Comparison of Cryopreservation Conditions on the Performance of NISTCHO Cells

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Abstract: The NISTCHO cell line is a recombinant Chinese hamster ovary cell line engineered to produce cNISTmAb, a monoclonal antibody that recognizes the fusion F glycoprotein on the surface of respiratory syncytial virus (RSV). These cells are invaluable as a standard reference material for developers of therapeutic monoclonal antibodies and serve as an educational resource in biomanufacturing training programs. This study investigates the performance of NISTCHO cells following cryopreservation at temperatures of -80°C and -150°C. Initial cell viability, maximum cell density in culture, and monoclonal antibody production were compared for cells cryopreserved for up to 30 weeks. Cells were thawed and cultured at two-week intervals to monitor their growth behavior, peak cell densities, and antibody production levels. Analysis of cell behavior in culture revealed no significant differences in cell growth or cell production between cells stored at -80°C and those stored at -150°C. These findings affirm that NISTCHO cells can be preserved at -80°C for up to 30 weeks without any adverse effects on their growth or monoclonal antibody production capabilities, an important finding for training and education programs that rely on -80° C freezers to store cell banks.

Keywords: NISTCHO, monoclonal antibody, biomanufacturing, cryopreservation

Introduction

The NISTCHO cell line, a collaborative creation by the National Institute of Standards and Technology (NIST) and Millipore Sigma, represents a significant advancement in biotechnological research tools. This open- access recombinant Chinese hamster ovary (CHO) cell line was derived from genetically engineering CHOZN™ cells [1] to express a monoclonal antibody (mAb) targeting a surface glycoprotein, the F protein of the Respiratory Syncytial Virus (RSV) [2]. The NISTCHO cell line was created using a glutamine synthetase (GS) selection system to select stable, high-producing clones by supporting their survival and growth in glutamine-free media. Monoclonal antibodies (mAbs) are the largest subset of protein biotherapeutics and represent the industry's fastest-growing segment due to their specificity and efficacy in treating a wide range of diseases. Currently, mAbs account for a significant proportion of new therapeutics, including treatments for cancer, autoimmune diseases, and infectious diseases, underscoring their critical role in modern medicine and the biotechnology industry. Monoclonal antibody therapeutics are twice as likely to succeed in clinical trials than small molecule drugs; they currently account for nearly a fifth of the FDA's annual new drug approvals, with an average of ten yearly approvals [3]. Monoclonal antibody-producing NISTCHO cells are an industry standard cell line capable of growing to high densities and producing a high mAb titer. They are an important reference cell line and provide a practical framework for studying cell growth dynamics and mAb production in a controlled, small-scale environment [4]. NISTCHO cells, like most mammalian cell lines, are cryopreserved at ultralow temperatures, such as in the vapor phase of a liquid nitrogen tank or dewar where the temperature is -150°C to -196°C. These conditions are considered optimal for storage, resulting in minimum cell damage and maximum cell performance upon

resuscitation [5]. Knowing how these cells behave under different cryopreservation conditions is important since teaching laboratories often have limited access to liquid nitrogen dewars and ultralow temperature freezers. For this reason, we conducted growth studies on NISTCHO cells that were cryopreserved at two distinct temperatures, -80°C and -150°C. The cells were compared after cryopreservation for 2, 4, 6, 8, 10, 14, 26 and 30 weeks. Following resuscitation, the cells were cultured in 30ml of medium in small-scale shake flasks and monitored daily for cell viability and cell density for nine days. The culture medium was then collected, and the mAb was purified and quantified to provide an estimated mAb titer for each culture. Comparative analysis of these metrics between the two cryopreservation conditions formed the crux of our investigation.

Methods

Cryopreservation

Working cell banks of NISTCHO cells passage 12 were established and stored at either -150°C or -80°C. Cells were cryopreserved in EX-CELL CD CHO Fusion Media (Sigma Aldrich) containing 7% DMSO. Each vial of NISTCHO p12 contained 10 million cells in a 1ml volume. Resuscitation of cells was performed using a Thawstar device (Biolife Solutions).

NISTCHO Cell Culture

NISTCHO cells were cultured in 30ml of EX-CELL CD CHO Fusion Media in 125ml disposable shake flasks. The initial seeding density was 3.33 x 10^5 cells/ml, and the cells were cultured over a period of 9 days in an environment maintained at 37°C, 5% CO_2, and agitated at 125rpm on a shaking platform. Cultures were sampled daily under aseptic conditions to monitor growth. Both the viable cell concentration and the percentage viability were assessed using a Luna fluorescence-based automated cell counter (ThermoFisher)

Cell harvest and mAb purification

On day 9, cell growth culture media was collected by centrifugation at 2500xg, 4°C, for 10 minutes, followed by clarification by 0.22u filtration. The cNISTmAb was isolated from 5ml of clarified medium using a 1ml Protein A Gravity Chromatography column (ThermoFisher Scientific Cat No. 21001). The binding and equilibration buffer used was a phosphate-based solution with glycerol and EDTA, adjusted to pH 8.0. The elution buffer was amine-based and adjusted to pH 2.0. Flow-through and wash fractions of 2 ml each were collected, along with 1 ml fractions of eluate. To each eluate fraction, 50 µl of 1M Tris (pH 9) was added for pH adjustment to 7.0. The concentration of mAb in each fraction was quantified using a Nanodrop spectrophotometer (ThermoFisher) at 280nm using the extinction coefficient for IgG [6].

SDS-PAGE

Samples from chromatography fractions were prepared by mixing with an equal volume of 2X sample buffer (Biorad). Samples were heated at 95°C for 2 minutes before loading onto the gel.

Electrophoresis was conducted using a Novex 4-20% Tris-Glycine gradient gel (Invitrogen). The electrophoresis was carried out at 100 volts in a 1X Tris/Glycine/SDS running buffer for 1.5h. Post-electrophoresis, the gel was stained with Coomassie Brilliant Blue stain. Kaleidoscope Precision Plus Protein standards (Biorad) were included as molecular weight markers to facilitate the identification of the heavy and light chains of the antibodies.

Results and Discussion

This study investigates NISTCHO cell growth characteristics, including cell viability and cell growth after extended cryopreservation at two different temperatures, -80°C and -150°C. The primary objective was to evaluate how cells sustain their functionality and viability post-cryopreservation. To this end, NISTCHO cells were banked in the cryopreservant DMSO in a -80°C freezer or a -150°C freezer. Vials of cells from

both cell banks were resuscitated at specific intervals and grown in culture with daily monitoring of the cultures over a nine-day period. Monitoring included the determination of cell density and the percentage of viable cells using an automated cell counter. Figure 1 demonstrates sample growth curves for 30ml cultures of NISTCHO cells that were seeded with cells cryopreserved at -150°C or -80°C for eight weeks. Cell counting was performed in duplicate, and the average cell concentration was plotted against the number of days in the culture. The growth curves represent typical proliferation patterns observed for NISTCHO cells grown in CHO Fusion Media in small-scale culture. The unique phases of NISTCHO growth, lag, exponential, plateau, and decline, are clear, as is the similarity of the growth dynamics of the cultures. This indicates that cells cryopreserved at -80°C for eight weeks have equal growth capabilities after resuscitation to those stored for the same period at -150°C.

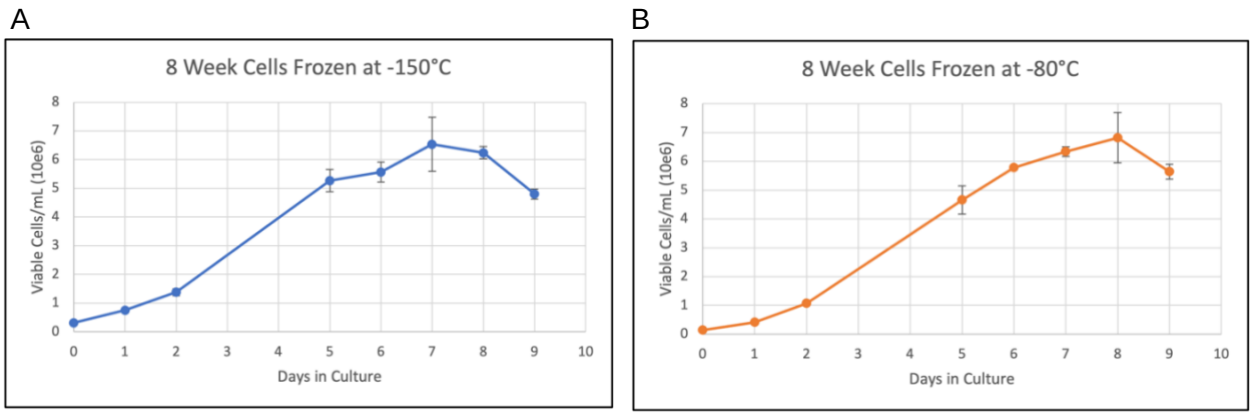

Figure 1. Comparison of NISTCHO growth curves of cells after cryopreservation for eight weeks at -150°C (A) or -80°C (B). Cell counting was performed in duplicate, and average cell concentration and standard deviation were calculated.

The study was extended to determine if longer periods of storage at -80°C affected the properties of the cells in culture. Figure 2 demonstrates a comparison of the growth profiles and cell viability measurements of NISTCHO cells stored at either -80°C or -150°C for 14 weeks and 26 weeks.
The data demonstrates that cells preserved at either temperature maintain robust growth with no significant differences in their growth trajectories or maximum cell density (Figs 2A and B). In addition, the higher cryopreservation temperature does not negatively affect the high level of cell viability throughout the span of the culture, with both cultures maintaining cell viability above 90% into the plateau phase of the growth curves (Figs 2C and D).

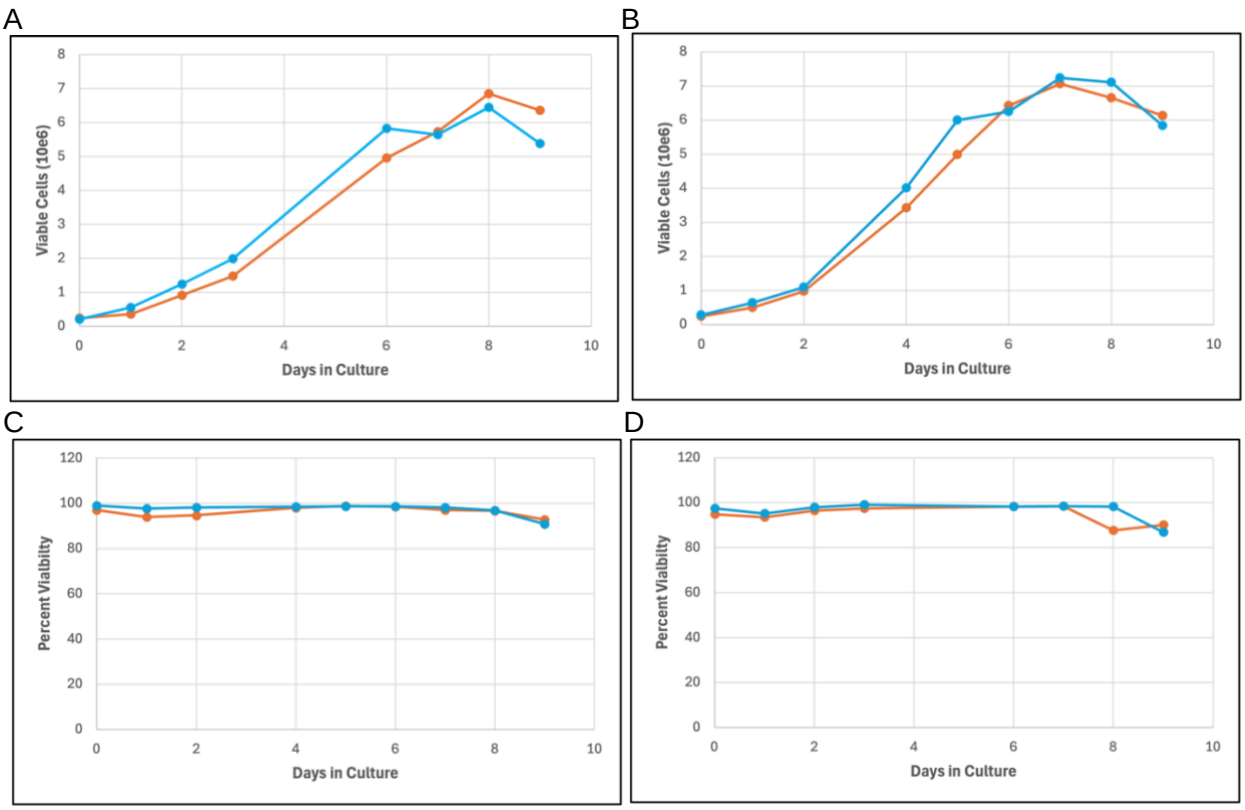

Figure 2. Comparison of NISTCHO growth curves and cell viability for cells cryopreserved for 14 weeks (A and C) and 26 weeks (B and D). Blue lines represent the cell density and viability measurements for NISTCHO cells stored at -150°C and orange lines represent the same for NISTCHO cells stored at -80°C

Building on this foundational data, we have compiled comprehensive growth curves that aggregate the results from growth studies from cells cryopreserved at both temperatures for 2, 4, 6, 8, 10, 14, 26, and 30 weeks. These combined growth curves provide a view of the cell behavior over the entire experimental period, enabling a broader comparison of cell behavior and performance in culture post-resuscitation. The proliferation patterns for cells frozen at the two temperatures are almost identical (Fig.3).

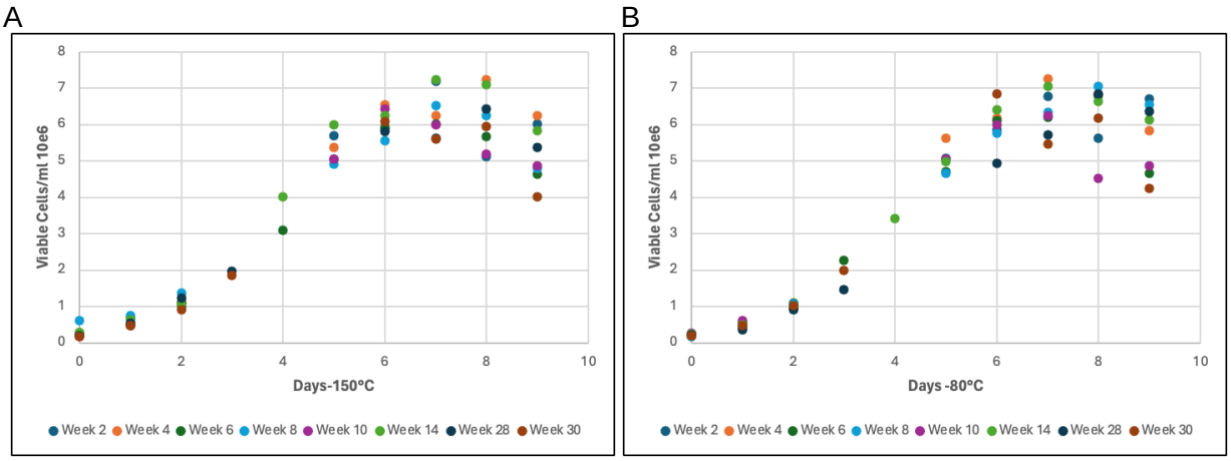

Figure 3. Combined growth curves depicting the growth of NISTCHO cells over nine days. Cells were initially seeded at a density of 3.33 x 10^5 cells/ml and cultured in 30 ml volumes. (A) The cells used to seed the culture were cryopreserved at -150°C for the stated number of weeks. (B) The cells used to seed the cultures were cryopreserved at -80°C

The comparative growth analysis of NISTCHO cells stored at -150°C and -80°C, demonstrated in Figures 1, 2, and 3, indicate that cells cryopreserved at -80°C, which is considered sub-optimal for most mammalian cell lines, perform as well as cells stored at the lower temperature. Notably, the initial percent cell viability immediately after resuscitation on day 0 (Fig. 2) is almost identical between the cultures. Maximum cell density remains very comparable throughout the study, indicating that cryopreservation temperature did not affect cell performance. This equivalence is particularly evident in the delineation of growth phases (lag, exponential, and plateau) within the scatter plots (Fig. 3), which are consistently observed in cell cultures from both conditions. The consistency across these data strongly suggests that NISTCHO cells exhibit a high degree of cryostability, which allows their metabolic and growth capacities to remain intact even after extended storage at -80°C.

The data challenge the prevailing assumption that lower storage temperatures are inherently superior for maintaining cellular integrity over long periods. Our data indicate that -80°C, a more commonly accessible and less costly cryopreservation option, does not compromise the growth characteristics of NISTCHO cells when cells are stored for up to 30 weeks.

NISTCHO cells in culture produce cNISTmAb and secrete it into the culture medium. Currently there isno commercially available functional assay, such as an ELISA, to easily quantify the concentration of cNISTmAb (i.e., titer) in the culture medium. To compare cNISTmAb titer in cell cultures from cells cryopreserved at each of the temperatures, the mAb was purified and quantified. Cell culture medium from each of the cultures was collected on culture day 9, and a representative 5ml sample was used to purify the cNISTmAb using protein A chromatography. The purified cNISTmAb was then quantified using spectrophotometry, absorbance at 280nm, and an estimated titer determined. Evaluation of Protein A Sepharose affinity chromatography purified antibodies typically indicate approximately 95% purity; hence, the vast majority of the purified material is the target protein. To assess the purity of cNISTmAb post chromatography, SDS-PAGE was performed under reducing conditions. In these conditions, the heavy and light chains of the mAb are separated and can be visualized as bands of approximately 50kDa and 25kDa.

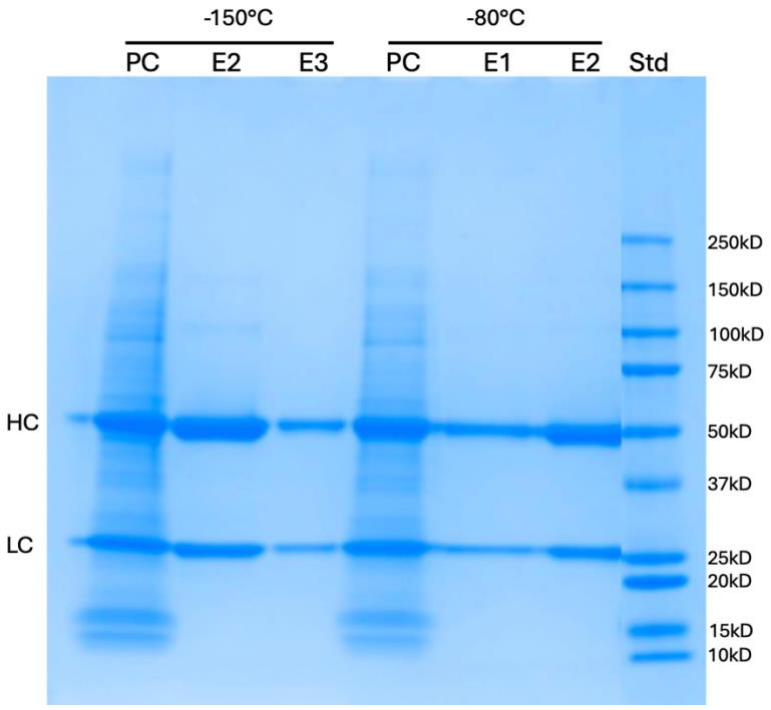

Figure 4. SDS-PAGE Analysis of clarified culture media and cNISTmAb after purification by Protein A Chromatography for NISTCHO cultures of cells preserved at -150°C or -80°C. -150°C storage PC: 6 ul Pre-Column clarified media, E2: 2ul of elution fraction 2 (1.650ug/ul), E3: 2 ul of elution fraction 3 (0.239 ug/ul), -80°C storage PC: 6 ul Pre-Column clarified media, E1: 2 ul of elution fraction 1, (0.605ug/ul), E2: 2ul of elution fraction 2 (1.285ug/ul). Std: Kaleidoscope protein standards. Bands corresponding to the heavy chain (HC) and the light chain (LC) of cNISTmAb are the predominant bands in each lane.

Figure 4 shows SDS-PAGE analysis of pre-protein A column material from cultures seeded with cells cryopreserved at -150°C or -80°C for two weeks and associated eluted fractions containing cNISTmAb for each. Lanes containing fractions from the column elution E2, E3, E1, and E2 show strong representation of the heavy chain (HC) and light chain (LC) components, bands at approximately 50 kDa and 25 kDa respectively, with only very light contaminating bands visible. These minimal contaminants are likely residual host cell proteins or culture media components. This data confirms the purity of the mAb and confirms that the vast majority of the protein in these eluted fractions represents the cNISTmAb.

The concentration of mAb in each eluted fraction was quantified using a Nanodrop spectrophotometer, with a conversion factor of 1 Absorbance unit equaling 0.73 mg/ml of IgG mAb. This conversion uses the extinction coefficient for IgG when determining the concentration of the analyte from the absorbance at 280nm [6]. From these readings, the total amount of cNISTmAb in the elution fractions was calculated and divided by the volume of culture medium loaded onto the column (5ml) to produce an estimated mAb concentration, i.e., titer in ug/ml.

Table 1 shows data collected for each cell culture performed and allows a comparison of the effect of long-term storage at each of the two cryopreservation temperatures used. Overall, the titer for the cultures remains constant over time, and levels are comparable between the twocryopreservation temperatures. Cells that were stored for 26 weeks -80°C have a slightly lower titer (294ug/ml versus 332ug/ml for -150°C), and the amount of mAb produced per cell (pg/cell) for both 26 weeks and 30 weeks is slightly lower for the -80°C storage cells. This could indicate a slight downward trend in cell performance for cells stored for

longer than the 30-week period of the study at -80°C. Future studies will analyze the performance of cells cryopreserved for one year at each temperature.

Table 1. Tabulated data for cell cultures cryopreserved from 2 to 30 weeks at -150°C or -80°C. Comparisons of % cell viability, maximum cell density, mAb titer (in ug/ml), and normalized mAb production (in pg/cell)

NISTCHO Cryopreservation -150°C					NISTCHO Cryopreservation -80°C				
weeks	Day 0 % Viability	Max Cell Density x 10_6 cells/ml	Titer ug/ml	mAb pg/cell	weeks	Day 0 % Viability	Max Cell Density x 10_6 cells/ml	Titer ug/ml	mAb pg/cell
2	90.20	7.19	320.00	44.51	2	97.70	6.77	312.00	46.08
4	98.80	7.24	308.30	42.51	4	96.40	7.26	292.30	40.26
6	92.90	6.00	284.80	47.47	6	99.00	6.26	305.30	48.77
8	98.30	6.53	276.90	42.41	8	98.60	7.06	253.50	35.99
10	97.00	6.43	285.40	44.39	10	96.50	6.26	276.80	44.22
14	99.10	7.24	259.00	35.70	14	97.00	7.05	278.00	39.43
26	97.40	6.44	332.00	51.50	26	94.90	6.85	294.00	42.90
30	98.70	7.11	260.10	40.40	30	100.00	7.33	259.80	35.44

Figure 5 illustrates data trends over the length of the study and clearly demonstrates the similarity of these trends for cells cryopreserved at -150°C and -80°C. Figure 5A demonstrates that initial cell viability in culture is comparable as the length of cryo storage increases, with cells from both conditions performing equally well. Figure 5B demonstrates a similar result for maximum cell density in culture. The stability of the cells in producing mAb after cryopreservation is shown in Figure 5C. mAb production is expressed as pg/cell to normalize for the variance in peak cell density between cultures. This normalization metric offers a more accurate depiction of cell productivity irrespective of the overall biomass, allowing for a direct comparison of mAb synthesis efficiency per cell under different conditions. Both cryopreservation temperatures yielded similar values for mAb production per cell over time, with a slight dip at weeks 26 and 30 for cells stored at -80°C. This suggests that the cellular machinery involved in mAb synthesis remains unaffected by the storage temperature.

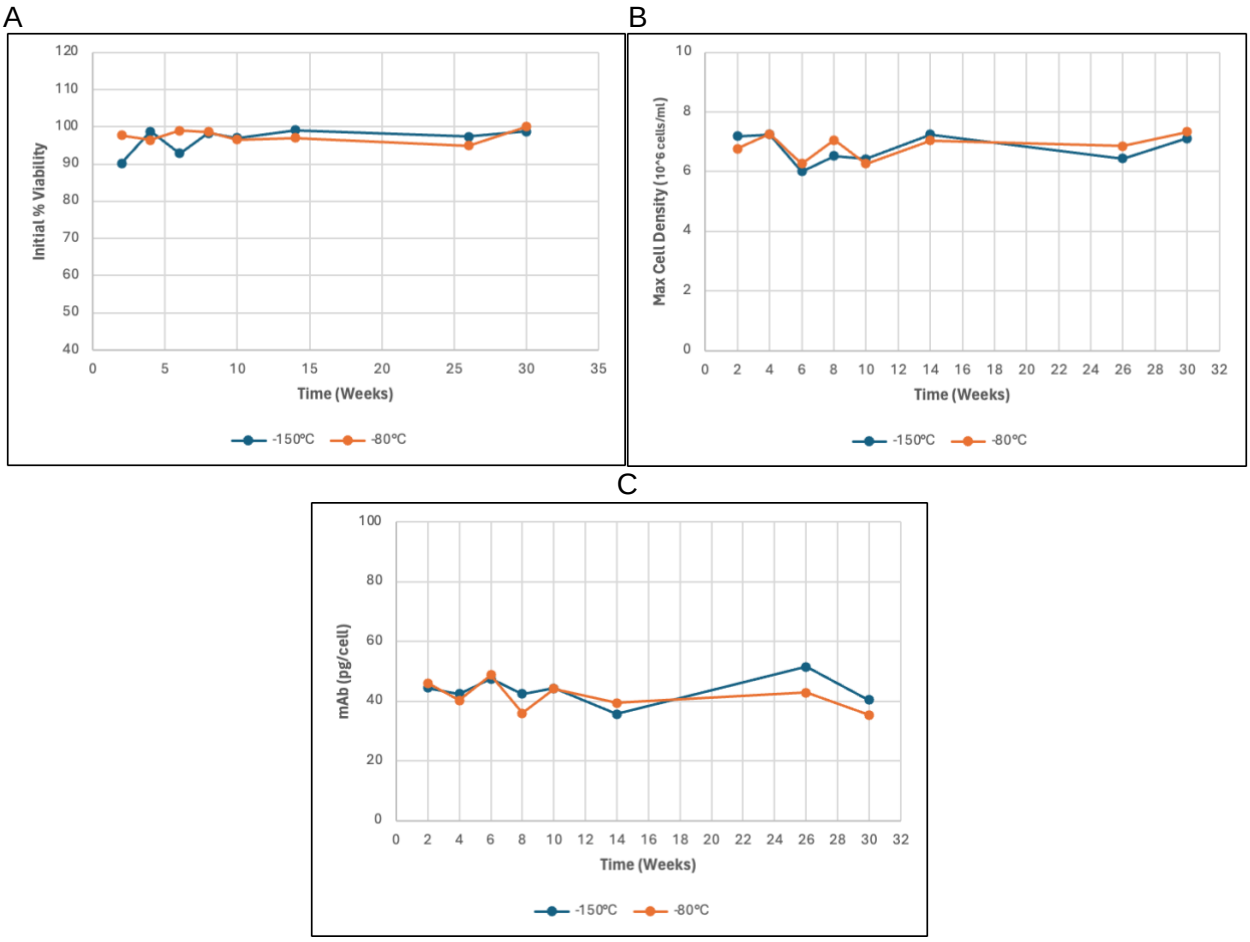

Figure 5. Data trends over the length of the study
A: The viability of NISTCHO cells immediately after resuscitation from cryopreservation at two different temperatures: -150°C and -80°C. B: Max Cell Density of NISTCHO cells in culture versus number of weeks cryopreserved. C: mAb Production (pg/cell) versus number of weeks cryopreserved. For each graph, the blue line represents cells cryopreserved at -150°C, and the orange line cells cryopreserved at -80°C.

For biomanufacturing, understanding individual cell performance under various conditions is not only crucial for scaling up processes but also essential in ensuring consistent product quality and yield in large-scale productions. This study's findings underscore the potential for using NISTCHO cells in scalable biomanufacturing frameworks, given their demonstrated stability and robustness under long-term cryopreservation [4]. Moreover, NISTCHO stability at higher cryopreservation temperatures promotes their adoption and use in a wider variety of laboratory environments, especially those that rely on -80°C freezers for cell bank storage.

Conclusion

The primary objective of this investigation was to assess the impact of different and potentially suboptimal cryopreservation conditions on the viability, growth, and monoclonal antibody (mAb) production of

NISTCHO cells. Specifically, we examined the effects of storing these cells at -80°C and compared the data with cells preserved at -150°C over 30 weeks. Our findings demonstrate that NISTCHO cells cryopreserved at these temperatures exhibited comparable growth patterns when revived and grown in 30ml shake flask cultures. Key metrics such as initial cell viability and peak cell density did not differ significantly between the two storage conditions. This indicates that the lower temperature of -150°C offers no distinct advantage over -80°C in preserving these parameters.

Furthermore, the consistent mAb production across both temperatures suggests that the cells' functional capacity to synthesize antibodies remains intact, irrespective of the cryopreservation temperature. This is particularly noteworthy as it highlights the robustness of NISTCHO cells under varying storage conditions, making them a reliable resource for biomanufacturing processes where cell stability and consistent output are needed.

The implications of these findings are significant for biotechnological applications, especially in settings where cost and energy efficiency are considerations [5]. Moreover, institutes that will use NISTCHO cells for education and training purposes, such as community colleges commonly store cell lines at -80°C if they do not have access to more expensive freezer options such as -150°C or liquid nitrogen dewars. The outcomes reported here will assure these laboratoriesthat storage at -80°C for up to 30 weeks does not compromise cell performance.

The ability to maintain cell viability and functionality at -80°C without compromising the production of cNIST monoclonal antibodies (mAbs) supports the integration of these cell lines into biomanufacturing curricula. Students can gain hands-on experience with these cells, understanding the critical aspects of cryopreservation, cell culture, and mAb production [6]. Furthermore, this adaptability allows institutions with more modest facilities to engage in cutting-edge biotechnological education and research, ensuring that a lack of access to specialized equipment does not disadvantage students.

In conclusion, this study supports the use of -80°C freezers as a viable and efficient option for the long-term cryopreservation of NISTCHO cells. The data supports more economical and accessible biomanufacturing practices with this NISTCHO cell line producing cNISTmAb. Future studies extending beyond 30 weeks could provide deeper insights into the long-term viability and functional stability of cryopreserved cells, potentially expanding their utility in the biotechnology field.

Acknowledgments We extend our deepest gratitude to members of the biotechnology department at Montgomery County Community College department for their tireless support and contributions throughout the research process. We also would like to thank the National Institute for Innovation in Manufacturing Biopharmaceuticals, NIIMBL, Dr. Brad O'Dell from the National Institute of Standards and Technology, and Dr. Lori Kelman from Montgomery College for their advice and support. This work was performed under the following financial assistance award: 70NANB21H085 from the US Department of Commerce, National Institute of Standards and Technology, and NSF ATE award # DUE 2055036, Principal Investigator Dr. Sandra Porter. The Corresponding Author would like to acknowledge that authors J.T. Fredericks and S. Milligan contributed equally to the study.

Disclosures The authors declare no conflicts of interest.

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