White Paper

Guidelines for Developing a High Cell Density Cryopreservation Process

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Biopharmaceutical manufacturers are leveraging several approaches to intensify processes with a goal of improving efficiency and productivity and reducing costs—all while ensuring the highest quality standards. The upstream workflow offers several opportunities to apply next generation technologies and methods to achieve these important goals. In this white paper we discuss integration of high cell density cryopreservation (HCDC) in the seed train and explore key considerations for developing a cryopreservation process including choice of cryoprotectant and freezing techniques.

Advantages of the HCDC Process

A typical biomanufacturing process begins with a cryopreserved vial from a working cell bank created from a master cell bank (**Figure 1**). The vial of cells is thawed, and the biomass expanded to a level sufficient to inoculate the bioreactor. Intensification of this upstream process using HCDC enables the seed train to be shortened and offers greater workflow flexibility.

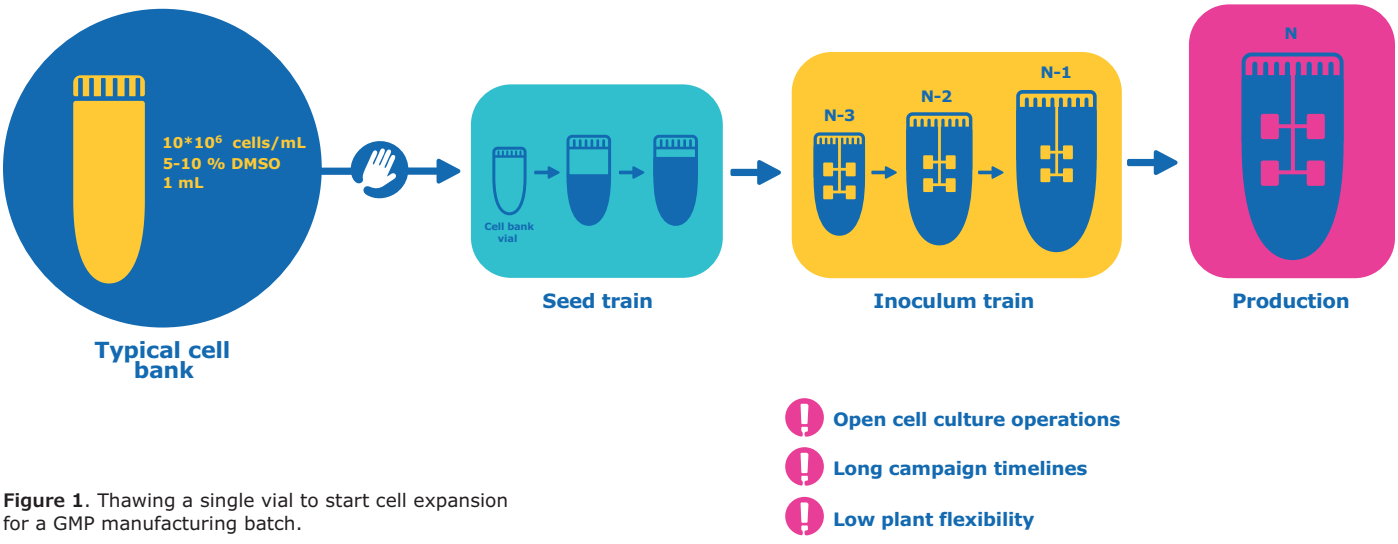


Figure 1. Thawing a single vial to start cell expansion for a GMP manufacturing batch.

In HCDC, cells are frozen in higher volumes with viable cell densities (VCD) up to 50–100 x 10^6 VC/mL in single-use bags.

Table 1 summarizes how much time can be saved with HCDC. Using a standard vial of cells, 24–25 days are required before production stage inoculation can take place. In contrast, if a 150 mL bag containing 50 x 10^6 cells/mL is used, the seed train can be shortened by 10 to 11 days.

In addition to time savings, HCDC offers the advantage of closed processing that reduces contamination risks. With HCDC, there are no open cell culture operations, which can also lead to a lower room classification for GMP manufacturing.

HCDC also offers better reproducibility in seed train expansion, which is not only important for manufacturing, but also in R&D and process development. Bioreactor confirmation runs should ideally have comparable starting points; with HCDC, a bioreactor can be run to generate the biomass for inoculation of the confirmation bioreactors, creating this starting point.

Use of HCDC also allows decoupling of cell expansion and batch production activities so that distribution of cells from a central expansion facility to global decentralized production facilities is possible. The upper box of **Figure 2** show a conventional production process in which every production campaign starts with a vial of cells. If HCDC is incorporated into the process, one vial is thawed and expanded, but then several bags are generated that can be used in multiple production processes (**Figure 2**, lower boxes).

	[10^6 VC/mL]
Inoculation VCD	0.5
VCD – End of batch	6

	Standard Vial	Bag
VCD [10^6 cells/mL]	10	50
Volume [mL]	1	150
Cell count [10^6 cells/mL]	10	7,500

Bioreactors	Volume [L]	Cell count for inoculation [10^6 VC]	Run time (without lag-phase) [d]	
N (production bioreactor)	15,000	7,500,000	3.5	
N-1	1,250	625,000	6.9	
N-2	105	52,083	10.4	
N-3	8.70	4,340	13.8	
N-4	0.72	361	17.3	10.4 days saved
N-5	0.06	30	20.7	
N-6	0.005	2.5	24.2	

Table 1. Summary of time savings with use of HCDC.

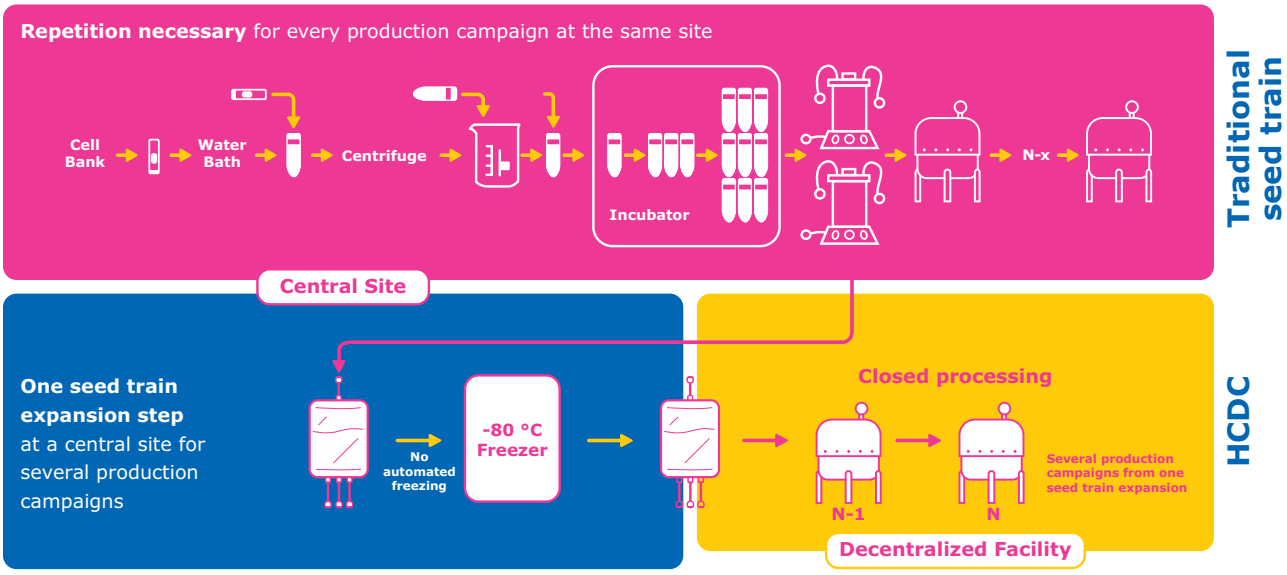
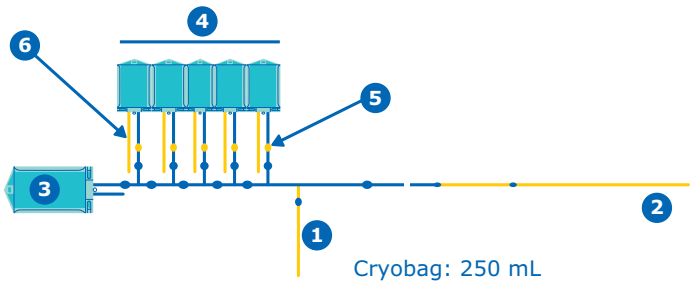


Figure 2. Comparison of a conventional seed train and an intensified process with HCDC.

Both the cell culture medium and the bag assembly are essential for successful HCDC. While different cryomedium formulations can be evaluated for use in HCDC, a best practice is to ensure the chosen formulation for expansion, freezing and subsequent expansion works well with the production medium. The goal is to maximize growth, minimize any lag phase when moving from one medium to the other and minimize cell damage during freeze and thaw.

Figure 3 illustrates a single-use assembly designed for HCDC process. The cell suspension line functions as the connection to the bioreactor while the one liter waste bag is used for flushing the tubes when switching from filling the bags with the required medium to filling the bags with the cell suspension. As soon as one bag is filled, it can be securely sealed and disconnected using our NovaSeptum® crimping tool, ensuring a closed and sterile environment for your cell culture. Additionally, there is a line on each cryobag that can be connected to a bioreactor after thawing for inoculation.

Figure 4 summarizes the process used to fill the bag assembly. A container with the cryomedium is connected to the assembly; this medium will contain a concentrated cryoprotectant, which will be diluted with the addition of the cell suspension to the cryomedium in the bags. Once the bag is filled, it can be easily disconnected and placed into a freezer in a supportive case. To use a bag for inoculation, simply remove it from the freezer, thaw it, and connect it to the bioreactor; the cells can then be added either with a pump or gravity.



1. Cryo medium filling line
2. Cell suspension line/connection to bioreactor
3. 1 L waste bag for flushing of lines
4. Cryobags
5. Metallic pinch pipe for disconnection with the NovaSeptum® crimping tool
6. Line for connection to bioreactor after thaw

Figure 3. Schematic of the Mobius® HCDC R&D assembly.

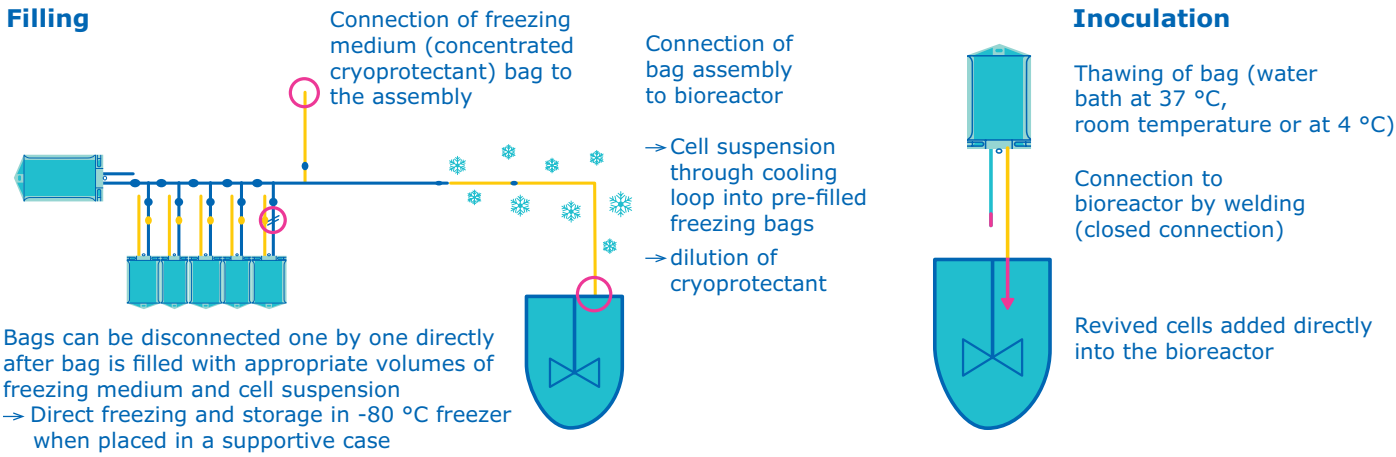


Figure 4. The HCDC workflow.

Customizing an HCDC Process

While the HCDC process should be customized based on the cell line being used, the following studies evaluated dimethyl sulfoxide (DMSO) concentration and freezing techniques.

Impact of DMSO Concentration

DMSO is typically added to the media used for freezing cells at a concentration ranging from 5–10% to protect them from the harsh conditions. Cell banking typically uses 2 mL vials that are filled via pipette in a relatively quick process; this means that the cells have limited exposure to the DMSO prior to being frozen. With HCDC, volumes in the range of 100 to 250 mL are used for cell banking and as such, the filling process takes longer to complete. The cells are therefore exposed to the DMSO for a longer period of time, potentially increasing the risk of damage by the DMSO itself.

To inoculate the subsequent bioreactor vessel, the entire volume of the HCDC bag is thawed and added, including the DMSO. The small volumes used in conventional banking do not typically cause a problem as the dilution factor is very high. Additionally, small vials can be centrifuged to remove medium containing DMSO followed by resuspension of cells in fresh medium. This process minimizes the presence of DMSO in the upstream process that may influence cell health and growth, as well as drug quality attributes. With HCDC, there is no opportunity to centrifuge the cells as they are frozen in bags and the volume added to the bioreactor results in a much smaller dilution factor.

A series of studies were used to identify the optimal parameters for use of DMSO in an HCDC process. The following parameters were evaluated:

- DMSO concentrations from 0 to 15 percent
- Exposure times of cells suspended in the DMSO cryopreservation medium from zero to two hours
- Temperatures during which the cells were in contact with DMSO
- A variety of cell lines CHO-K1, DG44, CHO-S and CHOZN®, our proprietary cell line platform

Figure 5 presents viable cell density, viability and final IgG concentration produced by the CHO-S cell line stored in cryomedium as a function of the DMSO concentration, duration of incubation, and the cell line. The CHO-S cell line showed the highest sensitivity and deviation compared to CHO-K1, CHO-DG44 and CHOZN® cell lines (data not shown); the most robust conditions were observed with a DMSO concentration of 7.5 percent.

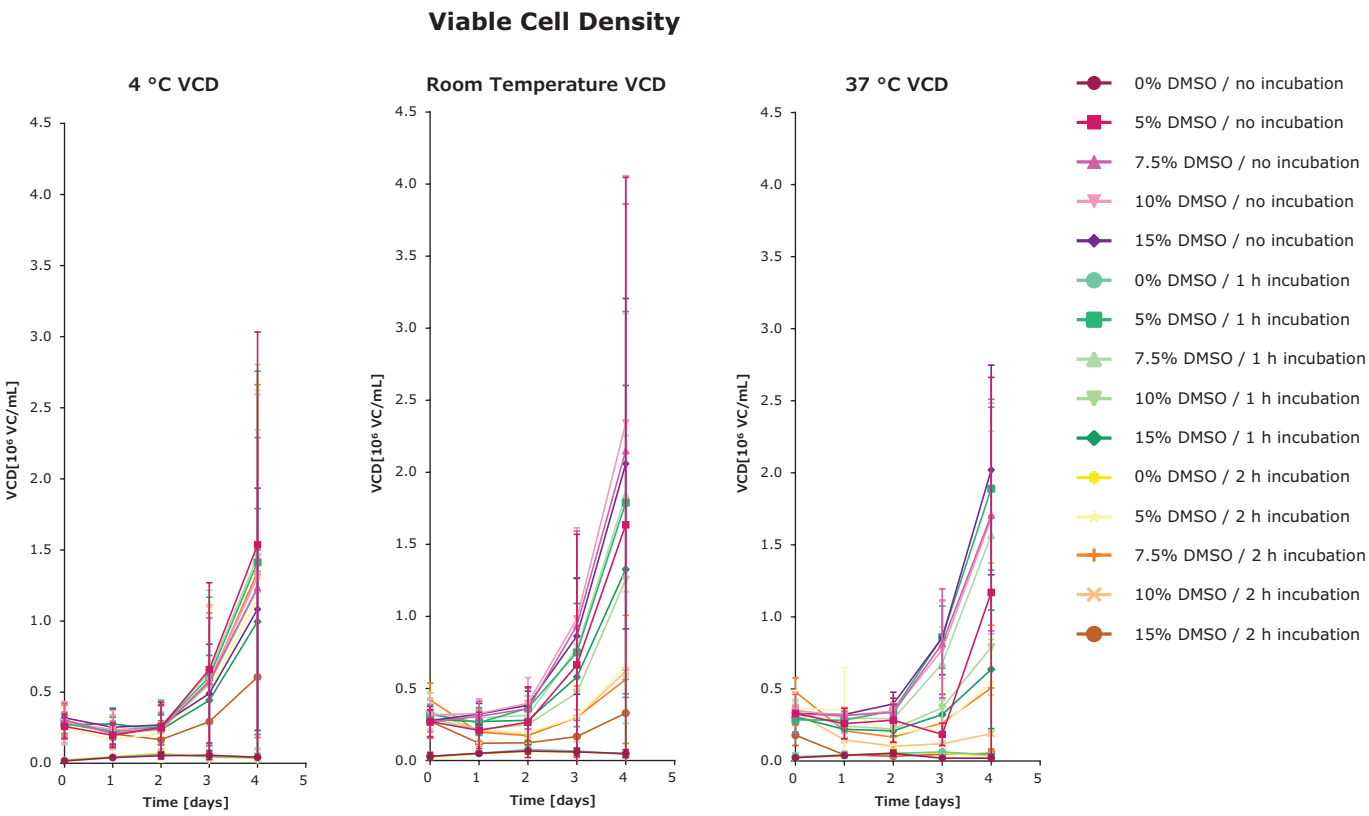


Figure 5. Impact of DMSO concentration and incubation time on viable cell density, viability and IgG concentration.

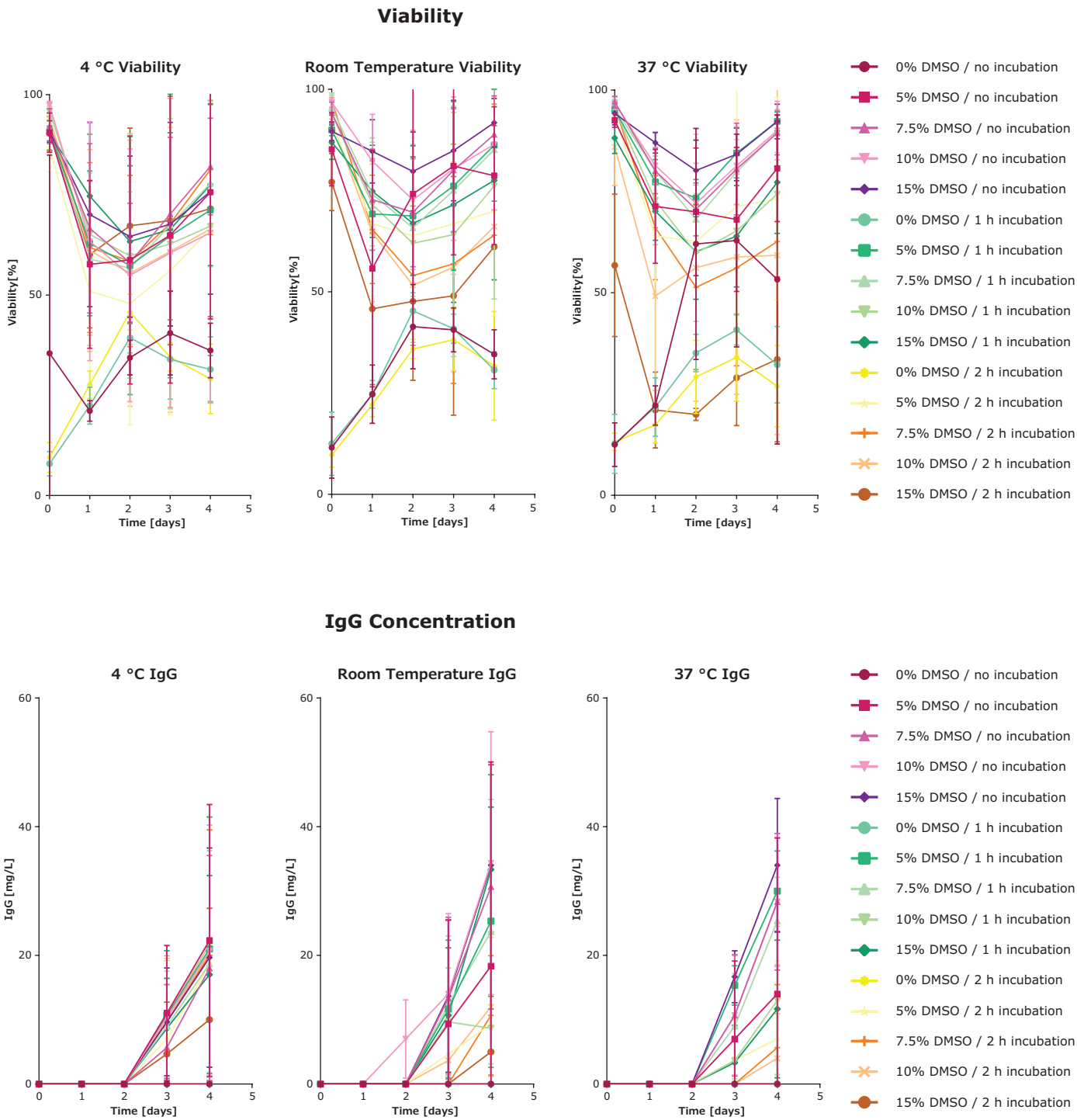


Figure 5. Impact of DMSO concentration and incubation time on viable cell density, viability, and IgG concentration.

Figure 6A shows the viability of one cell line following inoculation of a bioreactor with a cell suspension containing concentrations of DMSO ranging from 0 to 10 percent; viability decreased over time at concentrations above 1 percent when cells are in contact with DMSO. **Figure 6B** shows the toxicity threshold and indicated that a final concentration of DSMO should be approximately 0.5 percent in the bioreactor. At 7.5 percent DMSO, a 150 mL cryobag added to a 2.2 liter bioreactor equates to the desired a 1:15 dilution. As the bioreactor size that can be inoculated with a 150 mL bag exceeds the 2.2 liters, the final DMSO concentration in the culture vessel is negligible.

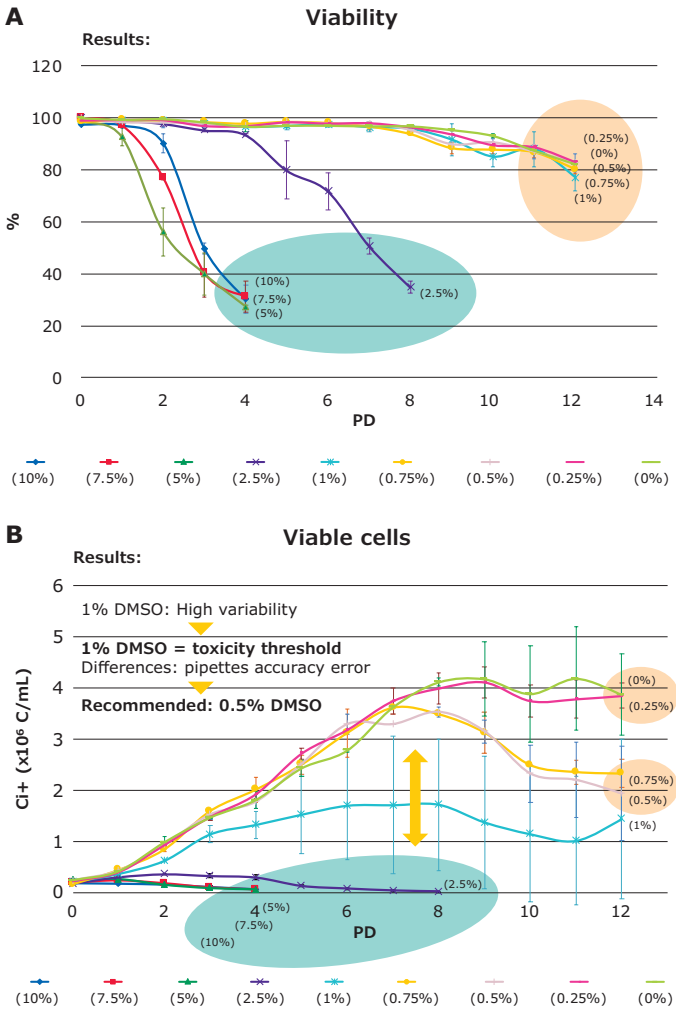


Figure 6. Impact of DMSO concentration (A) and the toxicity threshold (B).

Precooling the cell suspension prior to filling the bag may decrease the DMSO impact. Generating cell mass takes place at 37 °C and while this suspension can be transferred directly into the cryomedium, cells may be slightly more sensitive to DMSO. To further protect the cells, the temperature can be decreased to 20 °C or 4 °C prior to the transfer.

Impact of Freezing Technique

Standard protocols for freezing cells recommend freezing rates of about -1 to -2 °C/min to prevent cell damage. While this approach is easy to execute with small vials of cells, it is impractical for large numbers of larger volume cryobags. As such, the possible impact of uncontrolled freezing, and whether it is necessary to control the process, was evaluated. Results of the study in which two cell lines (CHO-S and CHOZN® cell line) were frozen using three different techniques are shown in **Figure 7**. Cells were either placed directly into a -80 °C freezer, frozen in a controlled rate freezer, or first placed in a CoolCell® container (Corning). The freezing technique had no impact on either cell line for any of the critical parameters measured.

It should be noted that while there was no impact on the two cell lines, it is important to evaluate the freezing technique for individual cell lines as introduction of DNA coding for a monoclonal antibody can transform cell behavior and impact the response to freezing.

A subsequent study evaluated the impact of the bag container itself on viable cell density, viability, and IgG concentration. CHO-K1 cells were grown in a bioreactor seeded from traditional vials. The bioreactor was run in batch mode until day three at which time perfusion mode was initiated. When the cells reached densities of $15x10^6$, $37.5x10^6$, $75x10^6$, $112.5x10^6$, and $150x10^6$ cells/mL, aliquots were removed to generate cryobags and vials, which would serve as a control. **Figure 8** shows a comparison of viable cell density, viability, and IgG concentration from cultures initiated either from vials or cryobags and indicates the bag had no impact on any of the metrics.

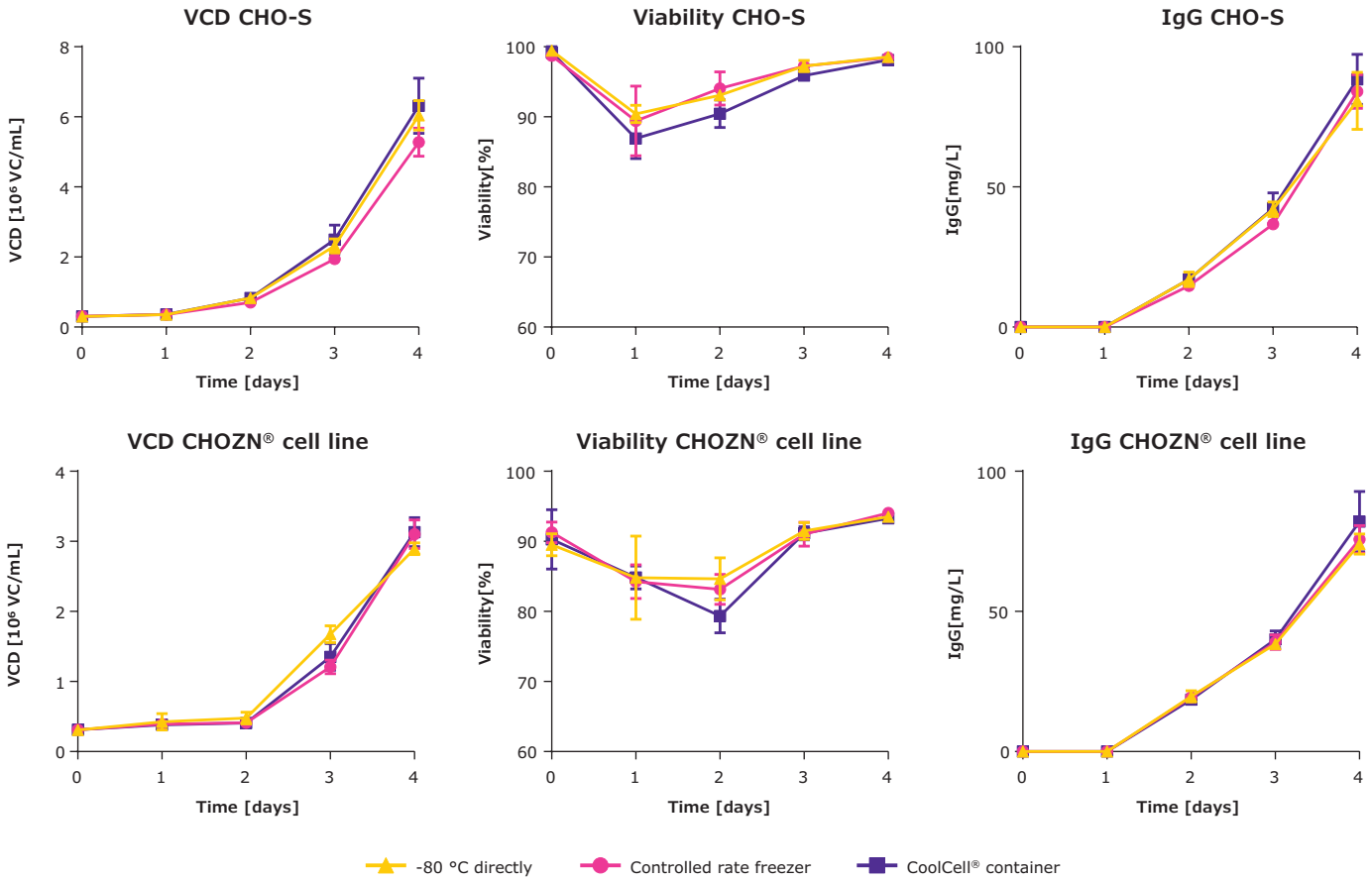


Figure 7. Impact of different freezing techniques on viable cell density, cell viability and IgG production from CHO-S and CHOZN® cell lines frozen using different techniques.

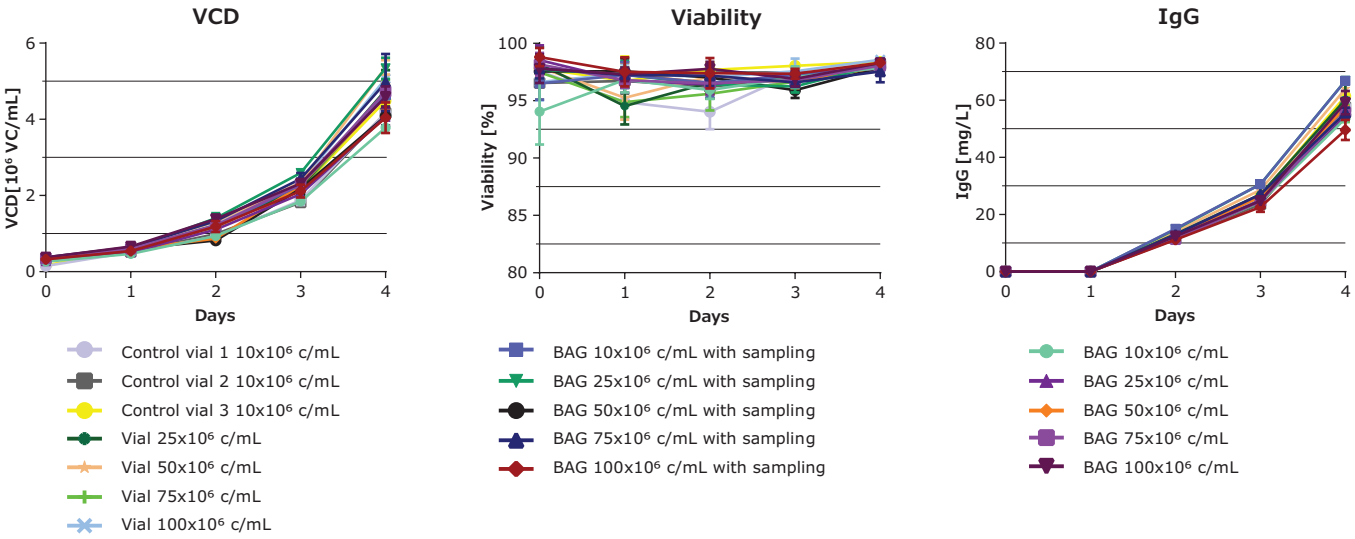


Figure 8. Comparison of viable cell density, viability, and IgG concentration from cells seeded from vials or cryobags.

To compare the intensified HCDC process and a conventional seeding process, cell growth and titer were evaluated in two bioreactors run in perfusion mode; one was inoculated with a seed train from an HCDC bag, one inoculated with cells originating from standard expansion from a vial. Following this, two more bioreactors were inoculated with the cells from the first two bioreactors and ran in perfusion with a steady state at the end (**Figure 9**). Both runs were comparable in terms of growth and titer, confirming that the HCDC application can be implemented without any negative effects on cell performance.

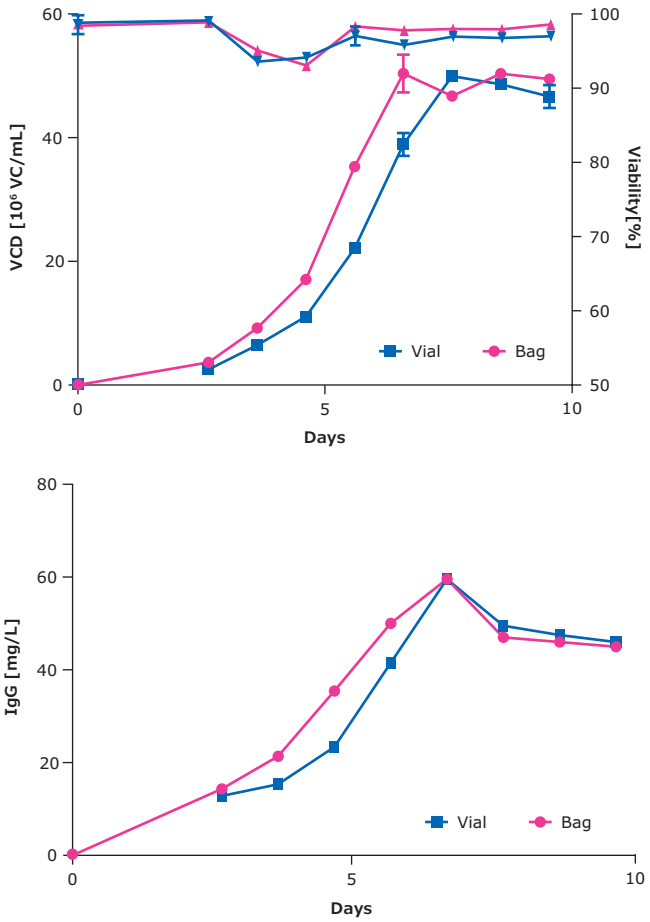


Figure 9. Growth and titer in simulated production bioreactor for CHO-K1 cells.

Conclusion

Use of HCDC is a proven strategy for intensifying the upstream workflow. As demonstrated in this white paper, HCDC can compress and accelerate the seed train and offers the advantage of closed processing. Bioreactors seeded with cells cryopreserved using HCDC bags can offer better reproducibility in seed train expansion and allows decoupling of expansion and batch production, enabling distribution of cells from a central expansion facility to global, decentralized production facilities.

While each cell line may respond to the HCDC process in a slightly different manner, guidelines for the recommended concentration of DMSO and the freezing process have been provided, offering a foundation for development of cell line-specific conditions.

Find the Right Products to Develop Your HCDC Process

Specially Designed Cell Culture Media for Expansion	
Cellvento® 4CHO-X Expansion Medium	**EMDMillipore.com/Seed-Train**
Specially Designed Single-use Assembly for HCDC	
Mobius® HCDC R&D Assembly	**EMDMillipore.com/Seed-Train**
Dimethyl Sulfoxide (DMSO)	
Dimethyl Sulfoxide EMPROVE® EXPERT Ph Eur,USP	**SigmaAldrich.com/catalog/product/mm/137117**

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Converge, Intensify and Evolve. Together.

Millipore®
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SAFC®
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