucose and glutamine feeding strategies, and potentially testing for impacts of the pH setpoint. A perfusion process may be better for the NS0 cell lines studied here as a strategy to decrease toxic and/or inhibiting metabolites and achieve an increased batch duration and productivity. Overall, Process A, B, and C presented here for NS0–59 and NS0–66 illustrated challenges associated with optimizing feeds, gassing, and agitation that can likely be addressed with further process development.

## Conclusions

In this study, we defined and shared process development studies that led to improved culturing of non-originator NISTmAb-producing cell lines. We identified critical process parameters and feeding strategies for the non-originator NISTmAb cell lines to improve cell health, culture longevity, and titer, and identified areas where further process improvements may still be made. For NISTCHO, we improved upon seed train growth rates by using shake flasks with optimized geometries for increased aeration and low shear, identified ideal feed percentage ranges for fed-batch protocols, tested the impacts of gassing strategy and agitation using single-use modular bioreactors, and developed fed-batch and perfusion operation strategies for a rocking bioreactor system. Additional improvements may still be made toward increasing the specific productivity of NISTCHO in both fed-batch and perfusion bioreactors. For NS0–59 and NS0–66, we demonstrated the challenges of shake flask culture, and consequently WCB generation and seed train expansion, that were overcome by using shake flasks with increased aeration and low shear. We also used DOE feeding studies to determine that L-glutamine was the only feed additive of the amino acids and a vitamin mixture, other than glucose, that led to consistent positive impacts on growth, viability, and productivity. Lastly, we presented the challenges of fed-batch bioreactor development for NS0–59 and NS0–66 and note that future process improvements could aim to optimize gassing strategy, agitation, and pH, and use feeding strategies to avoid accumulation of lactate and ammonium, which can affect both cell growth and quality attributes like glycosylation.[21] Future studies could include testing perfusion operations for NS0–59 and NS0–66 similar to perfusion operations of NISTCHO. This report helps the growing number of researchers studying non-originator NISTmAb-expressing cell lines as model cell lines by providing detailed process information for future work focusing on the impacts of major process changes, cell substrates, and/or integration of new manufacturing technologies on quality attributes of non-originator NISTmAb.

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## Disclaimer

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