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# A new seed-train expansion method for recombinant mammalian cell lines

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## Abstract

A new approach has been developed and used to minimize the time and more carefully monitor and control the seed-train expansion process of recombinant mammalian cell lines. The process uses 50 or 100 ml cryo-bags that contain frozen cells at high cell densities of $20 \times 10^6$ ml$^{-1}$ (100 ml bags) or $40 \times 10^6$ cells ml$^{-1}$ (50 ml bags). The frozen bag cell suspension is thawed and transferred directly into a bioreactor that has been modified such that pH, DO and temperature can be controlled at the initial volume of two liters (the working volume eventually increases to 12 l). The successful use of these cryo-bags and the modified 'inoculation' bioreactor to initiate a new seed train expansion of rBHK or rCHO cells is described herein. The interval between cell thawing and the accumulation of sufficient cell mass to inoculate a production reactor is reduced by at least 25 to 30 days compared to the conventional method that begins with the thaw of 1–2 ml cryo-vials. This 'one-step' technology leads to a much more consistent scale-up by reducing manual operations and avoiding subjective decisions during the scale-up phase. The cell metabolic rates and product integrity were similar to the control experiments. Furthermore, it was found that it is not necessary to include a wash step to remove DMSO prior to the inoculation.

## Introduction

In commercial manufacturing of recombinant proteins from mammalian cells the seed-train expansion of cryo-preserved cells is a vital step necessary to initiate a new production campaign. This scale-up process is critical since the quality of the inoculum often determines the success of the entire production campaign. Current seed-train expansion protocols involve thawing a 1–2 ml cell suspension in a plastic cryo-vial and subsequently transferring the suspension into T-flasks or spinner flasks that do not have active pH or DO control (these flasks are routinely incubated in a controlled $CO_2$ incubator). The cells are subsequently sub-cultivated based on cell growth into larger cell culture vessels (Whitaker et al., 1998). If enough cell mass is accumulated the cell suspension is pooled and used to inoculate a seed bioreactor that will be used to start a new production campaign. This practice requires manual operations and the use of many culture vessels, resulting in the risk of contamination. In addition, campaign-to-campaign variability may result from the lack of active pH or DO control during scale-up.

This article describes an alternative approach to the seed-train expansion of mammalian cells. Cells are frozen at high cell density (20–40 million cells ml$^{-1}$) in cryo-bags, previously used successfully in blood cell banking (Regidor et al., 1999). Frozen cell bag stability has been reported for more than 7 yr when used to store umbilical cord blood (Re et al., 1998), thus suggesting the possibility of using these bags for mammalian cell lines producing recombinant proteins. The cells are thawed and directly transferred into a dedicated 'inoculation' reactor that serves as the seed source for production scale campaigns. The freezing of large quantities of mammalian cells at high cell density has been demonstrated previously; Ninomiya et al. (1991) froze 25 ml of mammalian cell suspensions at cell densities up to 150 million cells ml$^{-1}$ using blood

banking bags. Here, this technique was streamlined in combination with a dedicated inoculation reactor in order to become transferable to a cGMP environment for the production of protein therapeutics.

A critical step of this operation involves freezing the cells in large volumes (>50 ml). Freezing an aqueous solution occurs in the several physically defined stages, and the first, supercooling, occurs when the temperature of the solution falls below its equilibrium freezing temperature. The temperature to which a solution can be supercooled depends on several factors, including sample volume, cooling rate, the amount and nature of impurities in the solution, and the characteristics of the container. For large volumes of solution, ice will usually nucleate heterogeneously. After ice crystal nucleation, ice crystal growth starts. As ice crystals grow, dissolved solutes are expelled from the crystal lattice, producing pure ice crystals surrounded by an increasingly concentrated solution, resulting in a concentration process. As a result of this concentration of solutes, an unfavorable environment around the cells arises. For example, the pH may shift and NaCl concentration may become extremely high. The pH shift is often due to the combination of partial eutectic phase separation and super-saturation of buffer components such as phosphate. The NaCl concentration may increase 24-fold in case of an isotonic saline solution is frozen to its eutectic temperature of –21 °C (Grout et al., 1990; Franks, 1991). The damage (also called freezing injury) which cells undergoing during the freezing process has been attributed to a number of factors (Fennema et al., 1973; Franks, 1985; Avis and Wagner, 1999) including the formation of intracellular ice crystals and thus mechanical stress, in addition to the effects of solute concentration mentioned above. Cryoprotectants such as dimethyl sulfoxide (DMSO) are used to minimize the damage. DMSO is capable of entering and exiting the cells easily during freezing and thawing and therefore results in less freezing damage (Siegel, 1999). A second way to minimize the damage to cells during freezing and thawing is to optimize the process parameters. Among many process parameters, the freezing and thawing rates are critical because they directly impact the formation of intracellular ice crystals.

To freeze a large volume of cells, the dimensions of the package are also important. Ice crystallization is a strongly exothermic event, so freezing a large volume of an aqueous cell suspension involves the removal of a large quantity of heat energy. The rate at which heat is removed from the cell suspension as it is frozen will therefore be a large factor in determining how rapidly ice can form. If heat removal is inefficient, the temperature in the center of volume may reach a plateau close to the melting temperature of ice in the solution. For a large package, this would cause a substantial differentiation in cell environment across the sample during freezing, as the freezing profile at the surface would be quite different from that at the center (Avis and Wagner, 1999). The thickness of the package may be more critical than the volume of the package. A thiner package is often preferred in order to reduce freezing damage to the cells.

The optimization of the reactor cultures using either 50 or 100 ml bags, the necessity of DMSO removal as well as long-term storage data and western blot analysis for product quality aspects are described herein. The article also focussed on the freezing and thawing optimization of the cell suspensions in cryo-bags.

## Material and methods

### *Cell lines and medium*

Two recombinant cell lines, a rCHO and a rBHK cell line were used in this investigation. Both cells produce a recombinant glycosylated protein and are cultured in a protein-free cell culture medium. For the BHK cell line the medium was based on a 1:1 mixture of DMEM/Ham's F12, for the CHO cell line a custom made medium was used. The medium containing only recombinant insulin (Humulin, Eli Lilly, Indianapolis, IN, USA) for the BHK cell line was manufactured by JRH (Lenexa, KS, USA). BioWhittaker (Walkersville, MD, USA) provided the medium for the CHO cell line, respectively.

### *Cryo-container preparation and freezing*

The cells cryopreserved in bags were taken from CHO or BHK perfusion reactor cultures. These cultures were maintained at cell densities of 20–30 × $10^6$ cells $ml^{-1}$. Two different size cryo-bags were used in this study: A 250 ml bag was used for the croypreservation of 50 ml aliquots of cell suspension and a 500 ml bag was used for 100 ml aliquots of cell suspension (Cryocyte$^{TM}$ 250 ml or 500 ml, Nexell Therapeutics Inc. Irvine, CA, USA). The use of the 50 ml bags involved a concentration step of the cell suspension (by centrifugation) to reach 40 × $10^6$ cells $ml^{-1}$ in

50 ml. The concentration step was not necessary for the 100 ml bags ($20 \times 10^6$ cells ml$^{-1}$ in 100 ml). These targeted cell numbers in a single bag ensure a starting cell density of $\sim 1 \times 10^6$ cell ml$^{-1}$ at a starting working reactor volume of 2 l, the inoculation volume of the bioreactor. This cell concentration of $1 \times 10^6$ cells ml$^{-1}$ is commonly used for cryopreserved mammalian cells (Lindl and Bauer, 1989; Morgan and Darling, 1993). The cells were frozen in the cell-specific medium listed above with 7.5% dimethyl-sulfoxide (DMSO, Sigma, St. Louis, MO, USA) as a cryoprotectant (Thrift et al., 2000). No additional proteins were added. The bags were placed in a aluminum cassette (Custom BioGenic Systems, Shelby Township, MI, USA) and held on ice during transport to be frozen in either a controlled rate freezer (CryoMed, Forma Scientific Inc., Marietta, OH, USA) or in a –40 °C freezer (Revco, Asheville, NC, USA) before being stored in the vapor phase of liquid $N_2$. The freezing time in the –40 °C freezer was at least 4 h.

Thermocouples were taped to the external surface of each of the bags (i.e. inside the aluminum cassette), and these thermocouples were connected to a Campbell Scientific 21x datalogger (Campbell Scientific, Logan, UT, USA) for data recording for the freezing rate experiments. The freezing profiles of 50 ml cell suspension aliquots in 250 ml cryo-bags were compared with the freezing profile of a 1 ml aliquot in a 2 ml cryo-vial. The 2 ml cryo-vials were placed into the CryoMed freezer, the chamber temperature was cooled to 4 °C and held there for one hour. The chamber temperature was then lowered to –40 °C, and held for 2 h. The temperature was then reduced to –90 °C for one more hour. Efforts to achieve a similar freezing profile for the bags were undertaken and the temperature control program is based on the empirical freezing equation of Plank (Heldman and Singh, 1981) that shows the relationship between the time taken to freeze and the ambient temperature. The equation has the form:

$$t = (D \times L / T_f - T_a) \times (P \times d/h + R \times d^2/k) \ , \quad (1)$$

where

$t$ = the freezing time [h];
$D$ = the density [kg m$^{-3}$];
$L$ = the heat of fusion [KJ kg$^{-1}$];
$T_f$ = the temperature at which the sample starts to freeze [K];
$T_a$ = the ambient temperature [K];
$P$, $R$ = terms related to the sample geometry [−];
$d$ = the thickness of the sample [m];
$h$ = the heat transfer coefficient [W (m$^{-2}$ × K)];
$k$ = the thermal conductivity of the frozen material [W/(m × K)].

Comparing two freezing experiments on the same sample, $t = f(T_a)$ since all other terms in the equation remain constant. Equation (1) is thus simplified to:

$$t = (x/(T_f - T_a)) \quad (2)$$

where

$$x = (D \times L) \times (P \times d/h + R \times d^2/k).$$

This equation was used in the experiments to obtain a tangential freezing rate.

*Thawing and inoculation procedure*

Cells were thawed in a 37 °C water bath. The cell suspension was transferred to a centrifuge tube, diluted with fresh medium (1:1) and washed via centrifugation. The supernatant was discarded, the cell pellet re-suspended in fresh medium and transferred to a bottle to inoculate the bioreactor. This wash step was used to remove most of the DMSO and is common practice in mammalian cell culture (Lindl and Bauer, 1989; Ninomiya et al., 1991; Morgan and Darling, 1993; Wu et al., 2000, 2001). In later experiments the previous DMSO wash step was not performed and the cells were directly transferred to the bioreactor. The first cell viability and count was performed approximately one hour after inoculation.

*The concept of the inoculation reactor*

The principle of the 'one-step' inoculation concept and the sizing of this reactor is shown in Figure 1. The inoculation reactor used for the seed-train can be sized accordingly, based on the volume of the production

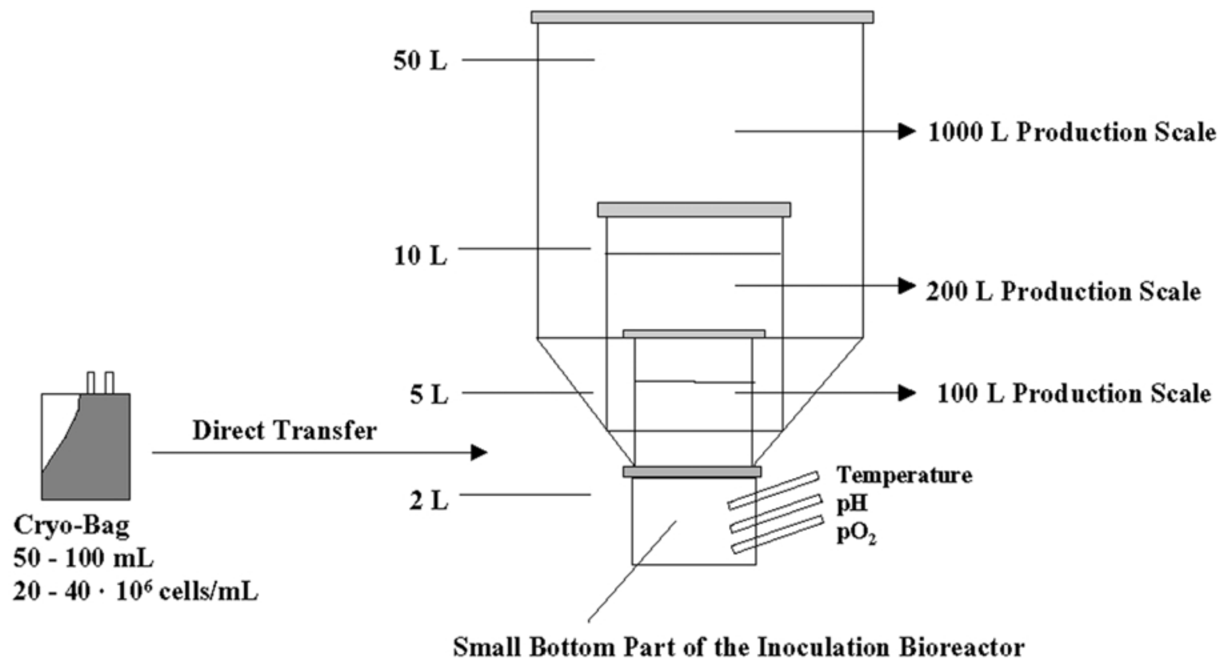


*Figure 1.* Schematic of the new 'one-step' inoculation procedure and sizing concept of the inoculation reactor: The cryo-bag is thawed and the cell suspension is directly transferred into the dedicated inoculation bioreactor. This reactor is started at an initial volume of 2 l. As the cells multiply, the volume is stepwise increased to the final working volume. After enough cell mass is produced the cells are used to inoculate a production bioreactor directly. The inoculation reactor can be sized for any size production reactor by simply modifying the upper part. The lower 'small bottom' part for the initial inoculation always remains the same.

bioreactor. The final volume of this inoculation reactor (VIR) can be determined by the formula:

$$V_{IR} = \frac{X_{PR} \times V_{PR}}{X_{IR}} [1] \qquad (3)$$

where

- $X_{PR}$ = the targeted initial cell density in the production reactor (in our case $1 \times 10^6$ cells $ml^{-1}$);
- $V_{PR}$ = the volume of the production reactor;
- $X_{IR}$ = the final cell density in the inoculum reactor.

The bottom portion of the reactor will be at least 2 l, and the volume is increased by increasing the diameter and the height of the upper part. Since VIR is not limiting, the same 'small bottom' concept can be applied to the design of larger bioreactors. The volume of the small bottom part is determined by the cell density and volume in the cryo-bag. Since in this study the small bottom part was always 2 l and the desired starting cell density was $1 \times 10^6$ cells $ml^{-1}$, 50 ml bags with $40 \times 10^6$ cell $ml^{-1}$ or 100 ml bags with $20 \times 10^6$ cell $ml^{-1}$ were used. The same type of formula (3) can be used to design a different small bottom part by changing the bag size and/or the cell density in the bag.

*Inoculation bioreactor operation*

Two different sized bioreactors were used in our studies.

(1) The 5 l working volume cultures were carried out in Applikon 7 l bioreactors (Applikon, Schiedam, The Netherlands), equipped with 0.5 $\mu$m stainless steel frit spargers (Mott Corp., Farmington, CT, USA) for aeration. The gas sparge rate was set to 50–100 ml $min^{-1}$. An external cell retention device was used during the perfusion period. Three flat-blade impellers at a speed of 60 rpm provided agitation. The minimum volume to cover the pH, DO, and OD electrodes was 2 l.

(2) The 12 l cultures were carried out in a modified 15 l inoculation fermentor. The reactor used the same stainless steel bottom portion as 7 l fermentor with the glass vessel and stainless steel lid of a 15 l Applikon reactor, connected by stainless steel flanges. Three flat blade impellers were used to agitate the

culture at 40 rpm. Aeration was provided through a 0.5 $\mu$m stainless steel frit sparger (Mott Corp., Farmington, CT, USA). The total gas sparging rate was set to 50–150 ml min$^{-1}$. An external cell retention device similar to the one for the 7 l system was used for this reactor as well.

Both bioreactor systems were controlled using a B. Braun DCU (Digital Control Unit, B. Braun Biotech, Melsungen, Germany) and a computer running a LabView based program (National Instruments, Austin, TX, USA). Dissolved oxygen was controlled at 50% air saturation and the pH was maintained at 6.80. Details of the general reactor setup is reported elsewhere (Chuppa et al., 1997; Heidemann et al., 2000). In the micro-sparged bioreactors, antifoam C (Sigma, St. Louis, MO, USA) was added at a concentration of approx. 20 ppm to the cell culture medium.

All cultures carried out in both the 7 and 15 l systems were seeded at an initial volume of 2 l. This is the minimum volume necessary to utilize the reactors pH- and DO-electrodes as well as temperature and optical density (OD) sensors (Aquasant, Bubendorf, Switzerland). Just after inoculation headspace aeration was used to avoid shear stress associated with gas sparging. The volume was increased stepwise to keep the cell density between 0.5 and $1.0 \times 10^6$ cells ml$^{-1}$. Headspace aeration was replaced with gas sparging when the culture working volume exceeded 3.5 l. Medium exchange with cell retention was started when the working volume was reached. The target cell density for each run was $20 \times 10^6$ cells ml$^{-1}$. Upon attaining $20 \times 10^6$ cells ml$^{-1}$, the viable cell density was kept approximately at this level by purging cells from the reactor based on the optical density probe readings.

According to formula (3) the 5 l bioreactor can be used to serve as the seed source for a 100 l production reactor were as the 15 l reactor (working volume 10–12 l) can be used as the inoculum for a 200 l production bioreactor (see also Figure 1).

*Comparison with 1 ml cryovials*

Conventional seed-train expansions were initiated from the 1 ml aliquots in cryo-vials. The initial cell accumulation phase was performed in plastic shaker flasks or roller bottles and a 15 l perfusion bioreactor (Applikon, Schiedam, The Netherlands) was inoculated to achieve an initial cell density of about $1 \times 10^6$ cells ml$^{-1}$. The cultivation conditions and the reactor set-up were the same as in the inoculation reactor cultures.

*Off-line analysis*

Sampling and off-line analysis was performed daily to determine cell and metabolite concentrations. Cell counts and viability were determined using a hemacytometer and the trypan blue exclusion method. In addition, the automated cell counter CEDEX (Innovatis GmbH, Bielefeld, Germany) was used. A YSI analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA) was used to measure glucose, lactate, glutamate and glutamine concentrations of samples. LDH and ammonia were measured using a Kodak Biolyzer (Eastman Kodak, NY, USA). A NOVA blood gas analyzer (NOVA Biomedical Corp., MA, USA) was used to measure the dissolved $CO_2$ level and to check pH and $pO_2$ values. Samples were also analyzed for product concentration.

*Chemiluminescent based western blot*

Western blots were performed to check the integrity of the product. The assay is a sensitive, generic, electrophoretic based method. It has been developed and optimized for assessing the target protein quality and potential for purification directly from fermentor harvests without any need for concentrating the sample. A desalted fermenter sample on SDS-PAGE followed by Western Blotting using in-house monoclonal antibodies. The key to this technique is use of a chemiluminescent-based detection system, which results in significantly higher sensitivity than conventional Western Blot detection techniques. Specifically, chemiluminescent substrates are used in combination with an amplification system consisting of an anti-mouse biotinylated secondary antibody that reacts with a horse-radish peroxidase conjugate avidin label. Routine detection to as low as 4 ng of target protein has been accomplished by this assay. Details of the method are described elsewhere (Zachariou et al., 2001).

## Results and discussion

*Cell freezing characterization*

The optimal freezing rates for mammalian cells were investigated with freezing rates that ranged from 313 °C min$^{-1}$ (plunging in liquid nitrogen) to

*Table 1.* The drop in cell viability in % compared with the unfrozen cell sample is shown for different freezing and thawing conditions. The best cell recovery was found with freezing in the range of 0.1–4 °C $min^{-1}$. Note that all frozen samples were thawed in a 37 °C water bath. For thawing the best recovery was obtained at high rates in a standard 37 °C water bath

| Freezing conditions (n = 3) | Drop in viability | Thawing conditions (n = 3) | Drop in viability |
|---|---|---|---|
| Freeze dryer, 0.1 °C $min^{-1}$ | 0±2 | 37 °C Water bath, 110 °C $min^{-1}$ | 5±2 |
| Controlled rate freezer, 0.7 °C $min^{-1}$ | 3±2 | 25 °C Water bath, 21 °C $min^{-1}$ | 7±2 |
| –40 °C freezer, 1 °C $min^{-1}$ | 3±1 | 20 °C Room temperature, 2 °C $min^{-1}$ | 18±2 |
| –70 °C freezer, 4 °C $min^{-1}$ | 6±2 | 5 °C Refrigerator, 1 °C $min^{-1}$ | 26±6 |
| Liquid $N_2$, 340 °C $min^{-1}$ | 33±10 | Freeze dryer, 0.1 °C $min^{-1}$ | 37±3 |

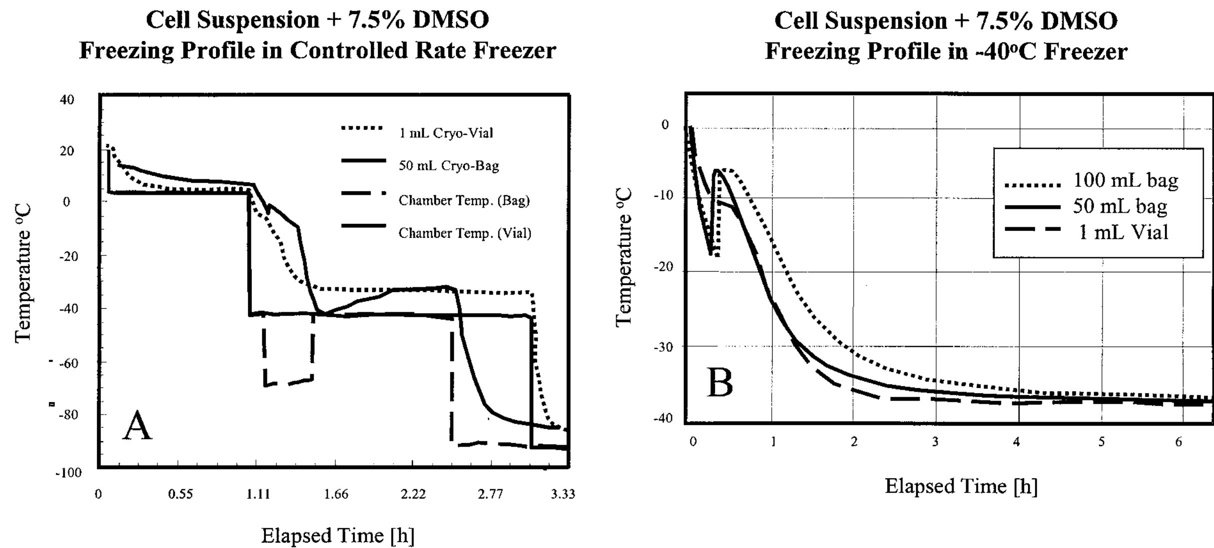


*Figure 2.* (a) shows the freezing profile of a 1 ml vial at –40 °C in comparison to a 50 ml bag frozen in the controlled rate freezer. By using the appropriate freezing program a cell bag can be frozen like a standard 1 ml cryo-vial. (b) Shows the freezing profile of 50 and 100 ml bags in the –40 °C freezer. No significant difference was noticed.

0.1 °C $min^{-1}$ (freezing sample in a freeze dryer). Table 1 summarizes the freezing results. Freezing rates were obtained by thermocouple readings. Specifically, freezing rates at 0.1–4 °C $min^{-1}$ resulted in good cell recovery. This corresponds with reports in the literature that often favors freezing rates of 1 °C $min^{-1}$ (Regidor et al., 1999). An optimized slow freezing profile averaging about 2 °C $min^{-1}$ using a controlled rate freezer was also favored for the cryopreservation of rat hepatocytes (Diener et al., 1993). Freezing the cells directly in liquid nitrogen (a freezing rate of 313 °C $min^{-1}$) had a negative impact on the cells as expected since the fast freezing rate is more likely to cause intracellular ice crystallization and cell damage (Avis and Wagner, 1999).

The freezing time for the cryo-bag (Figure 2b) and for the cryo-vial (Figure 2b) at –40°C was determined. Equation (2) (see the Materials and Methods section) was used to calculate the value of $x$ based on an estimated value of $T_f$. The objective was to produce a freezing profile for the cryo-bag similar to the freezing profile of cryo-vials. This was done by estimating the ambient temperature required for the cryo-bag from the values of $x$ and $T_f$. The following controlled rate freezer profile was used which is similar to the approach used by Diener et al. (1993):

(a) hold chamber at 4 °C for 1 h (only used in the freezing experiments, otherwise about 5 min);
(b) hold chamber at –40 °C until sample reaches –2 °C;
(c) hold chamber at –65 °C until sample reaches –35 °C;
(d) hold chamber at –40 °C for 1 hr and then:

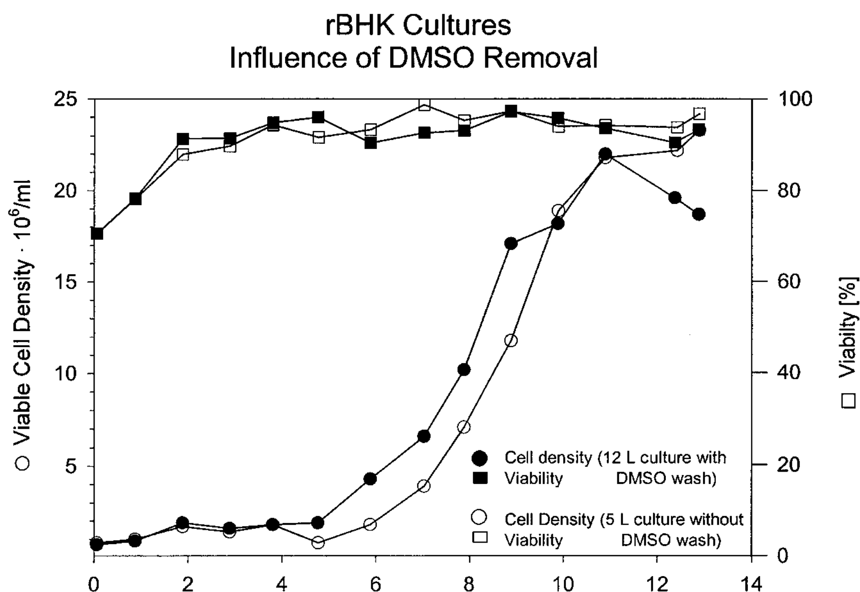


*Figure 3.* Influence of a DMSO removal step in the inoculation procedure: A DMSO removal step was performed prior to inoculation of the 12 l culture (white symbols). No DMSO washing was done prior to inoculation of the 5 l culture (black symbols). The viable cell density (circles) and the cell viability (squares) are given. Both cultures originated from the same lot of bags.

(e) hold chamber at –90 °C for 1 h.

Figure 2a shows the cryo-vial and bag temperature versus time, demonstrating the similarity in freezing times. The freezing profile of 50 and 100 ml cryo-bags in the –40 °C freezer was also investigated. The critical factor is not the volume, but the thickness of the package. The thickness of the package is directly correlated with freezing rate and thawing rate. It was found that the thickness of the package was almost the same, as the ratio of the aliquot to the bag capacity is the same, 1:5. Figure 2b shows the thermocouple readings for the 50 and 100 ml fill volumes. The figure shows that there is some lag-time associated with the larger volume, but this difference is not significant. The freezing rate is approximately 0.4 °C $min^{-1}$ (it should also be noted that there is a pronounced super-cooling effect after 20 min). The freezing profile shows that the freezing rate for 100 ml fill volume is within the optimal freezing rate range of 0.1 to 4.0 °C $min^{-1}$. It should be noticed that all cryo-bags used for the subsequent cultures described in this article were frozen in the –40 °C freezer.

*Cell thawing*

The thawing rates tested ranged from 110 °C $min^{-1}$ (37 °C water bath) to 0.1 °C $min^{-1}$ (thawing sample in a freeze dryer) respectively. As indicated in Table 1 thawing at 2 °C $min^{-1}$ or slower is damaging. Thawing in a water bath at 25–37 °C yields a thawing rate of 21–110 °C $min^{-1}$ and results in comparably good cell recovery. This is the optimal range for thawing mammalian cells as reported numerous times in the literature (Diener et al., 1993; Regidor et al., 1999; Wu et al., 2000, 2001). Table 1 summarizes all cell thawing results.

*Bioreactor cultures*

Several bioreactor cultures using this new seed-train expansion method were conducted. The 5 l bioreactor as well as the 12 l bioreactor system were used, both manufactured with the unique ‘small bottom’ part for the early inoculation phase. The reactor cultures focussed on the effect of bag size, DMSO removal, long-term stability data and overall cell performance.

*The influence of DMSO removal*

In addition to examining the overall feasibility of using this new scale-up method for mammalian cells the necessity of a DMSO washing step was also investigated. Eliminating the wash step further streamlines the cell expansion process. Figure 3 shows two rBHK bioreactor cultures; both seeded form the same lot of bags. A DMSO removal step was performed prior to inoculation of the 12 l reactor culture but not with

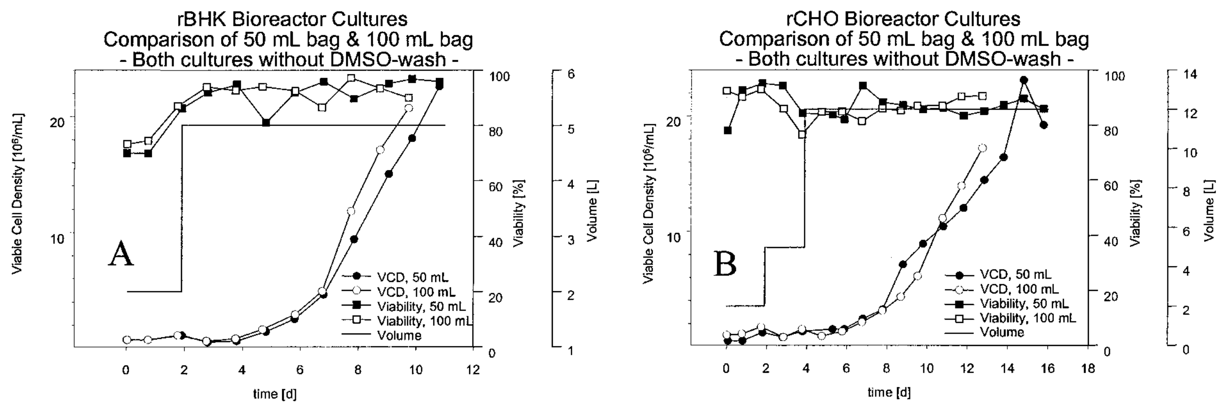


*Figure 4.* Influence of the bag size on one-step inoculation cultures. (a) Shows the cultures with the rBHK cell line. (b) Shows the results with the rCHO cell line. In both graphs the viable cell density (VCD) as well as the viability profile is given. No DMSO washing step was performed prior to inoculation for all cultures shown.

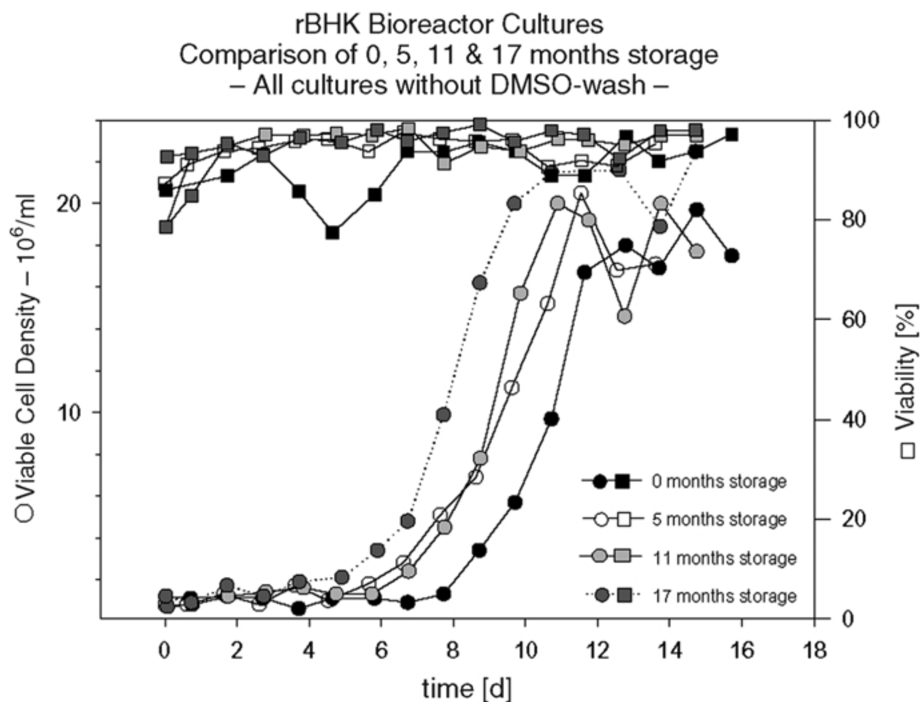


*Figure 5.* Long-term storage of rBHK cells in cryo-bags. Viable cell densities (circles) and the viability profile (squares) are shown. No DMSO removal step was performed prior to inoculation of all cultures.

the inoculation of the 5 l culture. This gave a dilution factor of 40 (50 ml in 2 l). The cell and viability curves showed no differences. In both cases the target cell density of 20 million cells $ml^{-1}$ was reached within 11 days, indicating that the omission of the DMSO washing step is acceptable.

*50 ml versus 100 ml cryo-bags*

During the cell freeze process for the 100 ml bags, the cell suspension was directly taken from a bioreactor, mixed with a 50% DMSO stock solution and then used to fill the bags. The resultant suspension had a final concentration of $\sim 20 \times 10^6$ cells $ml^{-1}$. Since no cell concentration step is necessary – in contrast to the 50 ml bags – to fill 100 ml bags, the use of these bags have major advantages in the cell banking process.

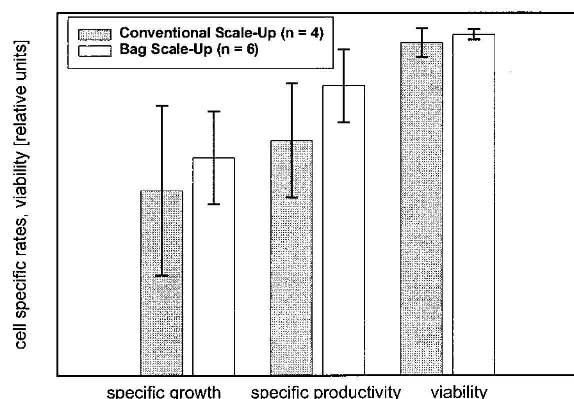


*Figure 6.* Average cell specific rates and cell viability data (all rBHK cell-line) from conventional seed-train expansions and cryo-bags. The error bars indicate the standard deviation.

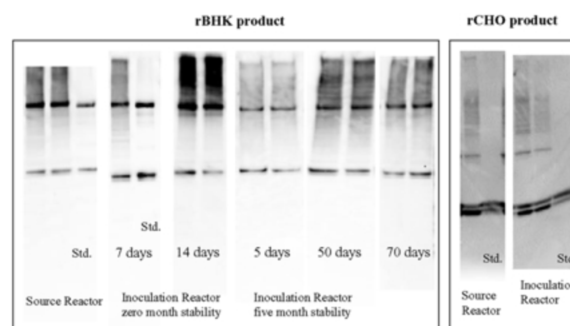


*Figure 7.* Western blot results for the rBHK product (left) and the rCHO product (right). Each set of blots shows reactor harvest samples from the source and the inoculation reactor. The source reactor contained the culture from which the cryo-bags were prepared. For the rBHK product, different time points form the zero and 5 months storage experiment are shown.

The 100 ml bags were also tested to determine if a DMSO removal step is necessary. Figure 4a shows two BHK 5 l bioreactor cultures, one started from a 50 ml bag, the other one from a 100 ml bag. In both cases the DMSO wash step eliminated. There was no apparent difference in the cell performance. Therefore, a 20-fold dilution of DMSO (to 0.4%) is acceptable if the 100 ml bags are used. The same type of experiment was also performed with a recombinant CHO cell line (Figure 4b) and the data indicate a similar feasibility for the CHO cells as well.

*Bag stability data and cell specific rates*

Figure 5 shows four rBHK cultures at different storage time points (0, 5, 11 and 17 months). The cell growth and viability profiles did not indicate any negative impact of frozen cell storage with time. The culture started with the 5 months storage bag was cultivated for 100 days without any problems (profile not shown). The product quality was similar to the control (detailed in the following section).

Key parameters were compared for the conventional scale-up (1 ml vials) and for the bag cultures. To get a fair comparison, only the initial bioreactor period (perfusion start until the target cell density of $20 \times 10^6$ cells $ml^{-1}$ was reached) were used for these calculations. Figure 6 shows no negative effect in cell viability, growth-rate or specific productivity. The cell-specific rates indicate no significant metabolic change if the new seed-train expansion method is used.

*Product quality data*

Western blot assays were performed in order to check the quality of the two different cell products (see Figure 7). The samples were taken during different time points of the one-step inoculation cultures and compared with samples from the conventional seed-train expansion cultures that served as the source for the bags. No difference or any sign of degradation was noticed in comparing the material from the source reactor and or the new one-step expansions. Also, long-term cultures of more than 70 days indicate no shift in product quality as shown by the blots of the 5 months storage experiment.

## Summary and conclusions

The successful use of cryopreserved cells in 50 or 100 ml bags to start a new seed-train expansion was demonstrated. A ‘modified’ inoculation bioreactor was built to serve as the seed reactor for production scale. The reactor can be sized to inoculate production reactors of different volumes (certainly in the range of 100–1000 l). To use this technology also for batch-type production plants using bioreactors of several cubic meters (>10 000 l) this inoculation bioreactor can be incorporated into their already existing scale-up train and therefore also enhance and streamline those processes.

The time to start a new production campaign using the 12 l inoculation reactor and this new ‘one-step’ inoculation concept is reduced to about two weeks, 60–70% faster than the old seed-train expansion method. This new scale-up procedure streamlines the entire

cell expansion process, significantly reducing labor-intensive small-scale cell culture operations and leads to a more consistent feed stream for the manufacturing of pharmaceuticals. It was found that a DMSO wash step is not necessary for the 50 or 100 ml bags, furthermore streamlining the inoculation process and also reduces the risk of contamination.

The freezing profiles and the successful reactor cultures of the cryo-bags demonstrate the use of a standard –40 °C freezer prior to storage in liquid nitrogen. Nevertheless, to obtain freezing profiles similar to those for 1 ml cryo-vials a controlled rate freezer should be used. This also contributes to a more consistent, reproducible and streamlined cell banking process.

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