Recent advances in optimal cell banking of mammalian cells for biopharmaceutical production

Mammalian cells are routinely used in the biopharmaceutical industry for production of recombinant therapeutic proteins. Cell banking to ensure preservation of these cells at low temperatures for an extended period of time is at the core of establishing a manufacturing process utilizing these cells and various strategies have evolved over time to ensure recovery of viable ‘and’ functional cells. This paper provides an overview of cryopreservation practices and highlights recent approaches that have been adopted to improve cryopreservation outcomes for these industrially relevant production cell lines.

Keywords: biopharmaceutical, cell bank, cell culture medium, cell recovery, CHO cells, cryopreservation, freezing, mammalian cell culture, monoclonal antibody, recombinant proteins

Recombinant therapeutics & mammalian cells

With over 200 biopharmaceutical products, biopharmaceutical sector still represents a significant and growing proportion of the overall pharmaceutical market [1]. Biopharmaceuticals differ from other pharmaceutical products in that they are created using biological processes, rather than being chemically synthesized. The most prominent biopharmaceuticals include monoclonal antibodies in the disease area of oncology (such as rituximab, trastuzumab and bevacizumab) and immunology (such as etanercept, infliximab and adalimumab), insulin and insulin analogs for diabetes, followed by erythropoietin-based products to stimulate the production of red blood cells in the treatment of chronic anemia.

Most biopharmaceuticals are termed as biologics wherein the biopharmaceutical is produced via living cells using recombinant DNA technology. Major mammalian cell lines used in the biopharmaceutical industry as host cells for production of biologics include Chinese hamster ovary (CHO) cells and mouse myeloma cells, including NS0 and Sp2/0 cells. Established technologies for expressing foreign genes in mammalian cells can be categorized into transient, inducible and stable expression platforms (Figure 1) [2–7]. Over time, these technologies have become increasingly important not only to understand the functional significance of genes and regulatory sequences but also as a primary method for production of biotherapeutics.

Defining cell banks

In order to produce these recombinant biotherapeutics, a cell line expressing the protein of interest is developed (Figure 1). Once a parental or host cell line is obtained [9], it is genetically engineered to express often repeated copies of the gene of interest to produce larger amounts of protein [10]. Once the cell line is engineered, the right cell substrate or clone with desirable production characteristics [8] (such as, good growth and productivity, good tolerance to waste metabolites, ability to maintain high viability and process protein correctly to confer desired product quality attributes) is selected (Figure 1) [11]. In a recent paper, Davies et al. [12] evaluated several CHOK1SV clones and in effect proposed to exploit genetic/functional variation...
Figure 1. Typical process flow for biopharmaceutical production using mammalian cells. Data taken from [2–6, 8].

in parental CHO cell populations to isolate clonal derivatives that exhibit superior, heritable attributes for biomanufacturing.

Continuous maintenance of these parental or production cell lines through the life cycle of a given biologic is impractical given the potential for genetic drift leading to loss of a cell’s original characteristics, risk of exposure to microbial contamination and the possibility of culture co-contamination. Therefore, a critical component in assuring the quality and safety of these biological products is the effective long term storage of the cell lines.

In addition to the routine preparation of frozen cell batches during research and development, typically, a two-tiered system is followed in the biopharmaceutical industry wherein both the Master Cell Bank (MCB) and the Working Cell Bank (WCB) are prepared and used for the initiation of the manufacturing process [5, 13]. Once an optimal production cell line has been engineered, an MCB is prepared from an aliquot of a single pool of cells (uniform composition), dispensed into multiple vials, and stored under defined conditions (usually -150ºC or below) (Figure 1). The WCB is derived from one or more vials of cells from the MCB, which are expanded by serial subculture (Figure 2). Normally, a 100-vial (10⁷ cells per vial) MCB and a 100–500 vial WCB is prepared. This two-tiered approach ensures preservation of the passage history and lineage of the original cell line or stock.

Generation of cell banks marks the beginning of the current GMP manufacturing process and each bank goes through a rigorous process of characterization and testing for the presence of microbial contaminants (bacteria, fungi, mycoplasma and endogenous and adventitious viruses) [8]. When using a two-tiered banking system, extensive characterization needs to be performed on the MCB. Because all WCBs would be derived from the well-characterized MCB, your WCBs may be tested in a more limited manner, focusing on adventitious agents to which the WCB could have been exposed during expansion from the MCB. Establishment of a two-tiered cell banking system – MCB and WCB – is expected for the licensed products, but given the devel-

Key term

Host cells for production of biologics: Mammalian cells such as CHO (Chinese hamster ovary cells) and NS0 (mouse myelomas) are preferred host cells for producing large proteins given their ability to carry out post-translational modifications (such as glycosylation).
development timelines, only an MCB may be available for early clinical trials and the WCB, created from the MCB, is then introduced during later stages of development. Perfusion culture may be utilized to attain high density and high viability cell culture for cell banking.

It is critical to ensure very early on the integrity, quantity and back-up storage for the MCB. Typically, MCB and WCB are stored in two or more separate locations within the facility or at a distant site for risk mitigation and to avoid loss of the cell substrate due to a local disaster or equipment malfunction. Access to these cell banks is also limited and controlled with appropriate record of the location, identity and inventory of individual ampoules of cells.

Several guidelines including points to consider are available from regulatory agencies (such as US FDA, Center for Biologics Evaluation and Research, the European Commission and more recently the International Conference on Harmonization) around appropriate standards for the preparation and characterization of cell banks to be used for production of biologics. International Conference on Harmonization Q5D provides a comprehensive approach to quality issues of mammalian cell substrates used to produce biotechnological products.

Overall, advantages of a well-characterized cell banking system are as follows: Characterization and testing of banked cell substrates confirm identity, purity and suitability for manufacturing use; banked cells for production of biological products provide a consistent source and assure that an adequate supply of equivalent cells exists over the lifespan of the product. Subsequent to establishment, stability of cell lines cryopreserved for a period of time also needs to be ascertained with respect to cell growth and production characteristics [5].

**Cryopreservation**

Cryopreservation techniques have emerged and evolved since the first successful cryopreservation
Key terms

Rate controlled freezing: Rate controlled freezing involves initial cooling of sample to freezing temperature; release of the heat of fusion; controlled post freeze cooling and accelerated post freeze cooling.

Non-freezing cold temperature: Low temperature of 0–10ºC may have a detrimental impact on the viability of mammalian cells due to events like oxidative damage, lipid phase transitions and ion imbalance across cell membranes.

Osmotic shock: Sudden change in solute concentration across the cell membrane, disrupting water flow across the membrane and leading to unfavorable events such as loss of membrane integrity.

of cells in 1949 [14], primarily from research that for large part focused on two cell lines: red blood cells and spermatozoa. Successful freezing of mouse embryos to -196ºC using a slow cooling rate of 0.3–2ºC/min was also demonstrated early on [15]. However, different cell types and lines may show varying sensitivity to different cryopreservation strategies and parameters leading to further protocol optimization.

Traditional protocols described in the literature entail freezing cells at <-80ºC and typically below the nominal glass transition temperature of pure water (-140ºC) [16,17] in 1-ml cryovials typically at cell concentration of 5–30 × 10⁷ cells/ml. More recently mammalian cells have been successfully frozen in larger size formats including 5-ml or 10-ml glass or plastic cryovials (cryovials or ampoules from, e.g., Nunc, Corning) and disposable bags (up to 150 ml) at cell concentrations as high as ~10⁹ cells/ml [6,18–21]. Banking larger number of cells, via freezing larger volume of cell culture and/or at a higher cell density enables faster expansion/scale up of cell culture post thaw.

Overall, success of a cryopreservation protocol depends on the rate at which the cells are cooled, the type and concentration of protective additive (cryoprotectant) used and the rate at which the cells are warmed up to revive from the frozen state. At a biophysical level, aim of these protocols is to reduce cryopreservation induced cell injury by modulating water transport across cell membrane and by minimizing intracellular ice formation [22].

Different cryopreservation strategies are described in the literature [23] (ultra-rapid freezing, controlled-rate freezing or rate controlled freezing, freezing with nonpenetrating polymers, vitrification and equilibrium freezing). Vitrification is a nonequilibrium method and may be regarded as a radical approach in which ice crystal formation is totally eliminated. Vitrification is a special form of polymer-assisted freezing procedure wherein neither extracellular nor intracellular ice forms and the entire sample vitrifies, in other words, transforms into a metastable, amorphous glass. Nevertheless, it requires an extremely high cooling rate alongside much higher concentrations of cryoprotectants when compared with slow freezing. Vitrification has been employed for cryopreservation of multicellular structures such as embryos and organized tissues (e.g., kidneys). Equilibrium freezing requires a sufficiently high concentration of cryoprotectant, and has not been very popular as a cryopreservation strategy for mammalian cells given the complexity around implementing protocols with high concentrations of the cryoprotectant during the freeze-thaw process. Controlled-rate freezing on the other hand requires lower levels of cryoprotectant. The cell sample mixed with freezing medium is first cooled to freezing temperature that allows for release of the heat of fusion, followed by controlled post freeze cooling between 1 and 5ºC/min until the temperature is below -40ºC, and finally cooled rapidly at rates greater than 5ºC/min below -40ºC to liquid nitrogen vapor phase temperature of <150ºC [7]. Spin drying CHO cells prior to cryopreserving at a controlled rate leads to rapid vitrification of cells resulting in improved survival post-thaw [24]. Chakraborty et al. [24] showed that spin-dried cells were successfully stored at cryogenic temperatures without using exceedingly high concentrations of cryoprotectants. This finding could also lead to new cryopreservation protocols to stabilize cells at temperatures higher than cryogenic temperature.

Cryoprotectants added to the freezing medium modulate solute concentration during cryopreservation. These additives, applied in conjunction with optimal cooling, protect cells against damaging solution effects, harmful volume changes and lethal, intracellular ice. Commonly used cryoprotectants include glycerol, dimethyl sulfoxide (DMSO), ethanediol and propanediol [7]. In a recent study, CHO cells were used to assess the induction of DNA breaks and chromosomal damage by three cryoprotectants: dimethyl sulfoxide, ethylene glycol and propylene glycol. It was found that dimethyl sulfoxide was not genotoxic; however, ethylene glycol and propylene glycol could induce in vitro chromosomal damage in eukaryotic cells [25].

Cryopreservation-induced cell injury

Nearly all cryopreservation protocols begin with reducing sample temperature to within the range of non-freezing cold temperature of 0–10ºC leading up to the liquid nitrogen temperatures of below -150ºC. Oxidative stressors presented to a cell in the cold, in conjunction with the general exposure of free radicals, can lead to multiple routes for the initiation of a molecular-based stress response to low temperature exposure. Distinct cell types would have different
sensitivity to stresses induced during cryopreservation. Some cell types may exhibit significant post-thaw death (30–70%) within 24–48 h.

Stresses associated with cryopreservation have been linked to structural changes in cell membrane, release of calcium, osmotic fluxes, generation of free-radicals and oxidative state of the cell, to name a few. It is important to note that molecular cell death following cryopreservation may kick-in only after several hours to days post-thaw leading to the term cryopreservation-induced delayed-onset cell death (CIDOCD) where cell viability is typically seen as elevated immediately post-thaw and then progressively decreases during the initial 24–48 h of recovery as apoptotic and necrotic events manifest, yielding a ‘true’ survival that is much lower than initially observed [26].

Multiple cell death pathways linked to apoptosis or necrosis may be activated inside a cell in response to cryopreservation [26]. Following induction, apoptosis (generally referred to as silent or physiological cell death in response to developmental and environmental signals such as exposure to nutrient deprivation or extreme temperatures) progresses via caspase (cysteine-aspartic acid protease family proteins) activation, mitochondrial release of cytochrome C, cell cycle arrest, alteration of membrane phospholipids, finally leading to nonrandom cleavage DNA into 180 kDa fragments by exonucleases, membrane blebs and apoptotic body formation (cell membranes remain intact) and the complete disassembly of cells. Necrosis on the other hand is a harsher and more disruptive form of cell death where the cell lysis is marked by membrane swelling and rupture, endonuclease activity leading to random DNA degradation and release of cytokines linked to active recruitment of immune and inflammatory responses. Necrosis has been observed under conditions of osmotic shock, exposure to toxic agents and also cryopreservation.

Recent strategies to improve cell recovery postcryopreservation

Kato et al. recently demonstrated freezing tolerance of mammalian cells expressing Aquaporin (AQP4), a water channel protein [27]. Under cooling rate of ~120°C/min (ultra-quick freezing) and in the presence of 10% DMSO, survival rates of both CHO and Madin-Darby canine kidney cells stably expressing AQP4 were significantly higher (~60 and 37%) compared with ~2% survival rate exhibited by host CHO and Madin-Darby canine kidney cells frozen under the same conditions. Furthermore, decrease in DMSO from 10 to 1% (corresponding to a decrease in osmolality from 1600 mOsm to 432 mOsm) was still sufficient in maintaining >50% survival rate in AQP4-CHO cells. The findings suggest that the expression of AQP not only enables a reduction in the amount of cryoprotectants for freezing, but also allows for selection and concentration of CHO cells transiently transfected with AQP4 by multiple cycles of ultra-quick freezing/thawing.

Given that DMSO may exhibit toxic side effects when used with certain cell types, disaccharides such as trehalose have been investigated as a cryoprotectant. Cryopreservation solution containing intracellular and extracellular trehalose has been shown to improve the recovery of stem cells after cryopreservation [28]. In addition to using trehalose for cryopreservation of cells, protection of cells using intracellular trehalose has also been demonstrated during dry preservation of mammalian cells. Loading trehalose into cells with a high capacity trehalose transporter (TRET1) improves survival of CHO cells during desiccation. The TRET1 was stably expressed in CHO cells (CHO-TRET1) and after partial desiccation, significant increases in viability and growth were observed for CHO-TRET1 cells incubated in trehalose solution relative to control CHO cells [29].

Recent developments have also led to improved cryopreservation media, wherein, in addition to the typical DMSO supplementation, more components are being included to attain the appropriate ionic environment necessary for cell maintenance during cryopreservation in an effort to make the medium composition more intracellular-like. In addition, the new-generation additives (Table 1) have been proposed and used to target the proteases and kinases involved in the cell death cascade that act by significantly reducing caspase activity, reducing membrane permeability and increasing membrane potential; thereby reducing overall cell death due to cryopreservation-induced stresses and improving the overall post-thaw performance survival rate. Molecules studied include Zvad-FMK which is a broad-spectrum irreversible inhibitor of caspase enzymes, Bax-peptide inhibitor, p53 tumor suppressor protein inhibitor (Pifithrinμ) and inhibitors of bleb formation in apoptotic cells (Pinacidil and Y-27632) [30].

Major concerns around viruses and lack of quality control led to the development of serum-free culture processes. Rodrigues et al. [37] assessed the impact of serum-free adaptation on monoclonal antibody production and found that medium supplement containing rhinsulin, ammonium metavanadate, nickel chloride and stannous chloride succeeded in adaptation of the Chinese hamster ovary-K1 cell line to serum-free conditions. Cell culture supernatant or spent medium, also known as conditioned medium typically contains useful growth factors (such as epidermal growth
Table 1. Recent strategies employed for successful cryopreservation of mammalian cells.

<table>
<thead>
<tr>
<th>Category</th>
<th>Molecule</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>Freeze medium additives</td>
<td>Recombinant albumin (animal component-free) [31]</td>
<td>For serum-free freeze medium formulation</td>
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<tr>
<td></td>
<td>Cholesterol [32,33]</td>
<td>Alters cellular membrane properties</td>
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<td></td>
<td>1-Deoxymannojirimycin [34]</td>
<td>Inhibitor of alpha-mannosidase, leads to an increase of intracellular free high-mannose oligosaccharides implicated in rescuing cells from freezing injury</td>
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<td></td>
<td>Rakkyo fructan [35]</td>
<td>Fructose polymer for serum-free freeze medium</td>
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<td></td>
<td>Sericin [36]</td>
<td>Silk protein for serum-free freeze medium</td>
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<td></td>
<td>Trehalose [28]</td>
<td>Disaccharide to replace DMSO as a cryoprotectant for DMSO-sensitive cell preservation</td>
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<td>Cell engineering</td>
<td>AQP4 [27]</td>
<td>Aquaporin, a water channel protein, expression in CHO cells (AQP4-CHO) to improve survival during ultra-quick freezing even in the presence of low levels of cryoprotectant</td>
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<td>TRET-1 [29]</td>
<td>Trehalose-transporter expression in CHO cells (CHO-TRET1) to enhance trehalose loading into the cells</td>
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<tr>
<td>Apoptosis inhibitors [30]</td>
<td>Bax-peptide inhibitor</td>
<td>Intracts with Bax and blocks caspase independent cell-death pathway</td>
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<td></td>
<td>Bongkrekic acid</td>
<td>Reduces membrane permeability by inhibiting mitochondrial permeability transition pore (PT-pore), and increases mitochondrial membrane potential</td>
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<td>Caspase-9 inhibitor</td>
<td>Inhibits caspase-9 activity</td>
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<td>Pifithrin-μ</td>
<td>Inhibits p53 tumor suppressor protein</td>
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<td></td>
<td>Pinacidil</td>
<td>ROCK-1 inhibitor: reduces apoptotic bleb formation</td>
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<td></td>
<td>SD-282</td>
<td>Inhibits p38 MAPK pathway mediated cell damage and apoptosis</td>
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<td></td>
<td>Y-27632</td>
<td>ROCK-1 inhibitor: reduces apoptotic bleb formation</td>
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<tr>
<td></td>
<td>zVAD-fmk</td>
<td>Increases mitochondrial potential; synthetic broad-spectrum irreversible inhibitor of caspase enzymes</td>
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factor, platelet-derived growth factor) and proteins secreted by the cells [38]. Supplementation with spent media of both the freezing medium and post-thaw growth medium has been shown to improve cryopreservation outcome for mammalian cells, especially in a serum-free and chemically defined basal media background [39].

In an effort to remove fetal bovine serum (FBS), serum-free freezing medium containing silk protein sericin as a cryoprotectant and consisting of PBS, 1% (v/w) sericin, 0.5% (v/w) maltose, 0.3% (v/w) proline, 0.3% (v/w) glutamine and 10% DMSO was studied with respect to cryopreservation of the P 3 U1 myeloma cell line and Chinese-hamster ovary cells. This medium containing sericin was shown to successfully cryopreserve both cell types as efficiently as the conventional medium of FBS containing 10% DMSO [36]. Supplementation with an animal component-free recombinant albumin has also been reported for its ability to replace serum and allow for efficient cryopreservation of an antibody producing CHO cell line [31]. Ogawa et al. [35] developed a serum-free cryopreservation method with rakkyo fructan, a fructose polymer, derived from the Japanese shallot as an alternative factor to serum. Fructan contributes to tolerance to frost and dehydration in plants by stabilizing the plant...
membrane. The rakkyo fructan and DMSO mixture was used in the cryopreservation of the mammalian cell lines CHO-DP12, a producer of recombinant antibodies, and HepG2, human hepatoma cells frequently tested in bioartificial livers. Following the freezing and thawing processes, CHO-DP12 cells retained their ability to produce recombinant antibodies and as did HepG2 cells for albumin and mRNA expression of cytochrome P450 enzymes, thereby demonstrating effectiveness of rakkyo fructan as a promising cryoprotectant that prevents mammalian cells from freezing stress similar to FBS.

Numerous biophysical and simulation studies have shown that the addition of cholesterol to membranes decreases membrane fluidity while increasing membrane thickness, bending modulus and lipid order, even without forming distinct domains, and plays an important role in the formation of lipid rafts. These indirect effects influence cellular behavior; for example, increases in membrane thickness caused by high levels of cholesterol are thought to help sort membrane proteins between the Golgi and the plasma membrane, and membrane structural changes can alter ion channel properties and influence protein signaling. Cholesterol content of a membrane and its impact on membrane fluidity is dependent on temperature and may affect cell survival during cryopreservation [32]. Purdy et al. [33] examined the positive effects of adding cholesterol to the membranes of CHO and bull sperm cells leading to increased survival after cooling to 5°C and after cryopreservation.

Intracellular free high-mannose oligosaccharides (HMOS) have also been shown to have cryoprotective effect on mammalian cells. 1-Deoxymannojirimycin, an inhibitor of α-mannosidase, leads to an increase in intracellular free HMOS, and was demonstrated to rescue PC-12 cells from freezing injury in a concentration and pretreatment time dependent manner. PC12 cells stored at -15°C for 2 h were able to recover fully with 1-deoxymannojirimycin at more than 25 mM after 48-h pretreatment and more than 3 mM after 72- and 96-h pretreatment [34].

Conclusion & future perspective
Successful cryopreservation of production cell lines plays a key role in establishing viable biopharmaceutical production processes. Historically, cryopreservation research has focused on examining the biophysical changes that are required to achieve the recovery of viable cells, linking those to different cryoprotection regimes. But more recently, advances have been made to comprehend changes occurring at the molecular level as cells are subjected to the freeze/thaw process. From a methodology standpoint, it would be valuable to develop and use protocols that enable high cell density cryopreservation (>1 × 10^6 cells/ml) frozen in larger volume formats (10–100s ml) to reduce the time taken for cell expansion in manufacturing seed train. It is also critical to continue working on strategies (new media additives and cell engineering among others) that build in robustness into freeze-thaw process and minimize post-thaw performance variability of the production cell lines. Moving forward, in this molecular-omics era, greater emphasis will be placed on maintaining genetic/epigenetic stability and assuring the authenticity of these cell lines will be placed on maintaining genetic/epigenetic stability and assuring the authenticity of these cell lines stored at low temperatures. Moving forward, low temperature preservation practices will also need to support and permit a greater understanding of ‘true’ biological capabilities in response to environmental impacts.

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Executive summary
- Recombinant biologics are produced using mammalian cells and are a significant portion of the pharmaceutical industry. Process of generating a highly productive cell line has been discussed.
- Cell banking or long term cryopreservation of cells is critical because continuous maintenance of these parental or production cell lines through the lifecycle of a given biologic is impractical given the potential for genetic drift leading to the loss of cell’s original characteristics, risk of exposure to microbial contamination and the possibility of culture co-contamination. Typical cryopreservation process flow and the two-tier strategy of MCB and WCB followed in the biopharmaceutical industry for banking of production cell lines have been outlined.
- Different cryopreservation strategies and the use of controlled rate freezing protocol for long-term preservation of mammalian cells.
- Cellular pathways (apoptosis and necrosis) and molecules involved in cryopreservation induced injury to the cell.
- Recent strategies employed to improve cell recovery postcryopreservation including: Development of serum-free and animal component-free freeze and thaw media; engineering cell lines to better withstand cryopreservation shocks; given that apoptosis is the major pathway implicated in cryopreservation induced cell death, addition of apoptosis inhibitors in cell culture media has improved cryopreservation outcomes.
Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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References

Papers of special note have been highlighted as:

• of interest; •• of considerable interest


• This paper presents the basic components underlying the current GMP Cell Banking system and highlights its importance as an integral part of the process of manufacturing biologic products.


• This paper presents a conventional seed train process flow in the biopharmaceutical industry and presents successful development of a process utilizing Chinese hamster ovary cells cryopreserved in large volume bags in place of the seed train process flow for initiation of cell culture manufacturing campaigns.


• This paper reviews the fundamental physiological basis and desirable attributes of production cell lines and efforts various researchers have undertaken to engineer dream cells for biopharmaceutical production.


• The paper provides an overview of core concepts in the area cryobiology—including biopreservation samples, processing steps and related quality and risk management systems.


**This paper presents fundamental biophysical events underlying cell freezing: including intracellular ice formation and osmotic stresses.**


- This paper summarizes the cell freezing principles and the five different approaches to applied cryopreservation: ultrarapid freezing and thawing, controlled-rate freezing, freezing with nonpenetrating polymers, vitrification, and equilibrium freezing.


- This paper reviews molecules involved in cryopreservation induced cell death and summarizes studies undertaken to use those molecular targets for preventing cell death.


**This paper provides an overview of stress factors associated with cryopreservation.**


