



Assessing the Stability of NISTCHO Cells in Long-Term Culture

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Abstract: NISTCHO is a recombinant Chinese hamster ovary cell line that has been genetically engineered to produce the monoclonal antibody cNISTmAb. This study investigates the stability of the NISTCHO cell line in long-term culture. Low passage number NISTCHO cells from a working cell bank were used to initiate a shake flask culture that was passaged over many weeks, accounting for approximately 129 cell doublings. Cells taken at two-week intervals during this period were used to inoculate fresh cultures, which were monitored over nine days for viable cell concentration, percent viability, and monoclonal antibody production. Results demonstrate consistency among growth curves over time with comparable peak cell densities and cell viabilities. Importantly, cNISTmAb production remained high, with culture titers remaining stable over the culture period and a high number of cell doublings. These findings demonstrate that the NISTCHO cell line has high stability and a sustained capability of producing cNISTmAb over extended culture periods.

Keywords: NISTCHO, Chinese Hamster Ovary, monoclonal antibody, stability, long-term culture

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Introduction

The NISTCHO cell line, developed by scientists at the National Institute of Standards and Technology, NIST, in collaboration with Millipore Sigma [1] is a Chinese hamster ovary cell line derived from genetically modified CHOZNTM cells [2] by introducing genes encoding a monoclonal antibody against surface glycoprotein F of the Respiratory Syncytial Virus (RSV) [3]. This monoclonal antibody, cNISTmAb, is a fully humanized IgG1κ antibody.

The development of the NISTCHO cell line and the production of cNISTmAb provides an important reference material for the biopharmaceutical industry and a standardized platform for research and development [4]. It also provides a much-needed resource for biomanufacturing training and education programs as a modern, industry grade cell line that can be used to develop small scale processes and relevant analytical assays for teaching.

Chinese hamster ovary cells are used extensively in the production of monoclonal antibody biopharmaceuticals [5]. In the commercial production process, these cells are grown in culture for as long as six weeks, in increasing volume seed bioreactors and finally a production bioreactor. In a traditional stainless-steel bioreactor process, the culture is expanded to a volume of 10,000L or larger [5]. During this culture expansion, the cells may undergo as many as sixty cell doublings. For this reason, biomanufacturers conduct stability studies on recombinant CHO cell lines to assess the genetic stability of the cells and the ability of the cell line to sustain high production levels of the monoclonal antibody.

In this study, the stability of the NISTCHO cell line has been assessed to ensure the consistency and reliability of cNISTmAb production over a number of cell doublings that exceed a typical commercial upstream processing strategy. Analysis of the growth performance of NISTCHO that have been grown in



long-term culture was conducted and the capability of the cells to continue to produce cNISTmAb was assessed.

The experiments conducted in the stability studies involved growing the NISTCHO cells in specific medium formulations. The cells were cultivated using EX-CELL Advanced CHO Production media, providing the necessary nutrients and support for cell growth and mAb production. The NISTCHO cells were developed using a glutamine synthetase (GS) selection system, where the GS gene is used as a selection marker, and the cells are grown in the absence of glutamine [6].

The stability studies conducted on the NISTCHO cell line involved growing the cells continuously in culture with passaging every 3 or 4 days; this allowed the culture to be maintained in the optimal cell density range for the cell line. For suspension cell lines such as NISTCHO passaging involves adjusting the cell concentration to a value in the low end of the optimal range for cell growth. Cells can be removed, and fresh media added, or a portion of the culture can be used to seed a new flask containing fresh medium. This allows the cells to continue to grow and double in the fresh medium without any detrimental effects of cell crowding. NISTCHO cell doubling time is between 24 and 26 hours, with three to four cell doublings between passages. In every two passages, a portion of the culture was cryopreserved. In the cryopreservation process, the cells are collected by centrifugation and resuspended in a freezing medium containing dimethyl sulfoxide. This process removes all medium from the original culture that contains cNISTmAb. The frozen cells from a particular passage number were then resuscitated and used to inoculate a fresh culture, which was monitored over an eight-day period. The culture medium was then used to purify and quantify the mAb to determine titer.

Methods

NISTCHO cell culture:

NISTCHO cells were grown in 125ml disposable shake flasks in Production Media (EX-CELL Advanced Fed-Batch Medium, Sigma Aldrich). They were grown at 37°C, 5 % CO₂, with shaking at 125rpm. The cultures were sampled, and growth was monitored over nine days. The viable cell concentration and percent viability were determined using a Luna fluorescence-based automated cell counter (Logos Biosystems).

Cryopreservation:

NISTCHO cells were cryopreserved as 1ml aliquots at a cell density of 1.3×10^7 cells/mL in an EX-CELL[®] CD CHO Fusion medium containing 7% DMSO. Vials were cryopreserved at -150°C.

Cell harvest

On day 8 of the cell culture, the cell suspension was transferred to a conical tube and centrifuged at 2500x g for 5 minutes at 4°C in a pre-chilled Eppendorf 5464R centrifuge. The supernatant was filtered using a 0.2 µm PES membrane filter unit. An appropriate volume of Halt protease inhibitor cocktail (100X) (ThermoFisher) was added to a final concentration of 1X. The clarified cell culture medium was stored at 2-8 °C for up to 4 days.

Purification of mAb

cNISTmAb was purified from clarified cell culture media using a 1ml protein A gravity chromatography column. 5 ml of clarified cell culture medium was loaded onto a pre-equilibrated column. Buffers and columns were provided by Protein A IgG Purification Kit (Pierce, Cat No.44667). 2mL flow-through and wash fractions were collected, and 1ml elution fractions were collected. A neutralization buffer (50ul of 1M Tris at pH 9) was added to each elution fraction to adjust the pH to pH 7. Protein concentration was determined using the Nanodrop spectrophotometer (ThermoFisher). For elution fractions, the mAb concentration was determined using the IgG extinction coefficient, i.e., conversion of 1 Abs unit = 0.73mg/ml of IgG mAb. The data collected from the Nanodrop were used to determine the mAb concentration per 1mL, i.e., titer of each culture.

SDS-PAGE

Chromatography fractions were analyzed on a Novex 4-20% gradient gel with Tris/ Glycine/ SDS Buffer (Invitrogen). Samples were combined with an equal volume of 2X sample buffer (Bio-Rad) and then heated to



95°C for 2 minutes before loading. Precision Protein plus standards (Bio-Rad) were used. Gels were stained with Coomassie stain.

Results and Discussion

A stability study was conducted on NISTCHO cells to evaluate the production consistency of cNISTmAb across multiple passages, from p:10 to p:46, encompassing 129 cell doublings. The experiment was designed so that NISTCHO cells were cultured continuously, and cryopreservation was performed every two passages. NISTCHO cells with increasing passage numbers were resuscitated, and a growth curve and titer determination were performed for each passage number. Vials of cells, with a concentration of 1.3×10^7 cells/mL, were thawed and cultured in 30 mL of production medium for eight days. This cultivation aimed to evaluate both the growth profile of the NISTCHO cells and the production of the mAb.

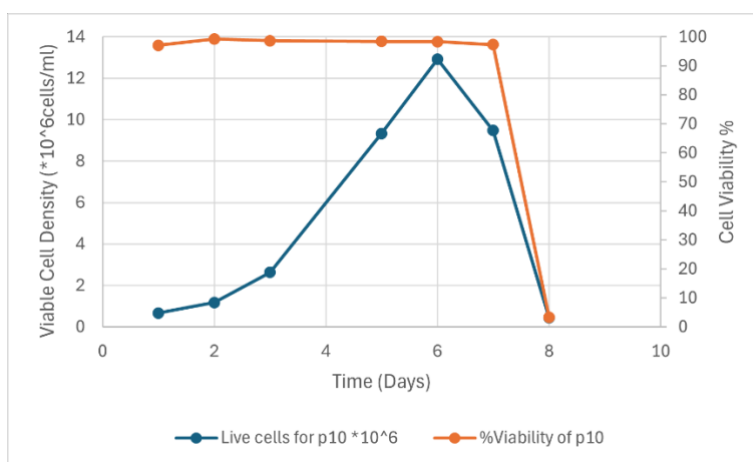


Figure 1. Growth curve of NISTCHO cells passage 10 with viable cell density on the left y-axis (blue line) and % viability (orange line) on the right y-axis. The x-axis represents the number of days in culture. Cell count was not performed on days 0 and 4.

The growth curve shown in Figure 1 depicts the proliferation of NISTCHO cells over the course of 8 days, with an initial lag phase and then an exponential phase peaking on day six at 12.92×10^6 cells/ml before the culture goes into a decline phase due to nutrient depletion. The percentage of viability cells remains high, greater than 90% up to day 6. This is the typical pattern for NISTCHO cells in a batch culture with no additional feed or nutrients added.

To look at cell performance for NISTCHO cells in long-term culture, cells from passages p18, p22, p32, p34, p36, p38, p42, and p46 were cultured and compared to p10. A vial of cells for each passage was resuscitated and used to inoculate 30mL of production media at a seeding density of 4.3×10^5 cells/ml. The cultures were maintained from day 0 to day 8 with daily sampling and monitoring. The peak viable cell density stayed within the range of 12 to 15×10^6 cells/ml throughout, indicating that cultures seeded with higher passage number cells performed as well as those seeded with low passage number cells. Figure 2 shows data for p10, p22, p32, p36, and p46 and provides valuable insight into the growth dynamics of the NISTCHO cells.

The initial viability of cells in a culture is a critical factor that significantly influences the outcome of cell culture experiments and serves as an indicator of the health and vitality of the cell population after cryopreservation. A high initial NISTCHO cell viability, such as the values observed in this study ranging from 94.4% to 99.4%, indicates that most cells are alive and capable of proliferating on day 0 of the culture. This is crucial because



cultures with high viability values have cells that are likely to maintain their functionality, replicate efficiently, and contribute to robust growth and proliferation throughout the culture period.

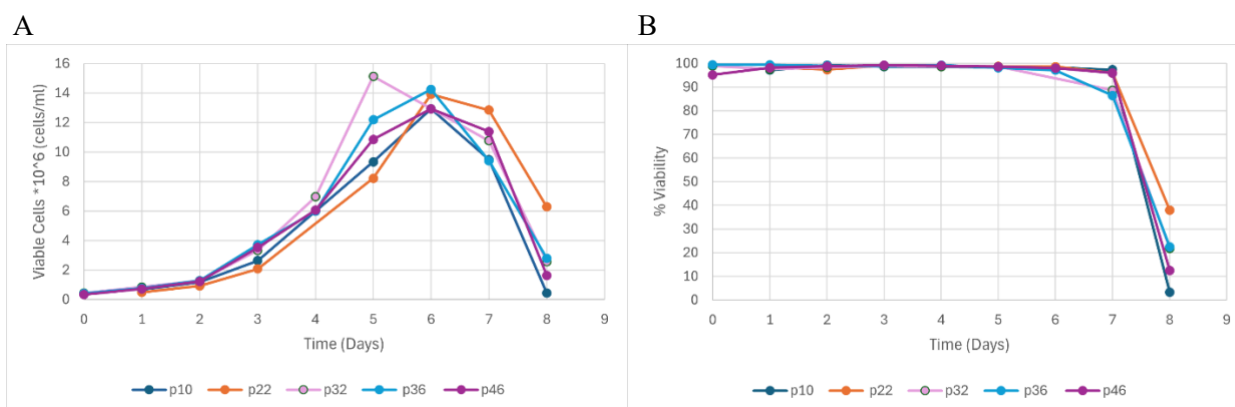


Figure 2. (A) Representative growth curves of viable cell concentration of cultures derived from cells with increasing passage number. All flasks were seeded with 1.3×10^7 cells by direct inoculation from cryopreserved vials of p10, p22, p32, p36, and p46 NISTCHO cells. Cell count was not performed on day 6 for p32. (B) % cell viability plotted against days in culture for cultures depicted in (A)

The growth curves of the NISTCHO cells across passages p10, p22, p32, p36, and p46 exhibit distinct patterns over time (Figure 2A). Initially, there is a gradual increase in cell density observed in all passages, indicating an initiation of cell proliferation. This early growth phase is followed by a period of rapid exponential proliferation, particularly evident in passages p32 and p36, where cell densities increase significantly from days 2 to 5. After day six, signs of a plateau or decline in growth become apparent across all cultures. The small differences in the growth curves represent natural diversity among cultures, and overall cell performance was comparable to the base passage, p10.

Table 1 contains data from nine NISTCHO cultures inoculated with NISTCHO cells of increasing passage number with the associated number of cell doublings for each passage number, with passage 10 representing the baseline for this experiment since these cells were used to initiate the culture. It is clear that neither the peak cell density of the cultures or initial % viability are compromised in cultures seeded with high cell doubling number cells; cells that have undergone approximately 129 doublings have comparable cell density to the original passage 10. Some variance between cultures is evident as is common in cell culture experiments.

Table 1. Impact of passage number (and associated number of cell doublings) on peak cell culture density and initial cell viability in NISTCHO cell cultures inoculated with cells of increasing passage number representing the increased number of cell doublings



Passage Numbers	Number of Cell Doublings	Peak VCD *10 ⁶ (cells/ml)	Initial % Viability
10	0	12.92	97.1
18	31	14.56	96
22	45	13.92	98.5
32	80	15.12	98.9
34	87	15.32	96.4
36	94	14.24	99.4
38	101	13.44	94.4
42	115	13.68	95.1
46	129	12.92	95.1

Protein A Affinity Chromatography Purification and Purity Assessment of cNISTmAb by SDS-PAGE Analysis

During the cell culture process, the cNISTmAb is secreted into the cell culture media, and the concentration of mAb in the medium is known as the titer, which is typically expressed as g/L or mg/ml. In this study, the cNISTmAb was purified from the culture medium and quantified to determine atiter estimation. Protein A affinity chromatography was used to purify the mAb. In this commonly used purification technique, the protein A ligand specifically binds to the Fc region of the antibody, allowing for the isolation and purification of the desired mAb product [7]. The mAb product can then be quantified using spectrophotometry [8].

For the cultures described above, the cell culture medium containing cNISTmAb was collected by centrifugation on day eight, and a portion of it was used to purify the cNISTmAb. The chromatography fractions were collected, and the fractions representing the eluate were analyzed using SDS-PAGE under reducing conditions (Figure 3). This technique separates proteins based on their molecular weights, allowing for the detection of the target mAb and any potential contaminants.

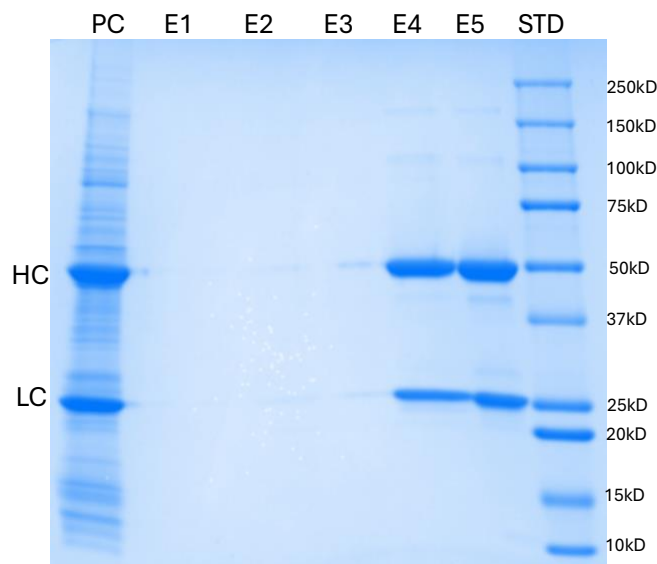


Figure 3. SDS PAGE analysis of protein A column chromatography fractions from p10 NISTCHO cell culture medium. PC contains the pre-column clarified culture medium, 6ul. E1:Eluate1, 10ul



(0.295 mg/ml). E2: Eluate 2, 10ul (0.225 mg/ml). E3: Eluate 3, 10ul (0.009 mg/ml) E4: Eluate 4, 1ul (1.771mg/ml). E5: Eluate 5,1ul (2.645 mg/ml).

In the SDS-PAGE analysis (Figure 3), the pre-column (PC) lane contains 6ul of clarified cell NISTCHO cell culture medium. Highly representative bands for the heavy and light chains of the cNISTmAb are visible with many contamination protein bands, representing CHO host cell proteins and media components. The heavy and light chains of the cNISTmAb are observed at around 50 kD and 25 kD, respectively. Lanes 2, 3, 4, 5, and 6 represent successive elution fractions from the purification process. Lane 2, Lane 3, and Lane 4 exhibit faint bands at 50 kD and 25 kD due to low concentration, and lanes 5 and 6, containing eluate fraction 4 and fraction 5 contain strong bands indicating that the cNISTmAb was eluted from the column in these fractions. To assess the purity of the mAb, high concentrations of eluate were loaded on the gel and faint bands representing contaminating proteins were visible around 170kD, 120kD, and 40kD. Despite the presence of these contaminants, the gel demonstrates a significant level of mAb purity in the region of 95% pure, post-protein A column purification. This underscores the effectiveness of the purification process in isolating the target mAb from the cellular components and other impurities.

Clarified medium (5ml) from each of the cell cultures described was used to purify the mAb by protein A chromatography.. The eluted fractions containing the mAb were analyzed using a Nanodrop spectrophotometer to measure the absorbance at 280nm. Using the extinction coefficient for IgG this value provided the concentration of the mAb in each fraction [8]. The volume of each fraction was measured, and the total amount of mAb in milligrams (mg) per fraction was calculated by multiplying the concentration (mg/mL) by the volume (mL) of the fraction. The total amount of mAb obtained was then divided by the initial 5 mL of clarified medium to determine the titer concentration.

Impact of Passage Number on growth performance and mAb Productivity

The evaluation of both peak cell density, titer, and monoclonal antibody (mAb) production per cell across the doubling numbers provides critical insights into the overall performance of the bioprocess. A successful bioprocess is characterized by achieving high peak cell densities while simultaneously maintaining optimal mAb production i.e. a high titer. In these experiments, the titer of each culture was estimated by purifying mAb from 5ml of clarified medium using protein A column chromatography.

Table 2 lists the estimated titer obtained for each culture and the mAb produced by each cell, taking into account the peak cell density of the culture. Culture titers remained high as the passage number increased and were comparable to the base p10 culture. mAb production per cell remained constant as the number of doublings increased, with p22 cells (45 doublings) and p42 cells (115 doublings) showing almost identical mAb production per cell, and overall cells that had undergone 129 doublings (p46) producing 35.45pg/cell compared to 40.63pg/cell for the original passage 10.

Table 2. Viable cell density observed for each culture of NISTCHO cells, with corresponding number of doublings of, culture titer, and the amount of mAb produced per cell.



Passage Numbers	Number of Cell Doublings	Peak VCD *10 ⁶ (cells/ml)	Titer (mg/ml)	mAb pg/cell
10	0	12.92	0.525	40.63
18	31	14.56	0.516	35.44
22	45	13.92	0.527	37.86
32	80	15.12	0.533	35.25
34	87	15.32	0.51	33.29
36	94	14.24	0.47	33.01
38	101	13.44	0.477	35.49
42	115	13.68	0.508	37.13
46	129	12.92	0.458	35.45

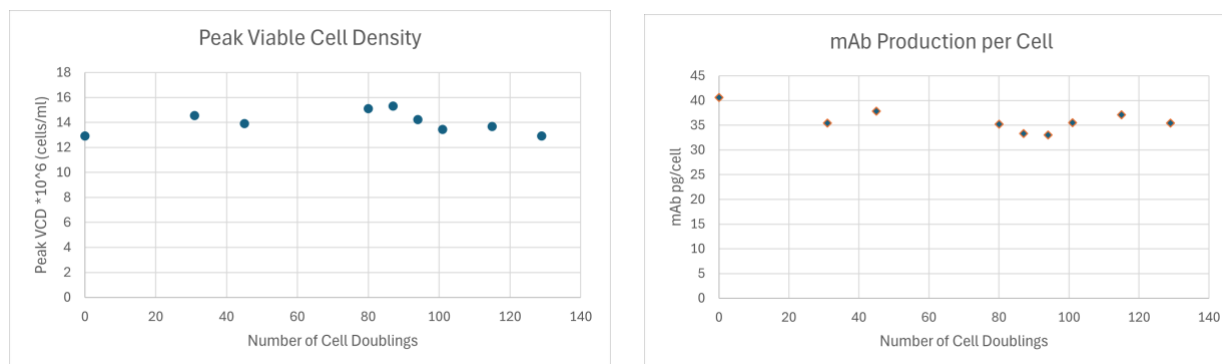


Figure 4. (A) The chart depicts peak viable cell density plotted against number of cell doublings of NISTCHO cells. (B) The graph illustrates the amount of mAb produced per cell in cultures inoculated with cells with increasing numbers of cell doublings

Figure 3 shows growth cell potential and mAb production over time. Data for peak viable cell density and mAb production per cell were plotted against the number of doublings of the NISTCHO cells used for culture. Despite an increase in the number of cell doublings from passage 10 to passage 46, the peak cell density for the cultures remains constant (Fig.4A), with a slight decrease starting around 100 doublings. The amount of mAb produced per cell (Fig.4B) also remains constant, with the highest level at p10 and comparable amounts of mAb produced for subsequent cultures. This data demonstrates the prolonged capability of the NISTCHO cell line to consistently produce cNISTmAb well beyond the number of doublings typically seen in upstream processing cultures.

Conclusion

Cell line stability studies are an important element of recombinant cell line characterization during biopharmaceutical research and development. The ability of a cell line to consistently express the transgene at high levels over a long period in culture is critical for meeting the high liter demands of commercial biomanufacturing. The findings in this study reveal that the NISTCHO cell line is a highly stable cell line when grown long-term in culture. Cells that had undergone approximately 129 cell doublings in culture produced comparable levels of mAb to those that were taken directly from the working cell bank. High passage number



cells also performed well in terms of the peak cell density they reached in culture and their ability to retain high % cell viability levels in batch cultures. These findings are of importance to biopharmaceutical companies that will use NISTCHO as a reference cell line in CHO cell development and characterization and to education and training programs that will adopt the NISTCHO in their courses.

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Disclosures The authors declare no conflicts of interest.

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