CRYOPRESERVATION OF CELLS

细胞的冷冻保存

**INTRODUCTION**

**引言**

Cryopreservationis the process of cooling and storing cells, tissues, or organs at very lowtemperatures to maintain their viability. The purpose of cryopreservation is tobank the cells and allow their future use in in vitro or in vivo applicationsfor which post-thaw function is sufficiently representative of the cells'prefreeze function. Cryopreservation also minimizes the risk of geneticmutation or development of subpopulations due to cell replication. Depending onthe application, sufficient postcryopreservation function may beassessed by the ability to divide, proliferate, differentiate, express genes,or to produce proteins, or by another specific functional property.

冷冻保存是将细胞、组织或器官在超低温环境下存储来维持其活力的过程。目的是将细胞储存起来并应用到后续的体内外实验中，因为复苏后的细胞仍可以保持冻存前的功能。冷冻保存还可以最大限度地降低由于细胞复制而导致的基因突变或亚群发育的风险。根据不同的应用，充分的冷冻保存后的功能可通过细胞分裂、增殖、分化、表达基因或产生蛋白质的能力或其他特定的功能特性来评估。

**PRINCIPLES OF CRYOPRESERVATION Overview**

**冷冻保存的原理概述**

Understandingthe role of water and the need to adequately remove it from cells or abrogateits ability to form ice crystals, which damage the cell membrane, is criticalto successful cryopreservation. When cells are frozen in aqueous suspension,often they are destroyed. However, in the 1940s Polge and others discovered thecryoprotective properties of glycerol. Since then several chemicals,generically called cryoprotectant agents (CPAs), have been identified. Themechanism of action of CPAs is complex and is not fully understood. However,according to the commonly accepted theory of colligative action, CPAs increasesolute concentration both within the cell and extracellularly, therebysuppressing ice formation. For this purpose, the so-called penetrating (orintracellular) CPAs [e.g., dimethylsulfoxide (DMSO), glycerol, propanediol, andmethanol] must be able to cross the cell membrane readily and penetrate thecell without significant toxicity.There also is a group of nonpenetrating (orextracellular) CPAs (e.g., sucrose and trehalose) whose mechanism of action isthought to be related at least in part to their stabilizing interaction withcell membranes. This property also may explain the cryoprotective activities ofcertain large molecular weight compounds such as hydroxyethyl starch andpolyvinylpropylene. Theoretical models of cryoprotection typically evoke thecolligative theory, but full explanation of CPA action is yet to beestablished.

了解水的作用以及将水从细胞中充分去除或消除其形成冰晶的能力以减少损坏细胞膜损伤，这对于成功进行冷冻保存至关重要。当细胞在水性悬浮液中冷冻时，通常会被破坏。在1940年代，Polge等人发现了甘油的防冻特性。从那时起，陆续鉴定出了几种通常称为冷冻保护剂（CPA）的化学物质。 CPA的作用机制很复杂，尚未完全了解。但是，根据公认的依数作用理论，CPA会增加细胞内和细胞外的溶质浓度，从而抑制冰晶的形成。因此，所谓的穿透性（或细胞内）CPAs [例如二甲亚砜（DMSO）、甘油、丙二醇和甲醇]必须能够轻松穿过细胞膜而没有明显的毒性。还有一组非渗透性（或细胞外）CPAs（例如蔗糖和海藻糖），其作用机制被认为至少部分与细胞膜的稳定相互作用有关。这种性质也可以解释某些大分子化合物例如羟乙基淀粉和聚乙烯丙烯的防冻活性。冷冻保护的理论模型通常唤起依数理论，但尚未建立对CPA作用的完整解释。

Analternative form of cell preservation, commonly called vitrification,wherebythe cell suspension is loaded with high levels of penetrating CPAs (oftenseveral in combination), induces a glass-like state in which cellular andextracellular water cannot readily form ice crystals. When cell suspensionsprepared in this way then are cooled very rapidly (cooling rates of100°–1000°/min or more) the extreme viscosity prevents osmosis, and the watermolecules are unable to form ice. This procedure has been widely used forcomplex structures including a variety of human, plant, and animal tissues andmay help preserve those cell preparations that have variable degrees ofcellular permeability or when standard cryoprotection cannot deliver the rangeof conditions required to optimally preserve viability in all the tissues'component cell types.

另一种细胞保存方式，通常称为玻璃化，即在细胞悬浮液中加入高浓度的穿透性CPA（通常是几种），诱导形成玻璃化状态，诱导细胞和细胞外水不易形成冰晶。当以这种方式制备的细胞悬液快速冷却（冷却速率为100°–1000°/ min或更高）时，极高的粘度可防止渗透，并且水分子无法形成冰晶。这个过程已广泛用于各种复杂结构的保存过程，包括各种人类、植物和动物组织在内，并且可以帮助保存具有不同程度的细胞渗透性的细胞制剂，或者也用于在所有组织的组成细胞类型标准冷冻保护程序无法提供其生存能力的条件范围内的最佳保存方案。

CPAs havebiological activities beyond their cryoprotective properties. Some, like DMSO,can affect the cell membrane, cytoskeleton, and gene expression and may betoxic to cells following prolonged exposure. Therefore, during development ofnew cryopreservation protocols analysts should perform a toxicity assay inwhich the cells are exposed to the CPA over a range of time intervals toevaluate loss of viability or alteration of functionality.

CPA具有超出其防冻特性的生物学活性。诸如DMSO等，可能会影响细胞膜、细胞骨架和基因表达，并且在细胞长时间暴露后可能对细胞有毒。因此，在开发新的低温保存方案的过程中，分析人员应进行毒性试验，将细胞在一定时间间隔内暴露于CPA，以评估活力丧失或功能改变。

**Key Elements of Cryopreservation Practice**

**低温保存的关键因素**

For anycellular sample or therapeutic product being cryopreserved, method developmentshould address the following elements:

对于任何冷冻保存的细胞样品或治疗产品，方法开发应解决以下要素:

PREFREEZE PROCESSING AND CHARACTERIZATION

预冷冻处理和鉴定

Optimizingthe condition of the cells immediately before cryopreservation is critical to asuccessful outcome. The nature and extent of prefreeze processing depends onthe state of the original cells harvested for preservation, the composition ofthe cell suspension, and the specific processing steps leading into cryopreservation.Prefreeze processing may include selection of subpopulations, ex vivoexpansion, or incubation with activating or priming factors.

冷冻保存之前对细胞条件进行优化是获得成功的关键。预冻处理的性质和程度取决于为保存而收获的原始细胞的状态、细胞悬液的组成及冷冻保存的特定处理步骤。预冷冻过程可能包括亚种群的选择、体外扩增或激活或启动因子孵育。

Precryopreservationcharacteristics and identity should be established during early processdevelopment. For cell banks in particular, the cell status and optimal growthconditions, as well as documented history (with traceability to a qualifiedcell bank or acceptable source), characteristics, and authenticity should be documented.Cell status and history typically are described in terms of the nature andnumber of manipulations and culture passages from the primary cells or originalisolate. Finite or primary cells usually are cryopreserved at an early passageto maintain integrity of the original tissue, but continuous cell lines may be clonedand expanded, ensuring a homogeneous cell population. It is recommended toprepare cell banks from a single preparation or expanded population of cells sinceit is often necessary to pool cells for freezing from multiple culture vessels.Cells from cultures with different passage histories and certainly from differentdonors should not be pooled. In both cases, analysts should maintain detailedrecords of the procedures.

在早期工艺开发过程中应确定好低温保存的特性。尤其是细胞库，应记录细胞状态和最佳生长条件，以及历史记录(可追溯到合格的细胞库或可接受的来源)、特征和真实性。通常根据来自原代细胞或原始分离株的操作或培养传代的性质和数量来描述细胞的状态和历史。有限细胞系或原代细胞通常会在早期传代时进行冷冻保存来保持原始组织的完整性，而连续细胞系可以通过克隆和扩增保持细胞群体同质性。建议从单一制备物或扩大的细胞群中制备细胞库，因为通常需要将细胞从多个培养容器中汇集起来进行冷冻。来自不同传代历史的培养细胞和来自不同捐赠者的细胞当然不应该混合在一起。在这两种情况下，分析人员均应保留操作过程的详细记录。

Toprepare for cryopreservation of cultured cells, cells should be harvestedduring exponential or the most rapid phase of growth and before the cultureenters stationary phase. Harvesting cells during this phase ensures that thecells are most viable and uniform. The optimal concentration of cells willdepend on the cell type, purpose, and best recovery. Typically this liesbetween 10 and 10 /mL for manufacturing cell banks but may be different forother purposes. Complete growth medium renewal a day before cell harvest alsocan be beneficial. Additionally, most cell suspensions benefit from washing bycentrifugation and resuspension in an isotonic medium to a specific cellconcentration. Prefreeze processing should not result in cells that arestressed before the start of the freezing process, or cell losses duringfreezing or after thaw will be higher than expected.

准备低温保存的细胞应该在指数生长或最快速生长阶段和培养进入平台期之前进行收集。在此阶段收集细胞可确保细胞最具活力和均一性。冻存细胞的最佳浓度取决于细胞类型、用途和最佳回收率。对于制造细胞库来说，这个值通常在106到107 /mL之间，但是出于其他目的可能会有所不同。在细胞收获的前一天完全更新生长培养基也会有益处。此外，大多数细胞悬浮液通过离心洗涤和在等渗介质中重悬来达到特定的细胞浓度。在冷冻过程开始前，预冷冻处理不应使细胞受到压力，否则在冷冻过程中或解冻后细胞损失将会高于预期。

Optimizingthe growth conditions of a cell line or primary cells is important to maintainhigh viability of the cells in culture. Typically, cells growing actively andin exponential phase have a low cytoplasm to nuclear volume ratio, which isconducive to successful cryopreservation with penetrating cryoprotectants.Suboptimal or improper culture conditions may result in lower viability andcell states that will be less robust for preservation and recovery. The culturemedium should be optimized and the same medium should be used throughout allexperiments, and each batch of animal-derived materials (e.g., serum) and otherculture reagents should be qualitied (e.g., see the 2010 WHO guidance and theFDA 2010 guidance referenced in the Appendix). If possible, it is recommendedto not use animalderived components in the culture medium particularly forcells used for therapy or as manufacturing substrates.

优化细胞系或原代细胞的生长条件对保持细胞在培养中的高活力很重要。通常情况下，活跃且处于指数生长期的细胞具有较低的细胞质与细胞核的体积比，这有利于穿透性冷冻保护剂成功进行冷冻保存。欠佳或不合适的培养条件可能会导致存活率降低和细胞状态变差，不利于冻存复苏。需要优化培养基，并且在所有实验中都应使用相同的培养基，对每批动物来源的材料（例如血清）和其他培养试剂进行鉴定（如见在附录中引用的2010年 WHO指南和2010年FDA指南）。如果可能的话，建议不要在培养基中使用动物来源的成分，尤其是用于治疗或作为生成底物的细胞。

Per the WHO 2010 guidance and based on a risk assessment, either theMaster Cell Bank (MCB) or the Working Cell Bank (WCB) must be tested foradventitious agents. Ideally, samples of cells should be tested for adventitious agentsbefore freezing. The specific testing regimen for potential microbial or viralcontamination of cells depends on the donor source, the culture history, andthe intended use. Detailed records of the cell history should be maintained asa basis for appropriate risk assessment to direct supplementary testing thatmay be required (e.g., exposure to bovine viruses in bovine serum albumin).Speci *USP*general chapter *Cellular and Tissue-Based Products*〈1046〉contains guidance about sterility and safetytesting requirements for cell therapy products.

根据WHO的2010年指南，并基于风险评估，必须对主细胞库（MCB）或工作细胞库（WCB）进行外来因子检测。理想状态下，冻存前应测试细胞样本中的不定因子。细胞潜在的微生物或病毒污染的具体测试方案取决于供体来源、培养史和预期用途。细胞历史的详细记录应作为适当风险评估的基础，以指导可能需要的补充检测（例如，暴露于牛血清白蛋白中的牛病毒）。针对特定用途产品（例如，细胞治疗或疫苗生产）的细胞或细胞供体测试的特定监管要求是基于过去必须包含或考虑的关键药物的经验。 USP通用章节“基于细胞和组织的产品<1046>”包含有关细胞治疗产品无菌性和安全性测试要求的指南。

REAGENTS AND CONTAINERS

试剂和容器

All cryoprotectants,containers, etc., should be fit for purpose as indicated in relevantregulatory guidances. Sterile, single-use, disposable plastic bags, cryovials, orstraws are customarily used for cryopreservation. Manufacturers' specificationsshould be carefully reviewed to ensure that the material used to manufacture thecryocontainer is appropriate for use at the storage temperature, is chemicallycompatible with the contents, minimizes the potential for leachables and extractables,and assures container closure integrity. If straws are used, then primary orsecondary containment during storage is important to prevent direct

contactof the preserved cells with liquid nitrogen. Cryovials should be selected basedon their ability to provide adequate cell bank integrity.

所有的冷冻保护剂、容器等，都应符合相关法规指南中规定的用途。无菌的、一次性使用的塑料袋、冻存管或吸管通常用于低温保存。应仔细审查制造商的规格，以确保用于制造冷冻容器的材料适合在存储温度下使用，并且与内容物的化学相容性好，最大程度减少浸出物和可萃取物，并确保容器密闭的完整性。如果使用了吸管，那么在储存期间的一次或二次封闭对于防止保细胞与液氮的直接接触是很重要的。应基于其提供足够的细胞库完整性的能力来选择冻存管。

Preservationof cells typically requires the use of specialized solutions that contain abase (typically an isotonic saline-based solution) with CPAs (most commonlyDMSO but sometimes glycerol) and sometimes proteins (fetal bovine serum, humanserum or plasma, conditioned medium, or human albumin). The optimum compositionfor different cell types may need to be determined.

细胞的保存通常需要使用含有碱基(通常是等渗盐溶液)和CPAs(最常见的是DMSO，有时是甘油)，有时是蛋白质(胎牛血清、人血清或血浆、条件培养基或人白蛋白)的专用溶液。不同细胞类型可能需要确定其最佳配比。

The typesof vials, labels, ink, or markers used should withstand extreme liquid nitrogentemperatures. The markings on the label should be legible and barcoded if possible. The minimum information on the label should include name ordescription of cell population, date of cryopreservation, lot number, andpassage number if needed. Since most cryolabels are very small, additionalinformation can be included on associated documentation. In certainapplications it may also be necessary to sequentially number vials within asingle lot as part of the minimum information on the label, to enable bettercontrol over movement of vials from a single bank, and to identify sectors ofthe bank which may have received different cryopreservation conditions.

所用的样品瓶、标签、墨水或标记的类型应承受极端液氮温度。标签上的标记应清晰易读，应编上条形码。标签上的信息至少应包括细胞的名称或描述、冷冻保存的日期、批号和传代号（如有必要）。由于大多数冷冻标签很小，因此可以在关联的文档中包含额外信息。在某些应用中，可能有必要对单个批次中的样品瓶进行顺序编号，以作为标签上最少信息的一部分，以更好地控制单个样品库中冻存管的移动，并识别可能已收到的样品库中样品不同的冻存条件。

ADDITION OF CRYOPROTECTANT SOLUTION

冷冻保护剂溶液的添加

Cryoprotectantsolutions typically are hypertonic and are not physiological. For example, a10% DMSO solution used commonly in cell preservation has a concentration ofapproximately 1.4 osmolarity (Osm/L). Cells introduced into this type ofsolution rapidly dehydrate as water leaves the cell in order to reduce the differencein osmotic potential between the inside and outside of the cell. DMSO slowlypermeates the cell to re-equilibrate. This may cause excursions in volume

that canresult in a loss of cell viability. Therefore, cryopreservation solutionscommonly are added to a cell suspension in stepwise additions or gradually(e.g.,using asyringe pump) or slowly dispensing down the side of the container to preventcell losses resulting from osmotic stress. The method for introducing or

removinga cryopreservation solution should be developed and evaluated for its impact oncell viability and functionality.

低温保护剂溶液通常是高渗的而不是生理的。例如，通常用于细胞冻存的10% DMSO溶液的浓度大约为1.4摩尔渗透压浓度(Osm/L)。随着细胞失水，引入这种类型溶液中的细胞迅速脱水，以减少细胞内外的渗透压差。DMSO缓慢渗透到细胞内使其重新平衡。这可能会导致体积偏移，从而导致细胞活力的丧失。因此，冷冻保存溶液通常是逐步添加或逐渐地(如使用注射泵)或从容器的侧面缓慢添加到细胞悬浮液中，以防止由于渗透压造成细胞损失。应开发引入或去除冷冻保存液的方法并评估其对细胞活力和功能的影响。

In thecase of DMSO, a large latent enthalpy of mixing results in sample heating whenthe two solutions are mixed. This heating can be high enough to damage the cells, so solutions that contain DMSO commonly are precooled before mixing.Prechilling the solution reduces heating associated with mixing of the solution, reducesthe osmotic volume changes that the cells experience, and reduces cell lossesassociated with exposure to DMSO. The time that cells are exposed to the cryoprotectant,prior to freezing, should be limited and the maximum time allowable, withoutdeleterious effects, should be determined during development work for routineuse.

对于DMSO，当两种溶液混合时，会产生较大的混合焓导致样品加热。这种加热的温度可能会高到足以损坏细胞的程度，因此通常将包含DMSO的溶液在混合前进行预冷。预冷可减少溶液混合过程中产生的热量，减少细胞渗透体积的变化，并减少细胞暴露于DMSO中的损伤。在冷冻之前，对细胞暴露于冷冻保护剂的时间应加以限制，并且常规研发工作中确定允许无有害影响的最大允许时间。

COOLING

冷却

Twodifferent types of freezing typically are used for cells: controlled-ratecooling (using programmable freezers) and passive cooling (including use ofinsulated containers). Controlled-rate freezers are attached toliquid nitrogen supplies. The temperature of the chamber should be controlledby increasing or decreasing the flow of cold nitrogen gas into the chamber according to apreprogrammed step. Controlled-rate freezing protocols typically involveseveral steps, each of which should be evaluated and qualitied for aspecific cell type.

细胞通常使用两种不同类型的冻存方式：控制速率的冷却方式（使用可编程的冷冻机）和被动的冷却方式（包括使用隔热容器）。液氮罐上附有冷冻装置，按照一个预先编好的程序增加或减少进入腔室的冷氮气流量来控制腔室的温度。控制冷冻速率的方案通常包括几个步骤，每个步骤都应该评估和限定特定的细胞类型。

The useof controlled-rate freezing provides more precise control of the freezingenvironment and therefore may provide more consistent (and higher) post-thaw recoveryfor cells that may have a narrow range of cooling rates associated with maximum survival or cells that are sensitive to the temperature at which ice forms in theextracellular solution. Temperature probes placed near the cells being frozen,or in a mock cell suspension that undergoes cryopreservation simultaneously, areused to monitor the freezing process and to provide process control. If releaseof the latent heat of fusion is delayed or poorly controlled, cells undergoing cryopreservationmay be damaged and may have diminished viability after thaw.

使用受控速率冷冻装置可更精确地控制冷冻环境，从而在细胞外冰晶形成时为冷却速率范围狭窄（与最大存活率相关）或敏感的细胞提供更一致（且更高）的解冻后恢复能力。将温度探针放置在要冷冻的细胞附近或同时进行低温保存的模拟细胞悬液中，用于监视冷冻过程并提供过程控制。如果融合潜热的释放被延迟或控制不当，则进行冷冻保存的细胞可能会受到破坏，并且复苏后的活力可能会降低。

Disruptionof the controlled-rate freezing during the protocol may occur and typically iscaused by failure of a valve in the controlled-rate freezer or cryogen. Protocolsfor handling disruption of the freezing process and backup plans should beprepared.

在试验过程中，受控速率冷冻可能会发生中断，通常是由受控速率冷冻器或制冷剂中的阀门失效引起的。应准备好冻结过程出现中断的处理方案和备用计划。

Passivefreezing involves placing a product in a freezer (about −80° or −150°) and permitting the sample to cool in anuncontrolled fashion. Insulation or specially designed boxes are used to slowthe cooling rate for the sample. The average cooling rate achieved for themajority of the process and the consistency of freezing curves should beevaluated and qualitied for purpose. In general, control of thethermal environment during freezing results in improved post-thaw recovery, butcertain cells exhibit comparable post-thaw recovery when they are passivelycooled.

被动冷冻涉及将产品放置在冰箱（大约-80°或-150°）中，并使样品以不受控制的方式冷却。用独立或专门设计的冻存盒来减慢样品的冷却速度。应评估并验证大部分过程中所达到的平均冷却速度和冻存曲线的一致性。通常，在冷冻过程中控制热环境可以提高解冻后的恢复能力，但是某些细胞在被动冷却后表现出类似的解冻后恢复能力。

CRYOGENIC STORAGE, SAFETY, AND TRANSPORT

低温存储，安全及运输

After thefreezing process has been completed, products are transferred fromcontrolled-rate or mechanical freezers to cryogenic storage units. Somemicrobial cell cultures can be suitably maintained in mechanical freezers butthis should be demonstrated. Sample warming should be minimized during transferof the cell product from the freezing device to storage. Cold tables orinsulated transfer devices can be used to minimize warming during transfer.Newly cryopreserved cells commonly are placed in a quarantine cryogenic storageunit before completion of testing for adventitious agents. After testing, cellsthat test negative for adventitious agents can be released for transfer intolong-term cryogenic storage units.

冷冻过程完成后，产品从控制速率或机械冷冻机转移到低温存储单元。一些微生物细胞培养物可以适当地保存在冰箱中，但应证实这一点。在将细胞产品从冷冻设备转移到存储设备的过程中，应尽量减少样品的升温。可以使用冷桌或隔热传输装置来减少传送过程中的温度升高。通常，在完成对不定因子的测试之前，应将新冷冻保存的细胞放置在隔离的低温存储单元中。在测试之后，将不定因子测试呈阴性的细胞转移到长期低温存储单元中。

Theinventory system (or repository) for the maintenance of the cryopreserved cellsshould be designed for easy access to minimize specimen handling, and thenumber of times per day that a repository is accessed should be limited becauseexposure to warmer temperatures may compromise cell viability and,consequently, longer-term stability. Cell banks (e.g., MCBs) or other cellcultures that are accessed infrequently should be stored separately from WCBsor other cell cultures that are accessed more often. Frequent retrievalfrom the cell bank/culture may cause shifts in temperature. This activitymust not compromise the longterm stability and performance of the infrequentlyused cell bank/culture. It is also valuable to divide a bank and store itin multiple locations to decrease risks due to a catastrophic event at a particularsite.

用于维护细胞低温保存的库存系统(或储存库)应设计为易于存取，以最大限度地减少标本处理，并且对每天进出储存库的次数应加以限制，因为暴露在较高的温度下可能会损害细胞的生存能力，从而影响细胞的长期稳定性。细胞库(如MCBs)或其他不常被访问的细胞培养物应与WCBs或其他经常被访问的细胞培养物分开存储。频繁从细胞库/培养物中提取可能会导致温度变化。这种活动不得损害不常使用的细胞库/培养物的长期稳定性和性能。划分区域并将其存储在多个位置，以减少在特定地点发生灾难性事件的风险，这也是很有价值的事情。

When storing cryopreserved cells, analysts should ensure that the storagetemperature does not rise above a critical temperature called the glasstransition temperature.For long-term storage of fastidious specimens such as cell lines and primarycell cultures, this critical temperature is not warmer than −130° for nonclinicalspecimens or not warmer than −150° forclinical material (to give an adequate margin of error) in the vapor phase ofthe liquid-nitrogen freezer. Liquidnitrogen freezers are prone to temperaturegradients in the vapor phase based on the shape and design of the freezer andthe level of liquid nitrogen. Although storing cryopreserved cells in liquidnitrogen prolongs longevity, hazards associated with unsuitable containers orcontainer use (e.g., exploding vials and rupturing bags) have prompted greateruse of nitrogen vapor phase storage. Liquid-nitrogen vapor phase provides amore convenient and safe environment for vial retrieval. If the liquid-nitrogenfreezer is suitably configured, the working temperature in thevapor phase is commonly −150° orcolder. The liquid-nitrogen freezer should be qualified, and thetemperature of the vapor phase should be routinely checked to ensure that thetemperature does not become warmer than −130° for cell lines or other frozen material or warmerthan −150° for material used for clinicalapplications (e.g., cell therapies).

当储存低温保存的细胞时，操作人员应该确保储存温度不超过一种称为玻璃化温度的临界温度。对于严格要求的样本，如细胞系和原代细胞，对于非临床标本，这个临界温度不高于- 130℃；对于临床样本不高于- 150℃(以提供足够的误差范围)。液氮制冷机的气相温度梯度是由制冷机的形状和设计以及液氮的浓度决定的。尽管将细胞保存在液氮罐中可以延长细胞寿命，但与不合适的容器或容器使用带来的相关危害(例如小瓶爆炸和袋子破裂)促使人们更多地使用氮气气相存储。液氮气相为小瓶的回收提供了更方便、更安全的环境。如果适当配置液态氮冷冻机，则汽相的工作温度一般在−150°或更低。液氮冷冻器应通过认证，并且应定期检查气相温度，以确保细胞系或其他冷冻材料的温度不高于- 130℃，临床应用材料(如细胞治疗)的温度不高于- 150℃。

Temperature-monitoringsystems should permit recording and storage of temperature history for qualitycontrol purposes. Storage units should be attached to alarms and facilitymonitoring systems. Critical storage units should be equipped with a multilevelalarm system to ensure backup in the monitoring and response.The storage unitsshould be routinely monitored for temperature failure caused by powerdisruption and any other potential malfunctions. In the event of equipment orpower failure, backup refrigeration should be available.

温度监控系统应允许记录和存储温度历史，以进行质量控制。存储单元应连接到警报和设施监视系统。关键存储单元应配备多级警报系统，以确保监视和响应中的备份。应例行监视存储单元的电源中断和其他潜在故障引起的温度故障。如果设备或电源出现故障，应提供备用制冷设备。

Properoperation of a repository requires monitoring of temperature andliquid-nitrogen levels and automatic filling. In addition, it is recommended tohave a backup for emergency cooling (e.g., empty backup cryogenic storage) incase of freezer failure.

储存库的正确操作需要监测温度和液氮水平，并进行自动灌装。此外，建议在冷冻机发生故障时备有应急冷冻装置(例如，空的备用低温存储器)。

Onlyindividuals who are trained for this purpose should access cryopreservedproducts or samples. In some cases, verification by a secondperson is required for source traceability. Personnel assigned to theimplementation of the protocols should be trained in standard operatingprocedures (SOPs). Sample tracking systems that incorporate computer softwareand sometimes barcoding for identification, logging, and tracking of frozensamples are particularly useful for large sample repositories and mayfacilitate rapid retrieval of samples and minimize time that the entirerepository is exposed to the risk of temperature excursions. In addition, it isrecommended that all changes to cryostorage inventories be recorded in logbooks near the storage unit.

只有经过这方面培训的人员才可以获得冷冻保存的产品或样品。在某些情况下，源的可追溯性需要由第二个人进行验证。负责实施的人员应接受标准操作程序(SOPs)的培训。结合了计算机软件的样品跟踪系统，有时还包括用于识别、记录和跟踪冷冻样品的条形码，这对大型样品存储库特别有用，还可以促进样品的快速检索，并将整个仓库面临温度波动风险的时间最小化。此外，建议在储存单元附近的日记中记录所有对冷藏库存的变更。

Productsand samples such as primary cells, cell lines, and cell therapy productsroutinely are shipped among sites of collection, processing, storage, and use.Cryopreserved cells typically are shipped in liquid-nitrogen vapor shipperswith temperature-monitoring systems to ensure that the unit does not becomewarmer than −130° forcell lines and −150° forclinical material during the shipping process. Shipping containers aresubjected to significant vibrations and mechanical stressesduring shipment and should be evaluated on a regular basis for proper function.Shipping validation studies should cover worst case scenarios and includetemperature monitors for critical materials.

产品和样品（例如原代细胞，细胞系和细胞疗法产品）通常在收集、加工、存储和使用地点之间运输。冷冻保存的细胞通常在带有温度监控系统的液氮蒸气运输箱中运输，以确保在运输过程中，细胞系的温度不超过−130℃，临床样本不超过−150℃。运输集装箱在运输过程中会遭受明显的振动和机械应力，应定期评估其功能是否正常。运输验证研究应涵盖最坏情况，并包括关键材料的温度监控器。

Cryopreserved cells, whether shippednationally or internationally, should be transported using local postal, USDepartment of Transportation, and International Air Transport Associationguidelines. Packages also should meet other regulatory requirements forquarantine, biosafety, and biosecurity. Cryopreserved cells should beretrieved, packed, and shipped in a manner that does not interfere with the integrityof the cells. For most cryopreserved cells, shipping in dry ice for shortduration may be adequate, but the shipping process should be validated, shownto have no adverse impact on the cells, and temperature monitors should beincluded. However, some cells may require shipping in liquid-nitrogen vaporphase (Dewars). Prevalidation of the shipping methods may be required to determine the best option and prevalidation riskassessment should be performed even if only one option for transport is being considered. With the shipment, shippers should include instructions for properstorage upon cell receipt.

低温保存的细胞，无论国内运输还是国际运输，都应该按照当地邮政、美国运输部和国际航空运输协会的准则进行运输。包装还应符合有关检疫、生物安全和生物安全方面的其他法规要求。冷冻保存的细胞应以不影响细胞完整性的方式进行回收、包装和运输。对于大多数冷冻保存的细胞，短期运输在干冰中可能就足够了，但是应该验证运输过程，表明对细胞没有不利影响，并且应该包括温度监视器。然而，有些细胞需要在液氮中（杜瓦瓶）运输。为了确定最佳选择，可能需要对运输方法进行预先验证，即使只考虑一种运输选择，也应进行验证前风险评估。发货时，托运人应在收货单上附上关于正确储存方法的说明。

THAWING

复苏

Cells frozenusing conventional methods (controlled rate or passive freezing) or byvitrification should be thawed as rapidly as possible, and the thawing processstarts as soon as the frozen sample is removed from storage. Slow warming ratesresult in recrystallization damage or exposure of the cells to highextracellular concentrations of CPAs, either of which can result in cell death.For each cell therapy product and cell line the most appropriate thawingprocedure (temperature, gradient, and time) needs to be developed. Theseproducts and cell lines typically are thawed in a warm-water bath or fortherapeutic cell preparations in a bead bath or thermoblock. The water bathshould be cleaned regularly and should contain sterile water or Water forInjection. The temperature of the bath also should be monitored. Many clinicallabs use plastic overwrap bags to hold the primary container during rapid thawto reduce the risk of product contamination in case the integrity of the innercontainer is impaired. Alternatively, warm bead baths (usually approximately37°) can be used to reduce contamination risks. Thawing rates should be asrapid as possible (>1°/s for most mammalian cells). Increasing bathtemperatures above 42° to increase the warming rate must be done with extremecaution because hyperthermic temperatures can damage cells, inducing necrosisor apoptosis.

使用常规方法(控制速度或被动冷冻)或玻璃化冷冻的细胞应尽快解冻，冷冻样本取出后立即开始解冻。缓慢升温会导致细胞重结晶损伤或使细胞暴露于外高浓度的CPAs，两种情况都可能导致细胞死亡。对于每个细胞治疗产品和细胞系，需要制定最合适的解冻程序(温度、梯度和时间)。这些产品和细胞系通常在温水浴中解冻，或在珠浴或热块中解冻用于细胞治疗的细胞制剂。水浴应定期清洗，并应含有无菌水或注射用水。水浴的温度也应该被监控。许多临床实验室在快速解冻期间使用塑料包装袋固定主容器，以减少在内部容器完整性受损时造成产品污染的风险。另外，温水珠浴(通常大约37度)可以用来降低污染风险。解冻速度应尽可能快(大多数哺乳动物细胞应＞1℃/s)。将浴液温度提高42℃以上以增加升温速率须格外谨慎，因为高温会损伤细胞，导致坏死或凋亡。

POST-THAW PROCESSING AND EVALUATION

解冻后处理和评估

Because cryopreservationsolutions are not physiological, it is not uncommon for some post-thawprocessing to be performed. For cells preserved in DMSO, cells typically arewashed or diluted immediately post-thaw because this CPA is harmful inparticular to frozen and thawed cells. Cells are more sensitive to expansionthan contraction, so CPA removal or dilution protocols must be carefullyoptimized to prevent cell losses from dilution or removal.

由于冻存液不是生理性的，所以一些解冻后的处理并不少见。对于保存在DMSO中的细胞，通常会在解冻后立即清洗或稀释细胞，因为这种CPA尤其对冷冻和融化的细胞有害。细胞对膨胀比收缩更敏感，因此必须仔细优化CPA去除或稀释方案，以防止细胞因稀释或CPA的去除而损失。

Quantifying theviability of cells post-thaw is important and may be performed by a variety ofmethods depending on the post-thaw requirements of the cells. Minimum viabilitylimits should be set based on experience and thawed products with viabilitiesbelow the set limits should be discarded. The process of cryopreservationsubjects the cells to significant stresses that can alter metabolic function,membrane structure, etc. Therefore, development and validation of suitablepost-thaw assays are critical. Post-thaw function is most commonly assessedusing physical integrity (e.g., membrane integrity), metabolic activity,mechanical activity (attachment or contraction), mitotic activity, or engraftmentpotential. The selection of assay depends strongly on the desired post-thawfunction of the cell. Membrane integrity is used most often. Today, dyes liketrypan blue are used less often to measure post-thaw physical integrity becausethe dye is difficult to validate on frozen and thawed cells. The method to testviability needs to be carefully selected and qualified for the particular celltype being measured with a protocol that specifies diluents and time.Fluorescent dyes are used with increasing frequency to determine cells'post-thaw physical integrity. Rigorous methods of post-thaw assessmenttypically involve multiple measures of cell viability and, in particular, atleast two independent assays to measure postthaw viability. For example, post-thawattachment and proliferation commonly are used to evaluate viability. As thecomplexity of the desired cell function after thawing increases, so do thedemands on post-thaw assessment. For example, post-thaw assessment of stemcells may require assay of membrane integrity as well as proliferation and theability of the cells to differentiate into different lineages post-thaw.Post-thaw assays should be carefully developed and validated to avoidmeasurement bias. A certain fraction of cells will lyse during freezing, andmethods of measuring cell recovery should include a complete assessment of celllosses (cells that have lysed as well as cells that are intact but not viable).Stability of cryopreserved cells can be assured by periodically thawing and testinga vial of the cells (also see ICH Q5D).

复苏后细胞活力的量化很重要，可以根据融化后细胞的需求通过多种方法进行。应根据经验设置最低生存能力值，并应丢弃生存能力低于设定限制的产品。冷冻保存过程使细胞承受很大的压力，可能会改变代谢功能，膜结构等。因此，开发和验证合适的解冻后检测方法至关重要。解冻后功能最通常的评估方法是物理结构完整性（例如，膜完整性）、代谢活性、机械活性（附着或收缩）、有丝分裂活性或移植潜能的评估。检测方法的选择很大程度上取决于所需的细胞解冻后功能。膜完整性是最常用的。如今，像台盼蓝这样的染料已很少用于测量复苏后的物理完整性，因为这种染料很难在冷冻和解冻的细胞上进行验证。需要仔细选择测试生存力的方法，并通过规定稀释液和时间的方案对特定细胞类型进行检测。而荧光染料的使用频率越来越高。严格评估解冻后细胞的方法通常涉及细胞活力的多种测量，特别是使用至少两个独立的测定法。例如，解冻后的附着和增殖通常用于评估生存力。随着解冻后所需细胞功能的复杂性增加，解冻后评估的要求也随之增加。例如，对干细胞进行解冻后评估可能需要测定膜的完整性、增殖以及解冻后细胞分化为不同谱系的能力。解冻后测定应仔细开发和验证，以避免测量偏差。一定比例的细胞将在冷冻过程中裂解，测量细胞回收率的方法应包括对细胞损失（裂解的细胞以及完整但不能存活的细胞）进行完整的评估。冷冻保存细胞的稳定性可以通过定期解冻和测试一小瓶细胞来进行（另请参阅ICH Q5D）。

Testing foradventitious agents after the preparation of MCBs and WCBs should be routine,and USP general chapters *Viral Safety Evaluation of Biotechnology Products Derived from Cell Linesof Human or Animal Origin <1050>*and *Virology Test Methods  <1237>* provide additional testing guidance (see also the FDA 2010guidance cited in the Appendix). Testing for mycobacterial contamination, whichmay not be isolated in standard sterility testing, also can be considered forsome cell substrates. Representative vials should be retrieved and tested forcontamination (bacteria, fungi, Mycoplasma, and viruses). Numerous well-establishedmethods are available for detecting Mycoplasma in cell cultures (see USPgeneral chapter *Mycoplasma Tests< 63>*).

制备MCB和WCB之后，应常规进行不定因子测试，而USP通用章节<1050>《人类或动物源细胞系的生物技术产品的病毒安全性评估》和<1237>病毒学测试方法提供了其他测试指南（请参见附录中引用的FDA 2010指南）。对于某些细胞底物，也可以考虑进行分枝杆菌污染检测，这种测试可能无法在标准无菌测试中分离出来。应当回收代表性的样品瓶并进行污染（细菌、真菌、支原体和病毒）检测。有许多成熟的方法可用于检测细胞培养物中的支原体（请参阅USP通用章节<63>“支原体检测”）。

The batch recordshould be detailed, including the history of the cells and all activitiesstarting from their receipt to release of the cell banks or products for use.The record should include detailed information about the cryopreservationprocess, including the procedure, the equipment used (with unique identifier),and a printed record of the freeze prolile. The viabilities of thecryopreserved cells should be monitored over time to ascertain theeffectiveness of the freezing process and the storage conditions. Theinformation captured in each batch file of a cell line must be traceable to theoriginal source, and all documents should be maintained and updated accordingto the quality management system in place.

批次记录应该是详细的，包括细胞的历史记录以及从收到细胞到释放细胞库或产品开始的所有活动。该记录应包括有关冷冻保存过程的详细信息，包括程序、使用的设备（具有唯一标识符）以及冷冻保存的打印记录。冷冻保存的细胞的活力应随时间进行监控，以确定冷冻过程和储存条件的有效性。在细胞系的每个批处理文件中捕获的信息必须可追溯到原始来源，并且所有文件都应根据现有的质量管理体系进行维护和更新。

**CRYOPRESERVATION OF HUMAN CELL THERAPYPRODUCTS**

**人体细胞治疗产品的低温保存**

Cellpreservation methods are used to ensure product stability during hold, storage,and transport steps for a wide range of human cell-based products (additionalinformation is found in *<1046>*). Cells can bepreserved in liquid suspension for up to a few days, but quantitative andqualitative changes in the cellular product invariably occur over time.Although preservation in the frozen state also affects the cellular product, itallows more predictable preservation of specific product characteristics overmuch longer time intervals.

细胞保存方法用于确保产品在各种人体细胞产品在保存、存储和运输过程中的稳定性（有关其他信息，请参见<1046>）。细胞可以在液体悬浮液中保存几天，但是随着时间的推移，细胞产物的定量和定性会发生变化。尽管在冷冻状态下保存对细胞也会有影响，但是它可以在更长时间内维持和预测细胞产品的特性。

For any given cell product, the decisionto use cryopreservation depends primarily on the timing of final product administration inrelationship to cell source collection and product manufacturing steps. Manypatient-specific autologous and allogeneic products are maintained in liquidsuspension, without cryopreservation, from starting cell source through finalformulation and are released as fresh products after a relatively short time.However, many clinical

applications require cryopreservation ofthe cell source, intermediate products, or final product. In these cases,cryopreservation can permit optimization of workflow during manufacturing,completion of lot-release testing, maintenance and management of a productinventory, transport of the product to the clinical site, and coordination ofproduct administration with the patient's medical or surgical regimen. Forexample, umbilical cord blood is cryopreserved and stored in public banks forsubsequent transport to, and temporary storage at, clinical transplant centers,where it is thawed immediately before infusion into a patient.

对于任何给定的细胞产品，是否需要冷冻保存主要取决于测定终末产品的时间与细胞源收集和产品生产的过程。从起始细胞来源到最终制剂，许多患者特异的自体和异体产品在较短的时间内作为新鲜产品释放出来，可以都保存在液体悬浮液中，无需冷冻保存。然而，许多临床应用是需要冷冻保存细胞来源、中间产物或最终产物的。在这些情况下，冷冻保存可以优化生产过程中的工作流程、完成批量放行测试、维护和管理产品库存、将产品运输到临床以及产品的给药与患者的医疗或手术方案相协调。例如，将脐带血冷冻保存并存储在公共细胞库中，以便后续运输到临床移植中心，并在临床移植中心临时存储，然后在将其输注到患者体内之前立即解冻。

Development of acryopreservation process for a clinical cell therapy must consider theconsequences for the product, the patient, and the overall feasibility of thetherapy. For the cell product, the manufacturer must ensure that expected celllosses because of cryopreservation and thaw occur in a manner that isreasonably predictable and must ensure that the final product administered tothe patient will meet specifications for cell number, viability, andfunctional characteristics.

开发用于临床细胞治疗的冷冻保存过程必须考虑对产品、病人和治疗整体可行性的影响。对于细胞产品,制造商必须确保由于低温贮藏和解冻产生的预期细胞损失是合理的可预见的，并且必须确保患者使用的最终产品符合细胞数量、生存能力和功能特征的规范。

For the patient,cryopreservation may affect the efficacy and safety of the final product. Forexample, the cryoprotectant DMSO is associated with risk of predictabledose-dependent gastrointestinal, cardiovascular, and neurologic toxicities thattypically are ascribed to histamine release. Residual DMSO in the final productshould be estimated or measured. DMSO is categorized by ICHas a Class 3 (relatively low risk) solvent or excipient in pharmaceuticalproducts, and amounts of up to 50 mg/day, or less, are considered acceptablewithout justification (see ICH Q3C). Cryopreserved cellular products frequentlycontain 10–20 times this amount unless they are washed after thawing. Evenhigher amounts of DMSO may occur with administration of multiple cryopreservedproducts, and this occurs commonly with autologous peripheral blood stem celltransplantation. A DMSO limit of 1g/kg recipient weight/day is commonly used inclinical cell therapy practice. Procedures to prevent DMSO toxicity also shouldbe considered. It is common clinical practice to premedicate patients withdiphenhydramine or other antihistamine agents to prevent DMSO toxicity. Productwashing methods by either manual (centrifugation) or automated methods also canbe considered but must be validated to ensure adequate postwash recovery andcell function.

对于患者而言，冷冻保存可能会影响最终产品的有效性和安全性。例如，低温保护剂DMSO与可预测的剂量依赖性胃肠道、心血管和神经毒性风险有关，这些毒性通常归因于组胺释放。最终产品中残留的DMSO应进行检测。DMSO被ICH归类为药品中的3类（相对低风险）溶剂或辅剂，正常认为摄入最高剂量在50 mg /天以内是可接受的（请参阅ICH Q3C）。除非它们在解冻后将DMSO清洗干净，正常冷冻保存的细胞产品含量大概是人体承受最高剂量的10-20倍。需要一次性使用多种冷冻保存的细胞产品时可能会产生更高浓度的DMSO，这种情况通常发生在自体外周血干细胞移植中。临床细胞治疗中通常使用DMSO限制为每天1g / kg受体体重。还应考虑采取防止DMSO毒性的措施。为预防DMSO毒性，临床上通常给患者使用苯海拉明或其他抗组胺药物对患者进行预处理。也可以考虑通过手动（离心）或自动产品洗涤的方法，但必须经过验证以确保充分的洗涤后细胞回收率和功能恢复。

Use ofcryopreserved cell therapy products may require the clinical site to receive,store, thaw, and perform other final preparation steps on the cryopreservedproduct. Feasibility assessment requires consideration of the site'scapabilities with regard to specialized personnel, training, equipment, andfacilities to execute those tasks.

冻存的细胞治疗产品的最终使用方案可能需要在临床上进行接收、储存、解冻以及执行其他最后准备步骤。可行性评估需要考虑现场执行这些任务所需的专业人员、培训、设备和设施。

Ifcryopreservation is planned as part of the manufacturing process, developmentteams must consider the effect of cryopreservation on cell number andcharacteristics and should require reliable methods for cell enumeration andassessment of cell viability and function. During development runs, more assaysoften are performed than will be required eventually for in-process and finalproduct testing. This is done in order to evaluate the effects of eachmanipulation of the product. Because some cells may be more susceptible thanothers to freeze–thaw damage, these studies should include assessment ofselective losses of important cell subpopulations within the product.

如果计划将冻-存作为细胞生产过程的一部分，则研发团队必须考虑冻存对细胞数量和特性的影响，并应使用可靠的方法进行细胞计数以及评估细胞活力和功能。在产品开发阶段，往往进行分析的细胞数量会比中间和终末测试所需的数量要多。这样做是为了评估产品每次操作的效果。由于相比较而言某些细胞可能更容易遭受冻融损伤，这些研究应包括评估产品中选择性的重要细胞亚群损失。

As described inthe Introduction, several critical processes influence the outcome of acryopreservation protocol. Following is a brief discussion of issues unique tocell therapies.

如引言中所述，几个关键过程会影响冻存方案的结果。以下是对细胞疗法所特有问题的简单论述。

**Prefreeze Processing**

**预冷冻过程**

If cells areharvested with plasma present, samples should be properly processed with ananticoagulant to prevent clotting. In addition, some cells are prone toclumping or aggregation when centrifuged, and some cell products may exhibitexcessive damage or loss of one or more populations within the product. Cellsharvested from adherent or nonadherent culture may include substantial numbersof dead or fragile cells. Therefore, centrifugation and wash steps should beoptimized and specific for the product's cellular contents, suspension volume,suspension medium, and container, along with appropriate evaluation of thecellular product before and after these manipulations. Prefreeze processingshould not result in cells that are stressed (e.g., cells that demonstrateelevated early apoptotic markers or temperature-shock responses) before thestart of the freezing process, or cell losses will be higher than expected.

如果收获的细胞中含有血浆，则应使用抗凝剂对样品进行适当处理来防止凝聚。此外，某些细胞在离心时易于结团或聚集，而一些细胞可能对其中的一个或多个种群表现出过度的损伤或损失。从贴壁或非贴壁培养中收获的细胞可能包括大量死细胞或比较脆弱的细胞。因此，应优化离心和洗涤步骤，并针对产品的细胞含量、悬浮液体积、悬浮介质和容器等进行优化，并在操作前后对细胞产品进行适当评估。在冻存过程开始之前，预冷冻处理不应导致细胞受到压力（例如，表现出早期凋亡标志物升高或温度休克反应的细胞），否则细胞损失将高于预期。

**Reagents and Containers**

**试剂和容器**

Clinical-grade reagents and containersshould be used whenever possible (see Ancillary Materials for Cell, Gene, andTissue-Engineered Products 〈1043〉). It iscustomary to use sterile, single-use, disposable plastic bags or cryovials thathave been qualitied for the specific cryopreservation process and subsequentstorage conditions. Cryopreservation media for cell therapy products usuallyconsist of isotonic saline-based solutions with one or more CPA, typically theintracellular cryoprotectant DMSO at 5%–10% final concentration with or withoutan extracellular cryoprotectant such as hydroxyethyl starch.The use of human-derived protein additives such as human serum albumin, serum,or plasma is common, but they may need extensive qualitication, so they shouldbe avoided if possible and alternatives should be evaluated. Additives such asanimal-derived heparin, citrate-based anticoagulants, and DNase sometimes areused. Many centers formulate their own cryopreservation media, but commercialcryopreservation media, which typically include 5%–10% DMSO and otherproprietary components, increasingly are used by cell therapy manufacturers toeliminate variability and the need for additional qualitication activitiesassociated with local formulation.

应尽可能使用临床级试剂和容器(参见“细胞、基因和组织工程产品的辅助材料”<1043>)。通常使用无菌的、一次性使用的袋子或冻存管，这些塑料袋或冷冻管已针对特定的冻存过程和后续的保存条件进行了鉴定。细胞治疗产品的冷冻保存介质通常由含有一种或多种CPA的等渗盐溶液组成，通常胞内冻存保护剂DMSO含量为5%-10%，加或不加胞外冷冻保护剂(如羟乙基淀粉)。通常使用人源性蛋白添加剂，例如最常见的人血清白蛋白、血清或血浆，但它们可能需要进行大量的鉴定，因此应尽量避免使用，并应评估其他替代品。有时会使用其他添加剂，如动物来源的肝素、基于枸橼酸的抗凝剂和核酸酶。许多单位制定他们自己的冷冻保存培养基，一般商业冻存液通常包括5%-10%的DMSO和其他专有成分，目前越来越多的细胞治疗厂家使用商业化的冻存液来消除变异性和本地配方相关的额外认证需要。

**Addition of Cryoprotectant Solution andCooling**

**添加冻存液及冻存**

Procedures for introduction or removalof a cryopreservation solution should be assessed before freezing to ensurethat cell losses resulting from this step are minimized. The cryopreservationmedium usually is added to the cell suspension in steps or gradually (e.g.,using a syringe pump) to prevent cell losses resulting from osmotic stress. Itis common to prechill the cryopreservation medium and keep the cell suspensionand the admixture chilled using cold packs, a frozen blanket, or a chilled worksurface to prevent heat-related cell damage during addition of DMSO. Once thecryopreservation medium is added, the cell suspension typically is transferredto the precooled chamber of a controlled-rate freezer. During the freezeprocess, a record of chamber and product temperature over time, or freezecurve, is generated for inclusion in the production record. Producttemperatures can be recorded from a probe placed on the product bag's outersurface or from the inside of a comparable product in a dummy bag or vial thatundergoes concurrent freezing.

冻存前应评估加入或取出冻存液的程序，确保将因该步骤导致的细胞损失降至最低。通常将冻存液分步骤或逐步地（例如，使用注射泵）添加到细胞悬浮液中，以防止由于渗透压变化引起的细胞损失。通常要使用冰袋、冰盒或冷冻的工作台对冻存液进行预冷，并将细胞悬浮液和混合物保持冷冻，以防止在添加DMSO期间因产生热量引起的细胞受损。一旦添加了冻存液，通常将细胞悬浮液转移到可控制冷冻速度的冻存盒中。在冷冻过程中，腔室和细胞随时间变化的温度记录或冷冻曲线会生成并整合到生产记录中。产品温度可以通过放置在产品袋外表面上的探针记录下来，也可以从同时进行冻存的类似的产品或模拟包装袋或冻存管中记录下来。

**Storage andTransport**

**储存和运输**

Cryopreserved cell therapy productstypically are stored and transported at temperatures of −150° or colder. FDArequires screening and testing for evidence of transmissible disease only forallogeneic donors of cell therapy products and not for autologous donations.However, many centers test autologous donors as well and segregate productsfrom autologous donors who are known to have transmissible diseases when theirproducts must be stored. A report of hepatitis B crosscontamination of cellularproducts within a liquid-nitrogen storage tank led to the currently commonpractices of storage in the vapor phase of liquid nitrogen and the use ofoverwrap bags to reinforce product containment. Liquid-nitrogen vapor phasestorage may be associated with vertical temperature gradients: products at thetop of the storage tank may have a warmer temperature than those stored at thebottom of the tank. Temperature gradients should be monitored, and verticaltemperature gradients should be minimized, e.g., by use of metal heat shunts.Overwrap bags may reduce the warming rate of the sample if used during the subsequentthaw process, and their use should be qualitied as part of the overallcryopreservation process validation.

冻存的细胞治疗产品通常在-150°C或更低的温度下存储和运输。 FDA要求仅对细胞治疗产品的异体供体进行传染性疾病筛查，而不对自体供体进行检测。但是，许多中心也对自体供体进行检测，并从已知有传染病的自体供体中分离产品。一份关于液氮罐中的细胞产品发生乙肝交叉污染的报告导致了当前在液氮气相中储存和使用外包装袋时加强产品密封性的普遍做法。液氮气相存储可能与垂直温度梯度有关：液氮罐顶部的产品可能比罐底部的细胞的温度高。应监控温度梯度，或使用例如金属热分流器将垂直温度梯度降至最低。如果在解冻过程中使用复温袋可能会降低样品的升温速度，应将其使用视为整个冻存过程验证的一部分。

Transport of cryopreserved cell therapyproducts usually is accomplished by using dry shippers containing absorbentmaterial that can be charged with liquid nitrogen to maintain vapor-phasetemperatures for up to 2 weeks if properly charged. Data loggers are used todocument the temperature history during transport. These shipping containersand procedures must be validated before cell therapy products for clinical useare shipped in the containers.

冻存的细胞治疗产品的运输通常是通过使用含有吸附材料的干燥的运输箱来完成的，这种材料可以添加液氮，从而保持气相温度长达2周。数据记录仪用于记录运输过程中的温度历史记录。在装运临床的细胞治疗产品之前，这些装运容器和程序必须要经过验证。

**Warming(Thawing)**

**解冻**

Although bedside thawing of cell therapyproducts before infusion has been a common clinical practice, the use oftrained personnel in a controlled laboratory environment is now recognized asthe preferred method for thawing because it allows a more standardized processand a higher degree of control when staff must respond to a container failure,which may require product salvage in a more sterile environment. Productthawing typically is done by immersion in 37° water baths and using overwrapbags to minimize product loss and contamination in case of primary containerfailure. Bags may be gently kneaded during thaw to reduce temperature gradientsacross the bag and to accelerate thawing. The product is removed from the waterbath when some ice is still present in the product but the majority of theproduct is thawed.

尽管在输液前在床边对细胞治疗产品进行解冻是一种常见的临床过程，但现在公认受过培训的人员在受控实验室环境中解冻是首选的方案。因为这样可以使工作人员在无菌环境中通过更标准化的操作过程和更高程度的控制细胞解冻过程，并对容器故障作出响应来产品回收。解冻通常是通过浸37°水浴中并使用复温袋来完成的，以便在主容器发生故障的情况下将产品损失和污染降至最低。解冻过程中可以轻轻揉捏袋子，降低袋子上的温度梯度并加速解冻。当产品中仍然存在一些冰而大部分产品已经解冻后，将产品从水浴中取出。

**Post-Thaw Processing**

**解冻后处理**

DMSO is toxic to cells in liquid suspension. Toxicity maybe reduced by diluting or washing the cell suspension before infusion orfurther manipulation. Because cryopreserved, thawed cells are more sensitive tovolumetric expansion when the cells transition from a hypertonic solution to anisotonic solution, the dilution and washing solutions and methods must becarefully designed and validated. Cell washing using either a conventionalcentrifuge or an automated device can result in additional mechanical stress tothe cells, so cell losses must be assessed by an appropriate method before aspecific method isimplemented in clinical practice.

DMSO对溶液中的细胞有毒。在输注或进一步处理之前，可通过稀释或洗涤细胞悬液来降低毒性。由于冻存过程中当细胞从高渗溶液过渡到等渗溶液时，融化的细胞对体积膨胀更敏感，稀释和洗涤溶液的方法必须仔细设计和验证。使用常规离心机或自动化设备进行细胞洗涤会给细胞带来额外的机械应力，因此具体在临床实践中，必须通过恰当的方法评估细胞损失。

**Quality Control Practices**

**质量控制过程**

Quality management of cryopreservation, storage, and thawof clinical cell therapy products must incorporate quality system elements commonto current Good Tissue Practices (cGTP) and current Good ManufacturingPractices (cGMP), including personnel qualification, facilitycontrols, document control, control of equipment and materials, label control,and use of validated SOPs (21 CFR 1271, 210, and 211). Quality controlpractices specific to clinical cryopreservation typically include assessmentand documentation of freeze curves for all products, retention of tubingsegments and vials for subsequent testing, and regular monitoring of post-thawproduct quality. Practices required by cGTPs and applicable to all human cell-and tissue-based products include measures to ensure accurate and completelabeling and records, to ensure that the correct product goes to the patient,and to allow tracking of the cell product from collection to infusion. Thepractical implications of these requirements are that labeling and records,including inventory systems, for cryopreserved products must be designed toprevent errors in identification of products. Identity verifications ofproducts moving into and out of cryopreserved storage are performed routinely,e.g., with two people checking the product label against records. If necessaryfor additional verification of product identity, the contents of a tubingsegment attached to the product bag can be thawed and tested before thaw of theentire product. ISBT 128, an internationally recognized system for labeling ofblood and cell-therapy products, incorporates use of consistent productnomenclature and barcoding of the product from donor source throughadministration (see Appendix).

临床细胞治疗产品的冷冻保存、存储和解冻的质量管理必须纳入动态组织操作规范（cGTP）和动态药品生产管理规范（cGMP），包括人员资格、设施控制、文档控制、设备控制以及材料、标签控制和经过验证的SOP（21 CFR 1271、210和211）的使用。针对临床冷冻保存所特有的质量控制措施通常包括评估和记录所有产品的冷冻曲线、保留用于后续测试的管段和小瓶以进行后续测试以及解冻后产品质量的定期监测。 cGTP要求的适用于所有基于人类细胞和组织的产品的规范包括确保准确和完整的标签和记录、确保将正确的产品运用于患者及允许细胞产品从采集到回输的过程的跟踪。这些要求的实际意义是，必须设计用于冷藏产品的标签和记录，包括库存系统，以防止产品标识错误。例行执行对进出低温保存的产品的身份验证，如果有必要对产品标识进行额外的验证，可以在整个产品解冻之前对附在产品袋上的管段中的内容物进行解冻和测试。ISBT 128是一种国际公认的血液和细胞治疗产品标签系统，通过将一致的产品命名和产品条形码的使用结合起来管理（请参阅附录）。

HEMATOPOIETIC STEM CELLS

造血干细胞

Studies of the response of hematopoietic stem cells(HSCs) to freezing began in the 1950s, and cryopreserved HSCs have been widelyused in clinical practice during the past 30 years. The most common method ofcryopreserving HSCs for clinical applications involves the use of 10% DMSO anda controlled-rate freezer set at a cooling rate of 1°/min. Another method, lesscommonly used, involves the use of passive freezing of the HSC product in a −80° mechanical freezer and the use of 5%DMSO + 6% hydroxyethyl starch solution. Cells commonly are frozen at densitiesof 30–50×106 cells/mL. Exceeding cytocrits of 20% (v/v) has beenshown to reduce cell recovery. Post-thaw assessment of the sample consists ofenumeration of nucleated cells, viable CD34 cells, and hematopoieticcolony-forming units, along with subsequent calculation of recoveries fromcorresponding prefreeze values.

造血干细胞（HSC）对冻存反应的研究始于1950年代，而冷冻保存的HSC在过去30年中已广泛用于临床。临床应用中最常用的HSC冷冻方法是使用10％DMSO和以1℃/ min的冷却速度设置的可控速率的冷冻机。另一种不常用的方法是将HSC产品被动冷冻在−80℃的冰箱中，以及使用5％DMSO + 6％羟乙基淀粉溶液保存。通常以30–50×106细胞/ mL的密度冻存细胞。研究表明，有超过20％体积比的细胞分裂会降低细胞回收率。样品的解冻后评估包括有核细胞、活CD34细胞和造血集落形成单位的计数，以及根据相应的预冻值计算回收率。

HSCs cryopreserved for clinical use may be obtained frombone marrow, mobilized peripheral blood, or umbilical cord blood (UCB). Eachsource has unique requirements for preservation. For example, peripheral bloodprogenitor cell products contain larger numbers of cells and may be frozen inmultiple bags with relatively high cell concentrations. Protocols for UCBpreservation may include the use of syringe pumps to introduce cryopreservationsolutions while minimizing osmotic stresses. For UCB, specialized solutionsoften are used after thaw to dilute or remove DMSO while minimizing osmoticstress for the cells.

临床使用的造血干细胞可以从骨髓、外周血或脐带血中获得。每种来源对冻存都有其特殊的要求。例如，外周血祖细胞（UCB）产品含有大量的细胞，并且可以在具有较高细胞浓度的多个袋中冷冻。UCB的保存方案可能包括使用注射泵引入冻存液，同时最大程度减少渗透压力。对于UCB，通常在解冻后使用专门的溶液来稀释或去除DMSO，同时最大程度降低细胞渗透压力。

MESENCHYMAL STEM CELLS

间充质干细胞

Research on the clinical use of mesenchymal stem cells(MSC) has grown rapidly since the mid-1990s. Reliable, safe, and efficientmethods of cryopreservation and storage are critical, especially for allogeneicoff-the-shelf MSC products manufactured in multiple product doses for treatmentof a large number of patients with a range of clinical indications.

自20世纪90年代中期以来，对间充质干细胞(MSC)临床应用的研究快速发展。可靠、安全和有效的冻存和储存方法是至关重要的，特别是对于用于治疗具有各种临床适应症患者的多产品剂量生产的异体间充质干细胞成品。

Because MSCs traditionally have been generated incultures that contain fetal bovine serum (FBS), cryopreservation media forthese cells often have incorporated FBS. More recently, alternatives togamma-irradiated FBS are being explored for the culture expansion beforecryopreservation, and cryopreservation has been successful in media thatcontain 5%–10% DMSO and other components without bovine sources of protein.Although there is no consistent method of post-thaw cell processing, someprotocols include dilution or washing of the cells to mitigate the effects ofDMSO. Emerging clinical applications for MSCs may require repeat dosing of thecellular product, a practice that requires attention to potentialimmunogenicity of components of the cryopreservation medium, e.g., FBS or otherproteins.

由于传统骨髓间充质干细胞是在含有胎牛血清(FBS)的培养基中产生的，因此用于这些细胞的冻存液通常含有FBS。近期，正在探索替代γ射线辐照的FBS进行细胞培养的方法，并且在含有5％-10％DMSO和其他不含牛蛋白质来源的成分培养基中，冻存已经取得成功。尽管没有统一的解冻后细胞处理方法，但可以采用某些方案包括稀释或清洗细胞以减轻DMSO的影响。骨髓间充质干细胞的新兴临床应用可能需要重复给药细胞产物，这种做法需要注意冻存液成分(如胎牛血清或其他蛋白质)的潜在免疫原性。

Post-thaw assessment of MSCs typically hasinvolved the use of membrane-integrity dyes such as trypan blue, surfaceantigen expression, and evidence that the cells are capable of multilineagedifferentiation. Because the mechanism of action of MSCs may involve theimmunomodulatory or trophic properties of the cells, post-thaw assessment alsoshould include relevant cell function.

骨髓间充质干细胞的解冻后评估通常包括使用膜完整性染料（例如台盼蓝）、表面抗原表达以及证实细胞能够进行多系分化。由于MSC的作用机制可能涉及细胞的免疫调节或营养特性，因此解冻后评估还应包括相关的细胞功能。

LYMPHOID CELLS

淋巴细胞

Lymphocytes are used for a variety ofclinical applications including immunotherapy to treat cancer, viral infection,and autoimmune disease. Therapy based on lymphocytes may consist of mixedlymphocyte populations or lymphocyte subpopulations that have been selected oractivated ex vivo, e.g., regulatory T cells, natural killer cells, andactivated T cells. As with hematopoietic cells, lymphocytes typically arecryopreserved using a 10% DMSO solution and a controlled cooling rate of1°/min.

淋巴细胞用于多种临床应用，包括治疗癌症、病毒感染和自身免疫性疾病的免疫治疗。基于淋巴细胞的疗法可以由已经体外选择或激活的混合淋巴细胞群或淋巴细胞亚群组成，例如调节性T细胞、自然杀伤细胞和活化的T细胞。与造血细胞一样，淋巴细胞通常使用10%的DMSO溶液冷冻保存，冷却速度控制在1℃/min。

Lymphocytes may undergo extensivepost-thaw apoptosis, which can influence the clinical efficacy of the cells.Highly purified populations of lymphocytes may exhibit higher levels ofpost-thaw apoptosis than mixed lymphocyte populations. Strategies such ascaspase inhibition and cytokine rescue have been used to diminish post-thawapoptosis of lymphocytes.

淋巴细胞在解冻后可能发生广泛的凋亡，从而影响细胞的临床疗效。高度纯化的淋巴细胞群可能比混合淋巴细胞群表现出更高水平的解冻后凋亡。caspase抑制和细胞因子拯救等策略已被用于减少解冻后淋巴细胞凋亡。

PRESERVATION OF HUMAN PLURIPOTENT STEM CELL LINES

人多能干细胞系的保存

Pluripotent stemcells (PSCs) are cells that appear to have the capacity to (1) undergoself-renewal and replicate indefinitely and (2) generate cells that arerepresentative of the three germ-layer tissues required to create all cells ofthe human body as demonstrated by the capacity to generate teratomas in immunedeficient mice. The two predominant types of stem cell line used for in vitrolaboratory research are human embryonic stem cells (hESCs) and human inducedpluripotent stem cells (hiPSCs). hESCs are derived from donated surplus blastocystsby isolation and culture of the blastocyst inner cell mass (the tissue thatwould have progressed to form the embryo). hiPSCs are created by artificialreprogramming of somatic cells (by the delivery of reprogramming factors usinga variety of methods) to yield cells that express the critical properties ofPSCs listed above. hiPSCs can be derived from a range of somatic cell typesusing an increasing range of methods to secure expression of certainreprogramming factors. A range of tissue-derived cultures are known to harborstem cell populations in vitro (e.g., subcutaneous fat, bone marrow, cord andblood of the fetal umbilicus, primordial germ cells from the fetal neuralcrest, and neural stem cell spheroid cultures) that have been shown to have alimited capacity for in vitro replication and have not been established asstable diploid PSC lines. Such cultures will not be covered in this chapter,and the following sections will deal specifically with hESC and hiPSC lines.

多能干细胞（PSC）是具有以下能力：（1）进行自我更新并无限复制，（2）产生代表创建该个体所需的三个胚层组织的所有细胞。如免疫缺陷小鼠中产生畸胎瘤的能力。用于体外实验室研究的两种主要类型的干细胞系是人胚胎干细胞（hESCs）和人诱导的多能干细胞（hiPSC）。 hESCs是通过分离和培养从捐赠的多余胚泡中的胚泡内部细胞团（已经形成胚胎的组织）获得。通过对体细胞进行人工编辑（通过使用多种方法传递重编因子）来产生hiPSC，这些细胞表达了上述PSCs的关键特性。hiPSCs可以从一系列体细胞类型中提取，使用越来越多的方法来确保某些重编程因子的表达。已知的一系列组织衍生物的体外干细胞群（例如，胎儿脐带的皮下脂肪、骨髓、脐带和血液、来自胎儿神经管的原始生殖细胞以及神经干细胞球培养），目前证实其在体外复制能力有限，尚未建立稳定的二倍体PSC品系。这种方法将不在本章中讨论，下面章节将专门介绍hESC和hiPSC系。

PSC lines are complex multicomponentcell cultures and may contain a variety of different cell populations withgreater or lesser degrees of differentiation or lineage commitment. However,the key property of a stem cell culture, self-renewal, must be sustained. Inaddition, the cells must retain the capacity to undergo asymmetric division toyield two different daughter cells: one that is a stem cell identical to theparent cell and one with a reduced degree of potency (i.e., ability to generatedifferent cell lineages).

PSC系是一种复杂的多组分细胞，可能包含多种不同的细胞群，或多或少具有一定的分化程度或谱系分化。但是，必须保持干细胞培养的关键特性，即自我更新。此外，细胞必须保持不对称分裂产生两种不同子细胞的能力:一种是与母细胞完全相同的祖细胞，另一种是分化潜力降低的子细胞(即具有产生不同细胞系的能力)。

**Development of Current Methodologies for PSC**

**PSC方法的发展**

Currently, individual labs have theirpreferred methodology, no single approach appears to dominate for routine use,and it seems possible to obtain acceptable levels of post-thaw viability andrecovery by either vitrification or controlled slow rate cryopreservation;however, a common method that results in better cell recovery is describedhere. Briefiy, this technique involves placing colonies of hESCs in avitrification solution composed of 20% DMSO + 20% ethylene glycol (EG) + 0.5mol/L sucrose after equilibration with lower-concentration DMSO + EG solutions.The colonies are loaded into straws and plunged into liquid nitrogen. Anothercommon method involves placing colonies in a solution consisting of 10% (v/v)DMSO and using either a passive freezing device or a controlled-rate freezerdesigned to achieve an average cooling rate of approximately 1°/min.

目前，各个实验室都有自己偏爱的方法，没有一种方法能在日常使用中占主导地位，而且似乎有可能通过玻璃化转变温度或控制慢速冷冻保存获得可接受的解冻后存活率恢复；目前是获得更好的细胞恢复的常用方法。简单地说，这项技术涉及将hESCs的细胞团放入由20% DMSO + 20%乙二醇(EG) + 0.5 mol/L蔗糖组成的玻璃化溶液中，然后用低浓度的DMSO + EG溶液平衡。细胞团被装入吸管中，然后放入液氮中。另一种常见的方法是将细胞团放置在含有10% DMSO的溶液中，并使用被动冷冻设备或控制速率的冷冻机，该设备设计的目的是使平均冷却速率达到大约1℃/min。

Vitrified IPS or hES samples havespecial storage and shipping requirements. All cryopreserved or vitrifiedsamples must be stored at temperatures below the glass transition temperatureof the sample, and for vitrified samples this is much lower (e.g., −150°) than fortraditionally cryopreserved cell samples. In addition, fluctuations intemperatures during storage or shipment can lead to crystallization andtherefore degradation of the sample. Because vitrified samples are sensitive tothese temperature fluctuations, they should be shipped at liquid nitrogentemperatures and should not be shipped on dry ice.

玻璃化的IPS或hES样品有特殊的储存和运输要求。所有低温保存或玻璃化的样品必须保存在低于样品玻璃化转变温度下，玻璃化的样品比传统低温保存的细胞样品保存温度要低得多(例如，- 150℃)。此外，储存或运输过程中的温度波动会导致结晶，从而导致样品的降解。由于玻璃化的样品对这些温度波动很敏感，它们应该在液氮温度下运输，而不应该在干冰上运输。

Challenges for the reliable and reproducible preservation of PSClines lie not just in the preservation process itself but also in thepreparation, cryoprotection, and recovery procedures. Specifically, methods ofharvesting and handling before cryopreservation may result in significant celllosses. Processing procedures should be made as reproducible as possible andwith the use of an SOP to enhance the reproducibility of preservation outcomes.Furthermore, the effective banking of PSC lines is challenged by the methods bywhich the cells typically are passaged and harvested for preservation (i.e.,individual colonies dissected and transferred as small colony fragments tofresh culture flasks for expansion or to preservation medium for freezing).First, in order to avoid extensive loss of viability in the earlier harvestedcells, cultures used to make a bank actually may require preservation in smallbatches over a working day. Second, preparation of PSC banks comprising smallerpools of cell colony fragments is not only time consuming and laborious, but italso makes it impossible to homogenize the preparation of cells beforealiquoting into vials, as would happen with more traditional methods ofpreserving cell lines. Thus, consistency between vials of PSC lines iscompromised. Third, freezing PSCs as colony fragments preserves the gapjunctions known toform between cells, potentially leading to intercellular propagation of icecrystals and extensive loss of viability.

对于PSC细胞系可靠和可重复保存的挑战不仅在于保存过程本身，还在于制备、低温保护和回收过程。具体来说，冻存之前的收获和处理方法不当可能会导致大量的细胞损失。处理程序应尽可能重复，并根据SOP操作以提高保存结果的可重复性。此外，PSC细胞系的有效储存受到细胞传代和保存方法的挑战。将单个细胞团分解为小细胞团碎片转移到新鲜的培养瓶中或放入冻存液中冷冻。首先，为了避免早期收获的细胞大量丧失存活率，用于储存的细胞培养物实际上可能需要在一个工作日内小批量保存。第二，由更小的细胞集落片段组成的PSC库的制备不仅费时费力，而且不可能像传统的保存细胞系的方法那样，在将细胞加入到培养瓶前进行混匀，这就导致PSC细胞系瓶间的一致性受到了影响。第三，以集落碎片的形式冷冻PSCs可以保留细胞间形成的缝隙连接，这可能会导致冰晶在细胞间传播并严重丧失细胞活力。

To avoid some of these issues, analysts can use enzymaticdisaggregation of colonies to simplify and accelerate cell harvesting and toenable cryopreservation of more homogenous, single-cell suspensions beforepreservation. To be prudent, some laboratories choose to perform enzymaticdisaggregation solely before cryopreservation but not for routine culture. Whenever possible,analysts should use non–animal-sourced enzymatic agents for cell dissociation.Another important characteristic of PSC lines is the common but variable incidenceof undirected differentiation that occurs within colonies and may varyconsiderably across the many colonies in a culture. Colonies with a highproportion of differentiated cells should be discarded because thedifferentiated cells are an undesirable component of a PSC culture and mayaffect the properties of the undifferentiated cells within the colony.

为了避免这些问题，分析人员可以使用酶解细胞团来简化和加速细胞的收获，并在保存前冻存更均匀的单细胞悬浮液。为了谨慎起见，一些实验室选择在低温保存前单独进行酶解，但不用于常规培养。只要有可能，分析人员应该使用非动物来源的酶制剂进行细胞解离。PSC细胞系的另一个重要特征是经常在细胞团内发生无定向分化，在一个培养的许多细胞团中可能存在比较大差异。分化细胞比例高的细胞团应丢弃，因为分化的细胞是PSC培养的不利成份，可能影响细胞团内未分化细胞的特性。

**Points to Consider in the Preservation of PSC Lines**

**保存PSC细胞系时应考虑的要点**

Analysts should consider a number of important factors in thepreservation of PSC lines that should be addressed at the levels ofpreparation, harvesting, banking, and testing of cryopreserved stocks.

操作人员应该考虑保存PSC细胞系的一些重要因素，应该在冻存的准备、收获、储存和测试等层面加以处理。

EVALUATION AND HARVESTING

评估和收获

Analysts should observe cell cultures on a regular basis. Culturesthat exhibit high levels of differentiated cells should be discarded. Anindividual investigator or banking facility should have a quality controlprogram that evaluates differentiation of cultures on a regular basis anddevelops threshold levels of acceptable differentiation in a culture. Theprotocol for harvesting followed by equilibration of the sample with CPAsshould be carried out rapidly in order to minimize cell losses because of harvesting.

操作人员应定期观察细胞培养情况，对于表现出高水平分化细胞应被丢弃。研究者或存储机构应该有一个质量控制程序，定期评估分化差异并制定可接受差异的阈值。为尽量减少因收获而造成的细胞损失，应迅速由CPAs平衡样品后实施收获。

THE BANKING PROCESS

存储过程

Conventional cell banking procedures require the development ofMCB and WCB. Cells in the MCB should be preserved at an early passage number(P10–P20) in order for experimental and cell line development work to beperformed on cells at the lowest possible passage number. The first WCB shouldbe established with the minimum passages to achieve the cell number required.To promote consistency, the WCBs subsequently can be replaced at the samepassage level from the MCB as required.

常规的细胞库存储程序需要开发MCB和WCB。MCB中的细胞应以早期传代数（P10–P20）保存，以便在可能的最低传代数下对细胞进行实验和细胞系开发。应该以最少的转运通道建立第一个WCB，以达到所需的存储单元数。为了提高一致性，随后可以根据需要在与MCB相同的通道级别上替换WCB。

VIABILITY

活率

Determination of post-thaw viability and function for PSCs can becomplicated and is often done incorrectly. The most common method of post-thawassessment is use of a membrane integrity dye. As a measure of viability, thefraction of cells with intact membranes is compared to those without. Anotherfairly common method is to quantitate PSC colony formations. The total numberof colonies seeded into a plate is counted and then is counted again after a certainincubation time. This method also evaluates other functional characteristics ofthe cells, such as their ability to attach and proliferate. Note thatprocedures such as harvesting and cryopreservation may induce apoptosis.Staining for early (phosphatidylserine on the cell surface) or later (AnnexinVI) apoptotic markers may provide insights into the general health of frozenand thawed cultures.

PSCs解冻后活力和功能的测定一般很复杂，而且常常容易做错。最常用的解冻后评估方法是使用膜完整性染料。作为一种生存能力的测量方法，将细胞膜完整的细胞与细胞膜不完整的细胞进行区分。另一种相当普遍的方法是定量PSC细胞团的形成。接种到平板上的细胞团总数先被计数，经过一定的孵育时间后再计数。这种方法还可以评估细胞的其他功能特征，如附着和增殖能力。值得注意的是，收获和冷冻保存等操作都可能诱导细胞凋亡。早期(Annexin VI,细胞表面磷脂酰丝氨酸)或晚期(碘化丙啶，PI)凋亡标记的染色可为冻存和解冻的样品总体健康状况提供依据。

HOMOGENEITY

均一性

As already described, preservation of colony fragments byvitrification can exacerbate the lack of homogeneity between vials or straws ofcells. When larger banks of PSCs are established, analysts should test vialsfrom early, middle, and late positions in the filling sequence of the cell bankfor viability, growth rate, and key markers as indicated in Viability.

如上述已经介绍的，通过玻璃化保存小细胞团块会加剧细胞在小瓶或吸管之间缺乏同质性。建立较大的PSC库时，分析人员应从细胞库填充顺序的早期、中期和晚期位置测试小瓶的活力、生长速率和活力的关键指标。

STEMNESS

干细胞特性

In order to check that a PSC line has not lost any of its stemcell characteristics during preservation, analysts should check expression of anumber of key stem cell-related markers. One extensive study analyzed 59 hESClines and a panel of 94 genes and resolved five stem cell-related molecules forwhich mRNA was expressed consistently in hESCs. These genes now are included incommercially available microfluidics gene cards that are specificallydesigned for investigating stem cell populations. If it is crucial todemonstrate that the PSCs have retained their pluripotency, then a number ofcharacterization tests can be performed, including teratoma formation inimmune-deficient mice, formation of trilaminar embryoid bodies, and directeddifferentiation to demonstrate that the culture can produce representatives ofeach of the three germ layer tissues that are required to form all of the cellsof the human body.

为了检查PSC品系在保存过程中没有失去任何干细胞特性，分析人员应检查许多关键的干细胞标志物的表达。一项大量的研究分析了59个hESC系和94个基因组，并解析了hESCs 中mRNA表达的5个与干细胞相关的分子。现在，这些基因已包含在商业可购买的微流体基因数据库中，该数据库专门设计用于研究干细胞群体。如果证明PSC保留了其多能性是至关重要的，那么可以进行许多表型测试，包括免疫缺陷小鼠中的畸胎瘤形成、三层胚状体的形成以及定向分化，证明培养的PSC可以产生代表形成人体所有细胞所需的三个胚层组织。

GENETIC STABILITY

遗传稳定性

In both iPSC and hESC lines, it is not unusual for clones ofabnormal karyotype to arise on extended passage and overgrow the culture. Thusanalysts should monitor cultures for such abnormal cells. Traditionally, thishas been performed by karyotypic studies of metaphase spreads of the cellsusing Giemsa staining.The occurrence of nondiploid cells, even at very low incidence,can be problematic. Guidance documents such as the 2009 International Stem CellBanking Initiative are helpful to determine if such cultures should be discarded.However, newer procedures such as array comparative genome hybridization andsingle-nucleotide polymorphism arrays provide much more detailed analysis ofgenetic stability and can be used in parallel with Giemsa banding to givegreater confidence in genetic stability.

在iPSC和hESC品系中，异常核型克隆在传代和培养中过度生长中比较常见。因此，分析人员应监控此类异常细胞的培养。通常通过姬姆萨染色对分裂中期细胞进行核形分析。非二倍体细胞的出现，即使发生率很低，也可能是有问题的。诸如2009年国际干细胞银行倡议之类的指导文件有助于确定是否应该丢弃此类培养物。然而，诸如比较基因组杂交和单核苷酸多态性阵列之类的较新方法提供了对遗传稳定性的更详细的分析，并且可以与Giemsa谱带结合使用，从而对遗传稳定性具有更大的信心。

BEST PRACTICES

最优方案

In addition to following good cell culture practices, analystsshould note the availability of a specific guidance that contains principlesand best practices in the procurement, banking, testing, and storage of hESCsfor research purposes (see the ISCB 2009 reference in the Appendix). Thisguidance is useful for both iPSC and hESC lines.

除了遵循良好的细胞培养规范外，分析人员应该还注意到一个具体的指南的可用性，其中包含用于研究目的hESCs的采购、存储机构、测试和存储的原则 (参见附录中的ISCB 2009参考)。本指南对iPSC和hESC都是实用的。

**CELL SUBSTRATES USED IN PRODUCTION AND CHARACTERIZATIONOF BIOTECHNOLOGY-DERIVED AND BIOLOGIC THERAPEUTIC PRODUCTS**

**用于生物技术衍生和生物治疗产品的生产和特性的细胞底物**

A wide variety of both recombinant and nonrecombinantcells are cryopreserved and used in the production and characterization ofhuman biologics and biotechnology-derived (B&B)products (more information can be found in ICH Q5D Derivation andCharacterisation of Cell Substrates Used for Biotechnological/BiologicalProducts). The major groups include cell lines derived from mammals (includinghumans), insects (primarily moths), and selected strains of bacteria and yeast.The most common microbial substrates used for production of humanbiotechnology-derived products are recombinant strains of Escherichia coli,Pichia pastoris, or Saccharomyces spp. (yeasts). Despite the high degree ofdiversity among cell types used to manufacture B&B products, there is asurprising degree of uniformity across cryopreservation practices and the sameprinciples apply to cells used for manufacture and those used in tests for adventitiousagents and product potency.

冻存各种重组和非重组细胞，并用于人类生物制剂和生物技术衍生产品（B＆B）的生产和表征（更多信息可在ICH Q5D“生物技术/生物产品所用细胞基质的衍生和表征”中找到）。这些细胞系主要包括来源于哺乳动物（包括人类）、昆虫（主要是飞蛾）以及选定的细菌和酵母菌株。用于生产人类生物技术衍生产品的最常见微生物底物是大肠杆菌、毕赤酵母或酵母的重组菌株。尽管用于制造B＆B产品的细胞类型之间存在比较大的差异，但在冻存中具有惊人的一致性，并且同样的原理适用于生产的细胞以及用于不定因素和产品效能测试的细胞。

Typically, a two- or three-tiered cell banking system is used formaintenance of manufacturing cell lines or microbial strains. For those thatuse a three-tier system, the first tier bank can be referred to as a research,seed, stock, accession, pre-MCB, or parent cell bank. The source of the pre-MCBcan be a research or development laboratory, or cells can be purchased from acommercial repository. It is advisable to characterize the parental cell bankprior to its use in cloning. The second tier (or first tier in a two-banksystem) MCB or Master Cell Stock (MCS) is prepared directly from this parentcell bank with minimal cell passages or generations. The MCB or MCS isextensively tested to confirm purity, phenotype, genotype, protein expression,or other important attributes. The WCB or Working Cell Stock (WCS) is derivedfrom vials of the MCB after successive passages in culture. The WCB is themanufacturing cell substrate that is scaled up through repeated subcultures to seedthe final production bioreactor, fermenter, or lot of culture vessels (e.g.,roller bottles). At each tier in the cell banking system, propercryopreservation is paramount to success in both product development andmanufacturing. In some cases, the end of production cells also may be bankedfor testing purposes as part of cell bank qualification.

通常，需要两级或三级细胞库系统用于维持细胞系或微生物菌株的生产。对于使用三级系统的存储库，第一级存储库可以称为研究库、种子库、原料库、接收库，MCB前期库或母细胞库。前期的MCB来源可以是研发实验室，或者从商业存储库购买细胞。建议在克隆前第二级（或两级系统中的第一层）MCB或主细胞库存（MCS）是直接从该亲代细胞库中制备的，细胞传代或代数最少。对MCB或MCS进行测试，以确保其纯度、表型、基因型、蛋白质表达或其他重要属性。WCB或工作细胞储备液（WCS）是在连续培养后从MCB小瓶中提取的。WCB是生产细胞的底物，可通过重复的传代培养扩大规模，用于最终生产的生物反应器、发酵罐或大量培养容器（例如滚瓶）。在细胞银行的每一级上，适当的冷冻保存对于产品开发和生产都是至关重要的。在某些情况下，也可以将生产单元的终末细胞存储起来以用于测试，并作为细胞库资格认证的一部分。

**Mammalian and Insect Cell Lines**

**哺乳动物和昆虫细胞系**

Mammalian cell lines are the cellular substrates of choice for theproduction of complex protein molecules. Mammalian cells possess the intrinsicbiological machinery required for posttranslational glycosylation of proteinsthat often is critical for stability and bioactivity in humans. Both diploidand heteroploid cell lines (including hybridomas) are used for production ofbiotechnology-derived therapeutics. Diploid cell lines are common vaccinesubstrates [e.g., WI-38 and MRC-5 (human fibroblast cell lines), BHK-21 (babyhamster kidney cell line), and MDCK (Madin-Darby canine kidney)]. Additionally,the African green monkey Vero cell line (a heteroploid cell line) is used forseveral US-licensed vaccines. Today, commonly used heteroploid cell linesinclude various recombinant Chinese Hamster Ovary and human embryonic kidneycell lines. Cryopreservation methods are fairly well standardized for thesecell lines. However, investigators may find it useful to investigate thetoxicity of different cryoprotectants and concentrations when they use a newcell substrate.

哺乳动物细胞系是生产复杂蛋白质分子的首选细胞。哺乳动物细胞具有蛋白质翻译后糖基化所需的内在生物学机制，而蛋白质通常对人类的稳定性和生物活性至关重要。二倍体和多倍体细胞系（包括杂交瘤）都用于生物技术衍生疗法。二倍体细胞系是常见的疫苗底物[例如WI-38和MRC-5（人成纤维细胞系），BHK-21（小仓鼠肾细胞系）和MDCK（Madin-Darby犬肾）]。此外，非洲绿猴Vero细胞系（一种多倍体细胞系）用于获得几种美国许可的疫苗。如今，常用的多倍体细胞系包括各种重组的中国仓鼠卵巢和人类胚胎肾细胞系。这些细胞系冷冻保存方法已经相当标准化。然而，当他们使用新的细胞底物时，研究人员可能会发现研究不同冷冻保护剂及其相应浓度的毒性是很关键的。

Insect cell lines have proven their capability for production ofvarious recombinant polypeptides. The most common cell lines are derived fromthe moths Spodotera frugiperda and Trichoplusia ni, and their established celllines are called Sf9 and Tn5 (or High Five), respectively. Production ofrecombinant proteins employs recombinant baculovirus infection for transfer ofheterologous genes. Although insect cell lines require different nutritionalfactors, lower incubation temperatures, and higher osmolarity than theirmammalian counterparts, the same essential elements of cryopreservation apply toboth groups.

昆虫细胞系已证明其具有生产各种重组多肽的能力。最常见的细胞系衍生自斜纹夜蛾（Spodoterafrugiperda）和毛滴虫（Trichoplusia ni），它们建立的细胞系分别称为Sf9和Tn5（或High Five）。重组蛋白的生产采用重组杆状病毒感染来转导外源基因。尽管与哺乳动物细胞相比，昆虫细胞系需要不同的营养因素子、更低的孵育温度和更高的渗透压，但两种细胞系都具有相同的冻存的要点。

In addition to *the guidance given in the Key Elements ofCryopreservation Practice*section, these additional points should beconsidered for animal cell line substrates:

除了在*低温保存实践部分的关键要素*中给出的指导，对于动物细胞系底物，还应考虑以下几点：

PREFREEZE PROCESSING

预冷过程

Analysts should ensure that cells arenot contaminated and are still viable. For diploid cells, analysts should growto a passage level to maintain diploidy and below the intended level for use.Analysts pool cell cultures and perform a cell count to determine the number ofviable cells available for banking. Harvested cells are centrifuged at arelatively low speed for a short duration, e.g., 100–200 × g for 5–10 min,preferably using a refrigerated centrifuge.

分析人员应该确保细胞没有被污染且任然可以存活。对于二倍体细胞，应保证生长到可传代水平保持二倍体，并低于预期使用量。实验人员收集细胞并进行计数，以确定可用于存储的活细胞数量。收集的细胞以相对较低的速度进行离心，且离心时间较短，如100-200×g离心5-10分钟，最好使用冷冻离心机。

CPAS AND CRYOCONTAINERS

CPAS和冻存盒

Themembrane-permeable CPA of choice is 5%–10% (v/v) DMSO diluted in fresh growthmedium. For certain sensitive cell lines, cell culture-conditioned medium canbe added to supplement the cryopreservation medium. DMSO must be sterile andtissue-culture grade (>99% purity). The most appropriate cryocontainer is a presterilizedpolypropylene screw-cap vial designed for cryogenic storage in vapor phaseliquid nitrogen.

在新鲜生长培养基中加入5％–10％膜透过性CPA DMSO。对于某些敏感的细胞系，可以添加条件培养基作为冷冻保存培养基的补充。 DMSO必须是无菌的，并且是组织培养级的（纯度> 99％）。最合适的冷冻容器是设计用于在气相液氮中低温储存的灭菌的聚丙烯螺旋盖瓶。

INTRODUCTION OF CRYOPRESERVATION MEDIUM

低温培养基的采用

Immediatelyfollowing centrifugation of cells, growth medium is removed from cell pellets,and cells are gently resuspended by slow addition of cryopreservation medium thatis often precooled for many cell types. The cell suspension is immediatelydiluted with an appropriate volume of cryopreservation medium based on viablecell count and targeted cell density (typically, cell banks are produced at aviable cell density of approximately 1 × 107 cells/mL). As mentionedin the previous section, cryopreservation medium is highly hypertonic, andexposure time should be limited. The

细胞离心后，立即从细胞沉淀中除去培养基，并通过缓慢添加预冷的冻存培养基轻轻重悬细胞。根据活细胞数量和目标细胞浓度（通常，细胞库活细胞密度大约1×107个细胞/ mL），立即用适当体积的冷冻保存介质稀释细胞悬液。如上一节所述，冷冻储藏介质是高渗的，应限制暴露时间。将最终的细胞悬浮液转移到一个容器中，在小瓶灌装期间可以在其中轻轻混合细胞，促进细胞库的均匀性。

COOLING AND CONTROLLED FREEZING AND STORAGE

冷冻、控制结冰和存储

WARMING AND VIAL THAWING

融化和解冻

Ingeneral, cells frozen at a slow cooling rate should be thawed as quickly aspossible to maximize cell viability. In order to ensure uniformity oftemperature, vials should be transferred directly from liquid-nitrogen vaporphase storage into a portable vapor phase (dry) shipper for transport to thelaboratory. If a liquid-nitrogen Dewar is not available, then vials cansometimes be packed in dry ice, but this process should be demonstrated assuitable because it can result in detrimental pH changes. After transfer to thelaboratory, vials are placed directly into a warm water bath (e.g., 37°, makingsure caps are not immersed), a bead bath, or a thermoblock. Note that insectcell lines should be thawed at 27°–30°. Vials should be agitated to facilitate uniformthawing of cells. Immediately after thawing, vials should be transferred intothe biological safety cabinet and sanitized before opening. Typically, growthmedium is slowly added to thawed cells while agitating to dilute DMSO and toslowly reduce the osmolarity of the post-thaw milieu back to a physiologicallevel.

通常，细胞必须慢冻快融，以最大限度提高细胞存活率。为了确保温度均匀，应将小瓶从液氮存储装置直接转移到便携式气相（干式）装置中，以运输到实验室。如果没有液氮杜瓦瓶，则有时可以将小瓶装在干冰中，但应证明此过程是合适的，因为它可能导致有害的pH值变化。转移到实验室后，将小瓶直接放入温水浴（例如37°，确保未浸入瓶盖）、珠浴或热块中。请注意，昆虫细胞系应在27°–30°解冻。小瓶应转动以促进细胞的均匀解冻。解冻后，在打开之前，小瓶应转移到生物安全柜中并进行消毒。通常，将培养基缓慢添加到解冻的细胞中，同时吹打以稀释DMSO，并将解冻后环境的渗透压缓慢降低至生理水平。

POST-THAW PROCESSING

解冻处理

Differentmethods can accomplish this step, but analysts should achieve suffcientdilution of the DMSO and return the cells to their normal isotonic growthenvironment. Manipulation of cells immediately post-thaw should be minimizedbecause of the stresses induced by the freeze–thaw process. For example,pipetting and centrifugation should be minimized.

不同的方法可以完成此步骤，但分析人员应充分稀释DMSO，并将细胞恢复到正常的等渗生长环境。由于冻融过程引起的压力，应尽量减少对解冻后即刻细胞的操作。例如，应尽量减少移液和离心。

RecombinantE. coli strains have a proven track record for production of a number ofcommercially viable biotechnology-derived products, including recombinantinsulin analogs, human growth hormone, and parathyroid hormone. Compared totheir mammalian and insect counterparts, recombinant E. coli strains arerelatively simple to grow and scale up to large volumes, e.g., 40,000 L.However, bacteria lack the sophisticated cellular machinery for building morecomplex protein molecules that require post-translational modificationssuch as glycosylation. Yeasts are unicellular, eukaryotic cells that can bemanipulated genetically to produce a wide range of recombinant proteins andpeptides with limited complexity. Despite the evolutionary distances betweenthe mammalian and microbial cell substrates, the same set of essentialcryopreservation elements described above apply, with the following uniquepoints:

重组大肠杆菌菌株在商业上生产许多生物技术衍生制品（包括重组胰岛素类似物、人类生长激素和甲状旁腺激素）方面有着非常好的表现。与哺乳动物和昆虫相比，重组大肠杆菌菌株相对易于生长并扩大规模，例如40,000L。但是，细菌缺乏复杂的细胞机制来构建更复杂的蛋白质分子，这些复杂蛋白分子需要翻译后修饰，例如糖基化。酵母是单细胞的真核细胞，可以进行遗传操作以产生相对复杂的各种重组蛋白和肽。尽管哺乳动物和微生物细胞之间存在进化差异，上述冻存方案仍然适用，并具有以下独特之处：

PREFREEZE PROCESSING

预冷冻过程

Culturesshould be propagated in shake flasks to late logarithmic or early stationaryphase using strain-specific growth conditions.

菌株应在摇瓶利用特定的生长条件繁殖至对数生长晚期或平台期。

CPAS AND CRYOCONTAINERS

CPAS和冻存盒

The cellwall or cell membrane-permeable CPA of choice is glycerol (although DMSO isalso sometimes used) at concentrations typically ranging from 5%–10%(v/v).Synthetic glycerol should be used for registration of commercial products alongwith other raw materials that are free of animal components.

选择可渗透细胞壁或细胞膜的CPA是甘油(尽管有时也使用DMSO)，其浓度通常在5%-10%之间。合成甘油应该和其他不含动物成分的原料一起用于商业产品的注册。

INTRODUCTION OF CRYOPRESERVATION MEDIUM

低温培养基的采用

Becausemicrobial cells possess cell walls for protection and support of cytoplasmiccontents, physical manipulation and osmotic shifts do not have the samenegative impact seen in animal cell lines. Immediately after centrifugation,cell pellets are vigorously resuspended in the cryopreservation medium (to adilution based onrequirements of the cell bank for number of vials and viable colony-formingunits/mL). This dilution step can be based on active measurement of cultureoptical density or other cell enumeration assays. The final cell suspension istransferred to a vessel in which the cells can be mixed during filling of finalcontainers to facilitate uniformity of the cell bank. Because of potentialtoxicity, analysts should limit the time of exposure to glycerol, despite therelative robustness of microbial cells. Vials can be filled manually byusing a hand-held pipetting device or by using an automated vial-fillingmachine.

因为微生物细胞拥有保护和支持细胞质内容物的细胞壁，所以物理操作和渗透压变化不会产生在动物细胞系中看到的损伤情况。离心后立即将细胞微球大力重悬在低温保存培养基中(稀释至细胞库对瓶数和活菌落形成单位/mL的要求)。这个稀释步骤可以基于培养物光密度的测量或其他细胞计数方法分析。最后的细胞悬浮液被转移到冻存管中，在加入最后的冻存管期间，细胞可以在其中混合，以促进细胞库的均匀性。由于甘油对微生物细胞潜在的毒性，分析人员应该限制接触时间，尽管微生物细胞耐受能力更强一些。小瓶可以手动填充通过使用手持移液器或使用自动瓶子灌装机。

COOLING AND CONTROLLED FREEZING AND STORAGE

冷冻、控制结冰和存储

Again,because of their inherent robustness, microbial cells do not require as strictcontrol of cooling rate as do animal cell lines. Consequently, the choice offreezing system or method has a smaller effect on the viability of the cellbank. Filled cryocontainers simply can be transferred into an approximately −80° freezer overnight followed by transferto vapor phase liquid nitrogen. Alternatively, microbial cell banks may not useliquid nitrogen storage but can be stored at

approximately−80°. However, if liquid-nitrogen storageis available, it is preferred for long-term storage (e.g., years). If acontrolled-rate freezer is employed, then it should be fully qualitied todeliver a uniform cooling rate that is within an expected range, e.g.,1°–5°/min.

同样，由于微生物本身的坚固性，它不需要像动物细胞系那样严格地控制冷却速率。因此，冷冻系统或方法的选择对细胞库的生存力影响较小。可简单地将冷冻容器转移到大约-80°的冰箱中过夜，然后转移到气相液氮中。或者，微生物细胞库不使用液氮存储，也可以存储在约-80°冰箱中。但是，如果可以使用液氮对于微生物长期储存（例如，几年）是比较好的选择。如果使用控制速率的冷冻器，那么它应该完全有能力在预期范围内提供统一的冷却速率，例如，1°-5°/min。

WARMING AND VIAL THAWING

融化和解冻

Controllingthe rates of cooling and warming is less critical for microbial cells, but thecells still should be thawed as quickly as possible using a warm water bath(e.g., 30°–35°). Temperatures can be adjusted to the incubation temperature ofthe strain.

对于微生物细胞而言，控制冷却和升温的速度并不那么重要，但是仍然应使用温水浴（例如30°–35°）尽快融化细胞。可以将温度调节至菌株的孵育温度。

POST-THAW PROCESSING

解冻处理

Growthmedium is added to dilute the thawed cells to a desired level of colony-formingunits/mL and to induce removal of intracellular glycerol. All cells are notcreated equal and are divergent by nature. A broad span of evolutionary timeseparates the various cell substrates used for manufacture of human B&Bproducts. Fortunately for the practitioner of industrial cell culture,cryopreservation strategies converge to a set of shared principles and methodsthat translate across evolutionary paths.

添加培养基将复苏的细胞稀释至所需水平的菌落形成单位/ mL，并诱导去除细胞内甘油。所有的细胞都不是生来平等的，它们本质上是不同的。大的进化时间跨度将用于制造人类B＆B产品的各种细胞基质分离开来。对于工业细胞培养的研究者来说，幸运的是，低温保存策略已经形成一套共享的原则和方法，这些原则和方法在进化过程中得到了应用。