Advanced Therapy Treatment Centres

UK review and recommendations on cryopreservation of starting materials for ATMPs

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UK REVIEW AND RECOMMENDATIONS ON CRYOPRESERVATION OF STARTING MATERIALS FOR ATMPS

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1. Introduction

Advanced Therapeutic Medicinal Products (ATMPs) are innovative therapies for human use, based on genes, tissues, or cells (as described in **Table 1**). They offer ground-breaking opportunities in the therapy of various diseases, including some that are currently difficult to treat or have no established therapeutic intervention.

While some ATMP approaches involve directly isolating immune cells and expanding their numbers, others involve genetically engineering immune cells to enhance their therapeutic properties (**Table 1**). When ATMP approaches use the patient's own cells as starting material, the transplant is termed autologous whereas if a donor's cells are used to manufacture therapies for one/many patient(s), the transplant is known as allogeneic. Two of the leading approaches in the ATMP landscape are Chimeric Antigen Receptor T-cell (CAR-T) and Tumour-Infiltrating Lymphocyte (TIL) therapies. CAR-T therapies have yielded promising results in treating blood diseases and TIL therapies are being explored for the treatment of solid tumours. The success of CAR-T therapies is reflected in the recent regulatory approval of two ATMPs in Europe; namely Yescarta® (Axicabtagene ciloleucel) and Kymriah® (Tisagenlecleucel). Additionally, Tecartus™ (Brexucabtagene autoleucel) and Abecma® (*idecabtagene vicleucel*) received conditional marketing approval in Europe as of December 2020 and March 2021, respectively.

Indeed, each decade brings an increasing number of ATMP clinical trials, suggesting a high rate of innovation[1]. With the potential to transform clinical care in an increasingly diverse range of diseases, the global market size of ATMPs was valued at USD 7.9 billion in 2020 and continues to grow[2].

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Table 1. Sub-classifications of ATMPs

TYPE OF ATMP	REGULATORY DESCRIPTION	REGULATION REFERENCE	EXAMPLE ATMP
Gene Therapy Medicinal Product	Includes an active substance that contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding, or deleting a genetic sequence. Therapeutic, prophylactic, or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence.	Annex I, Part IV, 2.1 of Directive 2001/83/EC[3].	Luxturna® is used to treat adults and children with loss of vision due to inherited retinal dystrophy, a rare genetic disorder of the retina. An Adeno-Associated Virus carrying normal copies of the RPE65 gene is injected into the eye to enable retinal cells to produce the missing enzyme.
Somatic Cell Therapy Medicinal Product	Contains or consists of cells or tissues that have been subject to substantial manipulation so that biological characteristics, physiological functions, or structural properties relevant for the intended clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor. Presented as having properties for, or used in or administered to human beings with a view to treating, preventing, or diagnosing a disease through the pharmacological, immunological, or metabolic action of its cells or tissues.	Annex I, Part IV, 2.2 of Directive 2001/83/EC[3].	Alofisel® is a medicine comprising mesenchymal stem cells removed from fat tissue of adult donors that is used to treat complex anal fistulas in adults with Crohn's disease (an inflammatory condition of the gut) by helping to reduce inflammation and support the growth of new tissue.
Tissue-Engineering Medicinal Product	Contains or consists of engineered cells or tissues. Presented as having properties for or used in or administered to human beings with a view to regenerating, repairing, or replacing a human tissue.	Article 2.1.b in Regulation (EC) No. 1394/2007[4].	Holoclar® is a stem cell therapy used to treat people with severe Limbal Stem Cell Deficiency due to corneal burns. Autologous human corneal epithelial cells, including stem cells, are expanded *ex vivo* and subsequently implanted back into the patient's eye.

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Combined Advanced Therapy Medicinal Product	Must incorporate, as an integral part of the product, one or more medical devices or one or more active implantable medical devices *and* cellular or tissue part must contain viable cells or tissues, *or* cellular or tissue part containing non-viable cells or tissues must be liable to act upon the human body with action that can be considered as primary to that of the devices referred to.	Article 2.1.d in Regulation (EC) No. 1394/2007[4].	MACI® (authorised for use in the EU in June 2013 but later suspended and withdrawn) uses a patient's cells to repair knee cartilage damage. Patient chondrocytes are expanded and seeded onto a resorbable collagen membrane. This is shaped to fit the cartilage defect and implanted.

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Despite this fast-paced innovation, there is a marked discrepancy between the number of ATMPs in clinical trial and those approved[5]. For instance, while there are more than 400 clinical trials in CAR-T therapies alone, a total of just 14 ATMPs have been given European regulatory approval; four of which have since been withdrawn. Additionally, there is a noticeable decline in market authorisation rate, with just two ATMPs being approved in 2019 and not even one in 2020 or 2021 (to September 2021).

Underscoring this delayed market access is the complexity of the ATMP manufacturing process and the challenges that come with upscaling. Cryopreservation – the preservation of biological samples at very low temperatures – forms an integral part of many ATMP manufacturing workflows[5]. However, establishing a strong cryopreservation protocol remains a challenge to market access.

Cryopreservation provides irrefutable benefit to the ATMP manufacturing workflow. From both a manufacturing and therapeutic perspective, extending the shelf life of ATMP starting materials and final products provides an unequalled degree of flexibility. For instance, multiple treatments from the same batch of starting material can be manufactured at different times and the timing of treatment can be easily adjusted[6]. Also, cryopreservation provides the chance to comprehensively test the starting material prior to manufacturing, resulting in enhanced patient safety.

While cryopreservation has already been integrated into the manufacturing workflow of approved ATMPs[5], a comprehensive, end-to-end solution for large scale ATMP production is still under development. As such, this document provides an overview of the current state of cryopreservation in ATMP workflows by addressing:

(1) Where cryopreservation fits into an ATMP workflow, the current rules and regulations, and the major challenges associated with cryopreservation logistics.

(2) The current scientific knowledge on whether cryopreservation of ATMP starting materials effects the efficacy and safety of the final product.

(3) How developing technologies are set to help optimize cryopreservation processes that are feasible for scalable manufacturing.

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2. Cryopreservation in an ATMP workflow

2.1 The cryogenic cold-chain within an ATMP workflow

The process flow for delivering traditional pharmaceuticals and therapeutics uses a centralised manufacturing approach, based upon producing large quantities of identical doses that are distributed through well-established pharmacies or other healthcare establishments[7]. However, since autologous ATMPs represent patient-specific products with a batch-size of one, they do not suit this archetypal supply chain model.

The inherent complexity and individuality of autologous ATMPs creates numerous challenges in standardisation and logistics. Yet, a general supply chain and manufacture model does exist, highlighting the major common steps across different types of ATMPs (**Figure 1**).

Briefly, the manufacturing and supply chain process flow begins once a patient has been referred, informed, consented, and assessed for the suitability of an ATMP treatment (and the suitability of the donor has also been addressed, where applicable). Initially, cells or tissues are taken from the patient or donor at a clinical facility. These starting materials need to be distributed to a manufacturing location that is often geographically remote from the clinical facility. Following ATMP manufacture, the materials must be transferred back to a clinical setting and administered to the patient.

Cryopreservation is used in the ATMP process flow to extend the shelf-life of biological materials and may be implemented twice in the process chain. The starting material, typically apheresis or tumour biopsy, can be cryopreserved after collection by an organisation licensed by the relevant competent authority for the processing and storage of cell and tissue products or specialist pathology laboratory that may or may not be located within the clinical facility. The cryopreserved starting material is then transported to a manufacturing site for transformation into a medicinal product[8]. In turn, the final product can be cryopreserved for storage and delivery to the clinical facility for administration. When procedures involved in cryopreservation – including cryopreservation itself, shipping, storage, and thawing steps – are linked, they form the cryogenic cold chain (**Figure 1**).

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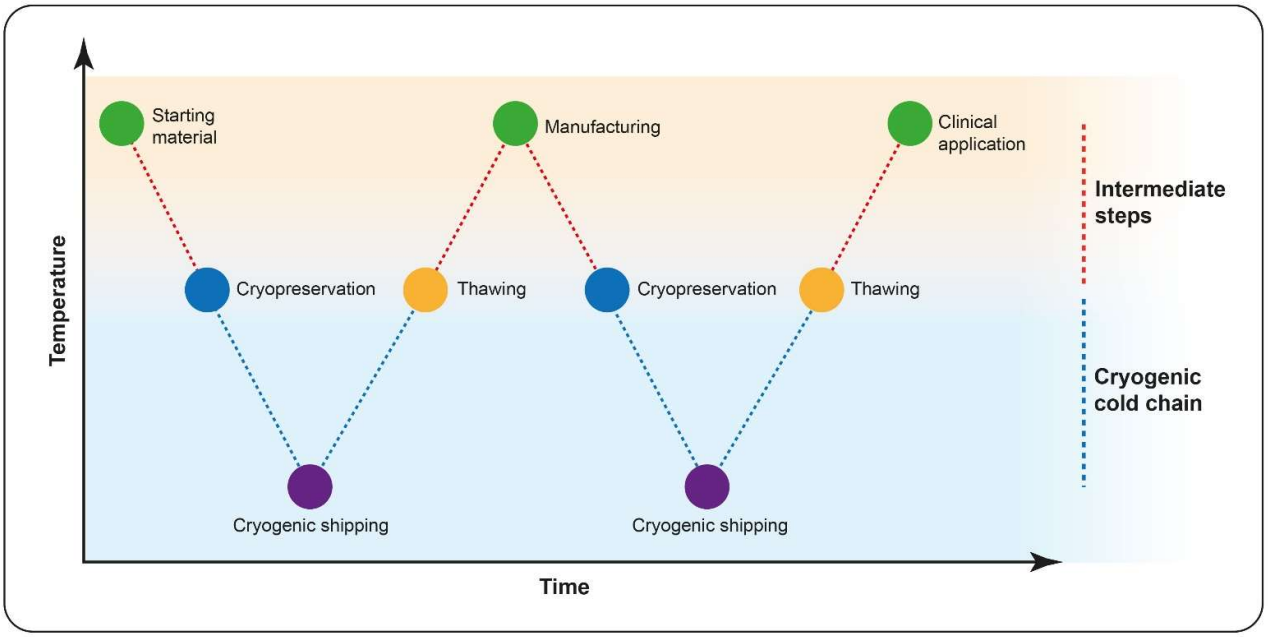

Figure 1. The major steps involved in the cryogenic cold chain. The dots represent crucial steps within an ATMP manufacturing workflow, represented on a schematic temperature versus time graph. The cryogenic cold chain steps are located lower on the y-axis (blue background) while the intermediate steps are higher on the y-axis (orange background).

2.2 Regulations and guidance for the collection and cryopreservation of starting materials

In the European Union and United States, ATMPs fall under the regulatory framework of biological medicinal products. However, compared to more traditional biological products, ATMP products are more complex and require more intensive follow up of efficacy, adverse events, and traceability requirements for the market release[9].

Regarding the collection of starting materials, both the EU and US legal framework provide comprehensive guidance and directives for ATMPs. For example, standards of "quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells" for ATMP manufacture in the European Union are regulated under the Tissues and Cells Directive (2004/23/EC) as transposed into Member State law[10]. In the UK, post-Brexit, this is now regulated by Statutory Instrument 2007 No. 1523: The Human Tissue (Quality and Safety for Human Application) Regulations[11]. In the US, this information can be found under the Code of Federal Regulations (21 CFR 1271) and the Public Health Service Act (Sections 351 and 361). Furthermore, procurement of human cells for the manufacture of ATMPs requires a

license in the UK governed by the Human Tissue Authority (HTA) or the Medicines and Healthcare Products Regulatory Agency (MHRA, under Blood Establishment Authorisation)[12]. The Pharmaceutical Inspection Co-operation Scheme (PIC/S) GMP Guide also outlines requirements for manufacture of ATMPs for human use, from control over seed lots and cell banks through to finishing activities and testing[13].

Compliance with Good Manufacturing Practice (GMP) is mandatory for all medicinal products that have been granted a marketing authorisation; a set of guidelines specific to ATMPs has been published by the European Commission to ensure their consistent and controlled production according to high quality standards, for the benefit and the safety of patients[14].

In addition, standards set out by professional accreditation bodies, such as the Foundation for the Accreditation of Cellular Therapy (FACT), provide guidance with reference to specific starting materials. For example, those wishing to use immune effector cells, including CAR-T cells, should refer to the FACT-JACIE (Joint Accreditation Committee of ISCT - International Society for Cell & Gene Therapy - & EBMT - European Society for Blood and Marrow Transplantation) Hematopoietic Cell Therapy Standards (HCT)[15]. However, while standardised pathways exist for the collection of blood cells, the process flow for solid tissues is less well defined and, in turn, needs to be an area of focus[16].

Where cryopreservation is involved, Good Tissue Practice (GTPs) considerations pertaining to sterility, reproducibility, efficacy, traceability, and safety are described in **Table 2**. These include selection of correct equipment for controlled rate cooling and storage, meticulous design of cryopreservation protocols (cooling cycle, time in formulation pre-freeze and post-thaw, storage conditions), careful choice of containers (type and volume), mindful selection of cryoprotective media (cryoprotectant(s) and any additive(s) used, at which concentration), and documenting each step of the process being performed by specifically trained personnel[8].

Cryopreservation-specific guidance related to best practices have been developed by renowned institutions including the International Society for Biological and Environmental Repositories (ISBER)[17] and the US Pharmacopeia (USP)[18]. These documents provide general information about developing cryopreservation protocols for biological materials. Moreover, the Parenteral Drug Association has recently published a comprehensive standard and guidance dedicated to “Cryopreservation of cells used in cell and gene therapies and regenerative medicine manufacturing” that consolidates information on ATMP cryopreservation[19].

Table 2. Factors to consider in the cryopreservation of starting materials for ATMPs

CONSIDERATION	DESCRIPTION
Cryopreservation Equipment	Cooling should be performed using a controlled-rate freezer. Liquid Nitrogen (LN_2)-dependent or LN_2-free equipment can be selected depending on several factors (see Part 4.2.2). In either case, the cryopreservation instrument should be equipped with environment monitoring systems and alarms, and contingency plans must be in place.
Cryopreservation Protocol	Cooling should be performed using an optimised temperature cycle. Additionally, cryogenic storage should be performed under specific conditions and with appropriate controls, for example, within a certain temperature range and with a process in place to limit temperature excursions.
Sample Containers	Sample containers need to be hermetically sealed, closed systems to reduce contamination risks as well as effective cryopreservation vessels. Typically, the starting material is collected in apheresis bags or collection bags and transported to the cell lab or manufacturing unit for further processing. After the addition of cryoprotectant, the starting material is transferred to cryobags for storage. Transfers are performed under aseptic conditions or using sterile connecting devices. Different cryobags capacities are available and the choice depends on the volume of the cryopreserved sample.
Formulation	Cryoprotectant(s) and any additive component(s) used in the formulation, as well as their concentration range must be tested and optimised. The time they are in contact with live cells should also be considered and minimised, both pre-freeze and post-thaw, since cryoprotectants may exert some level of cytotoxicity. Cell density and the final volume of sample are also crucial. Their optimisation for each sample/cell type leads to a variety of cell densities and final volume specifications for each product considered.
Quality Control (QC)	QC cryopreservation samples are small fractions of the bulk sample (small cryovials or tubing sections of cryobags). They are prepared and cryopreserved alongside the bulk sample and are necessary to evaluate whether the bulk sample remains within acceptable range following any damage done by cryopreservation.
Cryodata Integrity	Cryopreservation processes (freezing, storage, transport, and thawing) and associated metadata (e.g., temperature logs, sample identification) will need to be tracked and recorded to ensure Chain Of Custody (COC) and Chain Of Identity (COI). This requires a secure yet accessible data integrity system to ensure patient/donor identity and data collection and storage is accurate, reliable and private.
Process Validation	Process validation is the documented evidence that the manufacturing process can consistently produce a result within specific parameters. This applies to the cryogenic cold chain to ensure consistency and efficacy of the cryopreserved sample. Additionally, stability studies need to be carried out over the product's shelf life under appropriate storage conditions. Re-validation studies will also be necessary should any element critical to the process change outside of the originally defined ranges.

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As well as following Good Manufacturing Practice (GMP), it is essential that ATMP manufacturers transport all materials – from starting materials to the final product – according to Good Distribution Practice (GDP)[20]. Maintaining GDP compliance is essential for identifying and mitigating any risks involved in cryogenic transport. This includes ensuring that cryopreserved samples are appropriately handled, paperwork is maintained, and the Chain Of Custody (COC) and Chain Of Identity (COI) are upheld.

2.3 Guidance and challenges around cryogenic storage and logistics in the ATMP workflow

Maintaining cryogenic temperatures throughout the cold chain is an essential yet potentially challenging element of ATMP cryopreservation, both in terms of storage and transportation (**Figure 1**). Therefore, cryogenic storage tanks and shipping containers need to be regularly and rigorously tested and continuously monitored to minimise failures. Additionally, their quality must be monitored to identify any failures rapidly allowing intervention and rescue of the samples before critical temperature increases occur[21].

Even with appropriately tested and monitored systems in place, unexpected events may still happen. For instance, transportation delays due to weather events or problems at customs can lead to loss or damage to a sample if a contingency plan is not in place to maintain the sample at cryogenic temperature. Additionally, the precise hold time of LN_2 is influenced by a variety of factors, such as variable LN_2 evaporation rate between dry shippers or tilting and cannot be guaranteed.

The infrastructure and training that is needed to work with cryopreserved materials is typically extensive and requires the implementation of paramount Health and Safety (H&S) policies. Dedicated spaces and equipment, including large bulk tank feeders and storage tanks of liquid nitrogen (LN_2), specialised storage facilities, and shipping containers are all mandatory pre-requisites to implementing an effective cryogenic cold chain. Storage facilities need oxygen monitoring linked to a system that automatically shuts off nitrogen supply when oxygen depletion is detected. Additionally, good Heating, Ventilation and Air Conditioning (HVAC) technologies to control environmental conditions are critical to storage facility design. Furthermore, comprehensive personnel training in the handling and transportation of cryopreserved materials and LN_2 will need to be undertaken. The use of Personal Protection Equipment (PPE) and personal oxygen monitors is a particularly important training aspect since LN_2 has the potential to have severe, even fatal, consequences[21–23].

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3. Is cryopreservation a valid method for ATMP starting materials?

3.1 Process of cryopreservation of cells

The primary purpose of cryopreservation in the ATMP workflow is to maintain cell viability over extended periods of time and introduce greater flexibility in an otherwise very rigid workflow. Briefly, cells or tissues are reduced to temperatures below -120°C to induce a state of suspended cellular metabolism[24]. These extremely low temperatures stop all residual molecular mobility, preventing metabolic decline and disruption, and ultimately result in the preservation of cell viability post thaw.

There are two main methods employed in cryopreservation of cells and tissues; namely freezing (i.e., where the liquid phase changes to a solid crystalline phase) and vitrification (i.e., where liquid solidifies to a solid amorphous phase, without crystalline ice formation)[25].

Typically, cryopreservation in the ATMP workflow (starting material and final product) is carried out by controlled-rate, programmable freezing since:

- Slow cooling rates appear to be optimal at preserving cell viability and functionalities post-thaw, typically -1˚C/min for a wide range of somatic cells in suspension, and slower rates for more complex, multi-cellular structures[6,8,26].
- Each step can be recorded to demonstrate compliance with Standard Operating Procedures (SOPs) and current GMP regulatory requirements[25].

In slow freezing, the sample is mixed with a cryoprotective medium and a controlled cooling protocol applied. In line with decreasing temperatures, the cells progressively dehydrate once ice nucleates and as this ice fraction grows in the extracellular medium (~ -10˚C to -30˚C)[27,28]. Cell dehydration occurs because ice formation locks away pure water as ice, and the remaining non-frozen medium becomes increasingly concentrated with solutes, translating to an increase in osmotic pressure. The resulting difference in osmotic pressure across the cell membrane forces intracellular water out[27,28]. Some degree of cell dehydration during cooling serves to avoid ice crystals from forming *inside* the cells; a phenomenon termed 'Intracellular Ice Formation' (IIF). IIF causes extensive cellular damage and leads to cells not surviving post thaw. To mitigate IIF, further controlled cooling of dehydrated cells will eventually result in their vitrification – a phenomenon known as intracellular glass transition[24].

In vitrification, samples are cooled at extremely rapid rates (>1000˚C/min) by exposure of small samples to very low temperatures such that the glass transition state is reached without ice crystal formation. While there is lower risk of damaging ice crystal formation, vitrification is still challenging to achieve in practice, typically requiring toxically high concentrations of

cryoprotectants, and is limited to very small sample volumes/sizes[25]. Consequently, vitrification is a technique not yet used to cryopreserve starting material or finished products in the ATMP workflow, and will not be considered further in this document.

3.2 Starting material cryopreservation for CAR-T cell therapy

CAR-T therapy involves genetic modification of a patient's autologous T-cells to express a Chimeric Antigen Receptor (CAR) that recognises and targets a specific tumour antigen. CAR-T cell therapy is a remarkably promising treatment for cancer patients and has been successfully used to treat a number of blood cancers. In 2017, two CAR-T cell therapies received European regulatory approval and have since been used to treat nearly half a million patients[29].

The starting material for approved autologous CAR-T cell therapies is T-cells isolated from the peripheral blood of the patient. As interest for allogeneic CAR-T cell therapy grows, so does the search for more consistent, renewable, and readily available sources of starting material. Consequently, T cells differentiated from either Haematopoietic Stem and/or Progenitor Cells (HSPCs) – isolated from bone marrow, mobilized peripheral blood or cord blood – or induced Pluripotent Stem Cells (iPSCs) are now being investigated in research and clinical trials[30,31]. Both T-cells and HSPCs can be isolated from the blood by apheresis, although in the latter case, a drug (Granulocyte –colony-stimulating factor) is needed that causes the bone marrow to generate and release stem cells into the bloodstream (mobilisation)[32].

To enable off-the-shelf allogeneic CAR-T therapies in the near future, cryopreservation is a fundamental requirement. Cryopreserved products are potentially safer due to an extended time window for required testing and batch review to ensure compliance to specifications. Cryopreservation also provides an opportunity to solve a variety of logistical challenges related to ATMP starting material. For example, cryopreserved samples can be transported over greater physical distances, such as between collection and manufacturing centres, without the deterioration concerns associated with fresh samples. Additionally, cryopreservation allows for more flexibility in international transportation and makes it easier to accommodate unpredictable donor availabilities and/or clashing collection and manufacturing schedules[33].

3.2.1 Cryopreservation of T lymphocytes

Both of the European Medicines Agency (EMA)-approved CAR-T cell therapies (Yescarta® and Kymriah® rely on the collection and expansion of autologous T lymphocytes, following leukapheresis. In the Yescarta® manufacturing workflow, fresh starting material is normally shipped cold (non-cryopreserved) to the manufacturer. Conversely, the starting material for Kymriah® is cryopreserved within 24 hours and stored and shipped to the manufacturing facility

at temperatures below at least -120°C. At the appropriate time, the leukapheresis material is thawed under controlled conditions and then washed to remove cryomedium[34,35]. Cryopreservation has been a critical aspect of disseminating Kymriah® since apheresis starting material needs to be shipped internationally to a manufacturing facility and subsequent therapy transported back[36].

Cryopreservation is now an accepted and used method in the manufacturing process of CAR-T therapies. However, successful treatment relies on the initial delivery of viable Total Nucleated Cells (TNC) and CD3+ T cells, which in turn can be directly dependent on an effective cryopreservation strategy. Various cryopreservation methods and cryoprotectant solutions have been tried and tested to maximise the number of viable T lymphocytes post-thaw (**Table 3**).

Table 3. Studies investigating the effect of cryopreservation and cryopreservation media on T lymphocytes and Peripheral Blood Mononuclear Cells (PBMCs)

REFERENCE	AIM	STARTING MATERIAL	MAIN FINDINGS / CONCLUSIONS
Tyagarajan *et al.* (2019)[37]	Compare fresh and frozen leukapheresis material on production of tisagenlecleucel (Kymriah®).	Leukapheresis material	Comparable cell growth kinetics and product characteristics (composition, proportion of transduced cells, CAR expression levels and functional response [interferon-γ production in response to CD19-expressing target cells]) between fresh and frozen starting material.
Sadeghi *et al*. (2012)[38]	Evaluate the impact of cryopreservation on the rapid expansion of T-lymphocytes post-thaw.	Lymphocytes isolated from PBMCs	Cryopreservation of lymphocytes during the rapid expansion protocol did not affect cell viability.
Pi *et al*. (2020)[39]	Evaluate the freezing response of PBMCs (including T-cells) to cryoprotectants other than DMSO*.	PBMCs	DMSO-free cryoprotectants showed higher preservation capability for primary T-cells and helper T-cells, and high post-thaw recovery of many populations of PBMCs, but developing tailored cryoprotectants is necessary to optimise recovery of all the subsets of interest.
Panch *et al*. (2019)[40]	Explore the effect of cryopreservation of CAR-T starting materials on manufacturing and patient outcomes.	PBMCs	Observed a decrease in cell viability within 2 days of thawing the cryopreserved PBMC but did not observe a difference in cell expansion, transduction efficiency, percentage of CD3 cells, or CD4:CD8 ratios. Evaluation of thawed cryopreserved CAR-T products (n=79) where 50 had been manufactured using cryopreserved PBMCs showed no significant difference in the percentage of T cells, transduction efficiency, or CD4:CD8 ratios compared to fresh products. *In vivo* persistence and clinical outcomes were also similar.
Worsham *et al.* (2017)[41]	Study the effect of four different cryopreservation solutions compared to fresh, refrigerated samples on T-cells.	PBMCs	Using higher concentrations (10%) DMSO reduces the post-thaw viability of different CD4+ T-cell populations. Using lower concentrations of DMSO (5%) maintains T-cell function at levels similar to refrigerated, control samples.

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Weinberg *et al*. (2009)[42]	Explore the effects of cryopreservation on PBMCs.	PBMCs	No impact of extended storage in liquid nitrogen for ≤15 months on T-cell viability or recovery, or on results of lymphocyte proliferation and flow cytometric assays.

*DMSO: Dimethyl Sulfoxide

In one study, data from six single-centre CAR-T clinical trials were retrospectively analysed and manufacturing runs from 3 healthy donors were used to obtain novel data on fresh versus cryopreserved products[40]. As well as considering the effect of cryopreservation on starting material, they also addressed the consequences of cryopreservation of final CAR-T products.

Approximately half of the starting material samples were cryopreserved, and various parameters assessed two days after thawing. The total viable cell percentage was significantly lower in the cryopreserved fraction; however, the viable cell yield was sufficient to proceed with manufacture. Moreover, the cryopreserved samples did not show any significant difference in critical quality attributes (e.g., fold-expansion, transduction efficiency, percentage of T-cells) relative to the fresh samples. Subsequently, *in vivo* persistence and clinical outcomes in patients receiving fresh or cryopreserved/thawed (including products made from cryopreserved starting materials) CAR-T cells were similar[40]. Comparable outcomes have been observed in numerous other studies (**Table 3**).

Despite some differences in outcomes across studies, the approval of Kymriah® by regulatory agencies in the US, Europe, and Australia indicate that cryopreserved starting materials and CAR-T products are efficacious.

3.2.2 Cryopreservation of Haematopoietic Stem and Progenitor Cells

HSPCs offer a sustained source of T-cells for CAR-T therapies. Although none of the current approved CAR-T therapies use HSPCs as the starting material, this route is under active research to help make off-the-shelf allogeneic CAR-T therapies possible[30,31]. Besides, HSPCs are used directly for haematopoietic stem cell transplantation (HCT) where cryopreservation is a well-established process[6,43,44].

Various studies have examined the effect of cryopreservation on factors such as Haematopoietic Stem Cell (HSC) transplantation outcome, different cellular subsets in blood, and different types of starting materials (**Table 4**). Overall, short-term patient outcomes are comparable when using fresh or cryopreserved products. However, there are some concerns regarding the viability of different cellular subsets, as well as an increased incidence of transfusion reactions and bacterial graft contamination when using cryopreservation.

In light of these concerns, the standard practice in allogeneic stem cell transplant is not to cryopreserve but to infuse peripheral blood the same day or the day after collection[45]. Albeit a slow progression, there is a general trend toward the use of cryopreserved HSPCs, which may be partially attributed to the increase in favourable scientific data and, more recently, necessitated by the COVID-19 pandemic. Indeed, to ensure the integrity and delivery of HCT grafts and SARS-CoV-2 negativity of donors, the National Marrow Donor Program (NMDP) and European Society

for Blood and Marrow Transplantation (EBMT) have recommended the widespread use of cryopreservation[46].

Table 4. Studies investigating the effect of cryopreservation of HSPCs in haematopoietic stem cell transplants

REFERENCE	AIM	STARTING MATERIALS	MAIN FINDINGS / CONCLUSIONS
Shinkoda *et al.* (2004)[47]	Compare the outcome of bone marrow transplantation in twins using cryopreserved and fresh products.	Bone marrow	The outcomes were similar when either cryopreserved or fresh bone marrow were used for transplantation.
Eapen *et al.* (2021)[46]	Examine the effect of cryopreservation of related and unrelated donor grafts for transplantation for severe aplastic anaemia.	Peripheral blood (stem cells)	1-year survival rate was lower in the cryopreserved graft group, supporting the use of non-cryopreserved grafts for patients with severe aplastic anaemia.
Alotaibi *et al.* (2020)[33]	Determine the influence of cryopreservation of stem cell grafts on allogeneic HCT outcomes.	Peripheral blood (stem cells)	No differences in engraftment and survival between fresh and cryopreserved grafts for allogeneic HCT.
Hamadani *et al.* (2020)[48]	Determine the influence of cryopreservation of stem cell grafts using Post-Transplantation Cyclophosphamide (ptCY) on allogeneic HCT outcomes for patients with hematologic malignancy.	Peripheral blood (stem cells)	For patients undergoing ptCY-based allo-HCT, cryopreservation of donor allografts appear to be safe.
Berens *et al.* (2016)[49]	Explore the differential tolerance of cellular subsets in peripheral blood stem cells and donor lymphocyte infusions (DLI) to cryopreservation and thawing.	Peripheral blood (stem cells)	Significant differences were observed for post-thaw cell recovery of different cell subsets. Data suggests a lower tolerance of CD3+ cells for cryopreservation.
Fisher *et al.* (2014)[50]	Explore the differential tolerance of cellular subsets (CD34+ and CD3+) in peripheral blood stem cells to cryopreservation and thawing. As well as the impact of Granulocyte –	Peripheral blood (stem cells)	G-CSF-mobilized unrelated donor products may contain less CD3+ cells than non-G-CSF exposed products upon thaw and, when indicated, cell doses should be monitored.

	Colony-Stimulating Factor (G-CSF)-mobilized donor products.		
Parody *et al*. (2013)[45]	Determine the impact of cryopreservation of peripheral blood stem cells on overall patient outcome undergoing allogenic transplantation.	Peripheral blood (stem cells)	Cryopreserved and fresh transplant materials achieve similar patient outcomes; however, cryopreservation may influence the pattern of acute graft-versus-host disease.
Medd *et al*. (2013)[51]	Examine the medium to long-term outcomes following the transplantation of cryopreserved allogenic peripheral blood stem cell transplant.	Peripheral blood (stem cells)	Cryopreserved and fresh, related and unrelated, allogenic peripheral blood stem cells showed similar patient outcomes. However, cryopreservation may influence the pattern of acute graft-versus-host disease.
Lioznov *et al.* (2008)[52]	Evaluate the molecular impact of cryopreservation on allogenic peripheral blood stem cells and bone marrow grafts.	Peripheral blood (stem cells) and bone marrow	The potential deleterious effects of cryopreservation of peripheral blood stem cells increase after prolonged transportation and storage. Bone marrow transplants are not affected by these parameters.
Kim *et al*. (2007)[53]	Determine the effect of using cryopreserved and fresh peripheral blood stem cells from related allogeneic donors for transplants.	Peripheral blood (stem cells)	Overall, outcomes of using either cryopreserved or fresh materials for transplant were similar.

3.3 Cryopreservation of starting material for TIL

TIL therapies hold enormous promise for treating late-stage cancer patients and have been shown to mediate durable tumour regression, especially in metastatic melanoma patients. TILs are a heterogeneous population of lymphocytes composed mostly of T cells and Natural Killer (NK) cells. These cells are a natural part of the human anti-tumour immunity. Because they penetrate the stroma of a tumour to actively destroy it, TILs are potentially present in all solid tumours but generally in low numbers. As such, TIL therapy aims to increase the numbers of these immune system cells via *ex vivo* expansion to treat solid cancer patients[36].

The production of TIL therapies typically begins with procurement of a sample of a patient's tumour. Subsequently, tumours are processed (fragmented, disaggregated, or enzymatically digested), and placed into culture. TILs are expanded in culture in the presence of high dosage Interleukin 2 (IL-2) and further tested for tumour recognition. Tumour-reactive lymphocytes are then expanded via a Rapid Expansion Protocol (REP) to reach therapeutic doses and ultimately infused back into the lymphodepleted patient (**Figure 2**).

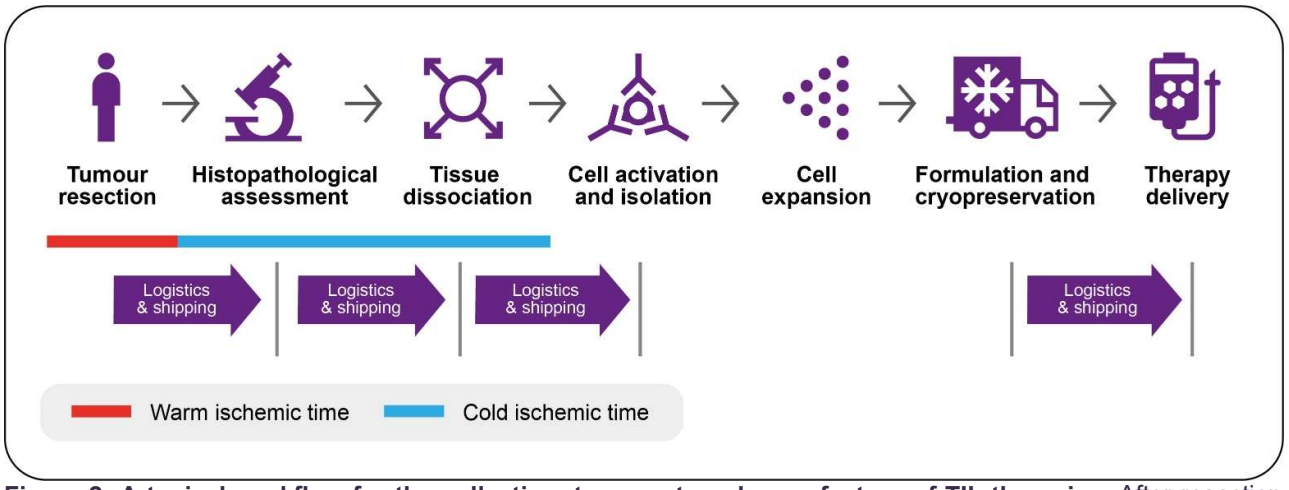

Figure 2: A typical workflow for the collection, transport, and manufacture of TIL therapies. After resection from the patient, the tumour is histopathologically assessed for diagnostic purposes. The remaining tumour sample can then be dissociated, TILs activated in culture and further expanded *in vitro*. Once the TILs therapeutic dose is reached, the therapy is formulated and cryopreserved. The therapy is then ready to be thawed and delivered to the patient, when needed. Warm (red) and cold (blue) ischemic time are highlighted for the initial steps of the workflow as well as the logistics and shipping steps.

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As with CAR T therapies, cryopreservation can occur at several steps during the manufacturing of TIL ATMPs (e.g., before the REP phase or after final product formulation)[38]. In fact, cryopreservation forms part of the manufacturing workflow of Lifileucel; a TIL infusion treatment for patients with advanced melanoma[54]. Furthermore, Iovance Biotherapeutics has more than 30 patents filed regarding TIL therapy, many of them encompassing the cryopreservation of the TIL infusion bag[55]. Nevertheless, the collection process of solid tumours as starting materials for ATMPs is less advanced than for liquid tissues. Indeed, there is currently no standardised approach for the collection of solid malignancies within the industry[36, 37]. Despite the range of protocols tissue banks have developed for the preservation of various solid tissues, most of current validated techniques allow for either the maintenance of structural integrity (embedding or snap freezing) or viability (fresh samples in cell media or other preservation media) but not both. If cryopreservation of the starting material were to be introduced, the time between tissue collection and initial processing and cryopreservation will need to be minimised since this plays a significant role in cell viability and yield[56] (**Figure 2**). Time and temperature control become extremely important in cases when the therapy is not manufactured locally at the hospital and the fresh tissue must be transported to the manufacturing site for further processing. These time periods are measured in two portions:

- **Warm ischemic time:** the time a tissue remains at the original body temperature after its blood supply has been cut off, but before it is cooled. This is not controllable.

- **Cold ischemic time:** the time from excision to the moment the tumour is cooled in cold storage (+4C). This is controllable.

Cryopreservation could remove the need for the immediate shipment of freshly collected tumours while maintaining the cell viability and sample quality in general. This would consequently also impact on the surgery schedule and the patient management[57]. Since TILs are typically present in low numbers, establishing an effective way to preserve the finite cell numbers prior to manufacture is a key issue in the development of TIL therapies.

Biopsy samples of limited size may be suitable for cryopreservation but larger tissues will first require further dissection or processing[8] which ideally should occur as close as possible to the collection site and prior to reaching the manufacturing centre. To date, there has been limited research into the cryopreservation of tumours for TIL starting material.

Recently, Liang *et al.* (2021)[58] sought to optimise the cryopreservation of small pieces of tumour biopsies from rectal cancer patients and explore how it affected the recovery of viable TILs. While the study indicated differential recovery of cell sub-types, the authors demonstrated robust recovery of viable TILs from cryopreserved biopsies. Another approach is a pathway involving the collection of tissues followed by disaggregation to generate a cell suspension that can be

cryopreserved[16,59]. Alternatively, Iovance Biotherapeutics has proposed a method for fragmenting the tumour tissue, incubating the fragments in cryopreservation medium, and cryopreserving the fragments by flash freezing[60]. Although this approach will overcome the difficulties of cryopreserving whole pieces of tumours, the effects on patient outcomes have yet to be investigated.

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4. How can we improve cryopreservation of ATMP starting materials?

4.1 Successfully integrating cryopreservation into ATMP workflows

Optimising a complete cryopreservation process and integrating this into an ATMP manufacturing workflow – as summarised in **Figure 3** – can be a complex task requiring significant investment (e.g., liquid nitrogen infrastructure, freezing, storage, robust environment monitoring and alarm system, shipping and thawing technology, implementing health and safety measures as well as complying to regulatory requirements and licencing such as HTA). It is also recognised that current practices of cryopreservation of starting material show some level of variability (e.g., in terms of sample volume, cell densities, cryoprotectant concentrations, primary packaging, controlled-rate freezing technology, cooling protocols). Development of specific guidance regarding best practices on how to prepare, cryopreserve, and recover material for ATMP purposes is underway[61] and should assist in the design of efficient cryopreservation workflows.

Nevertheless, lymphocytes and HSPCs appear to be quite robust to the freeze-thawing process (see section 3). Indeed, small variations in some parameters do not seem to dramatically impair post-thaw recovery, viability, and functionality in a way that would prevent these cryopreserved products from being used as starting materials for ATMP manufacture. Additionally, the Advanced Therapy Treatment Centres (ATTC), an Innovate UK funded project operating within the NHS framework, are making significant investments to address the unique and complex challenges associated with bringing ATMPs to patients. In particular, the SAMPLE programme (Standard Approach to atMP tissue colLEction) aims to standardise the way cell and tissue samples are collected, preserved, and transported.

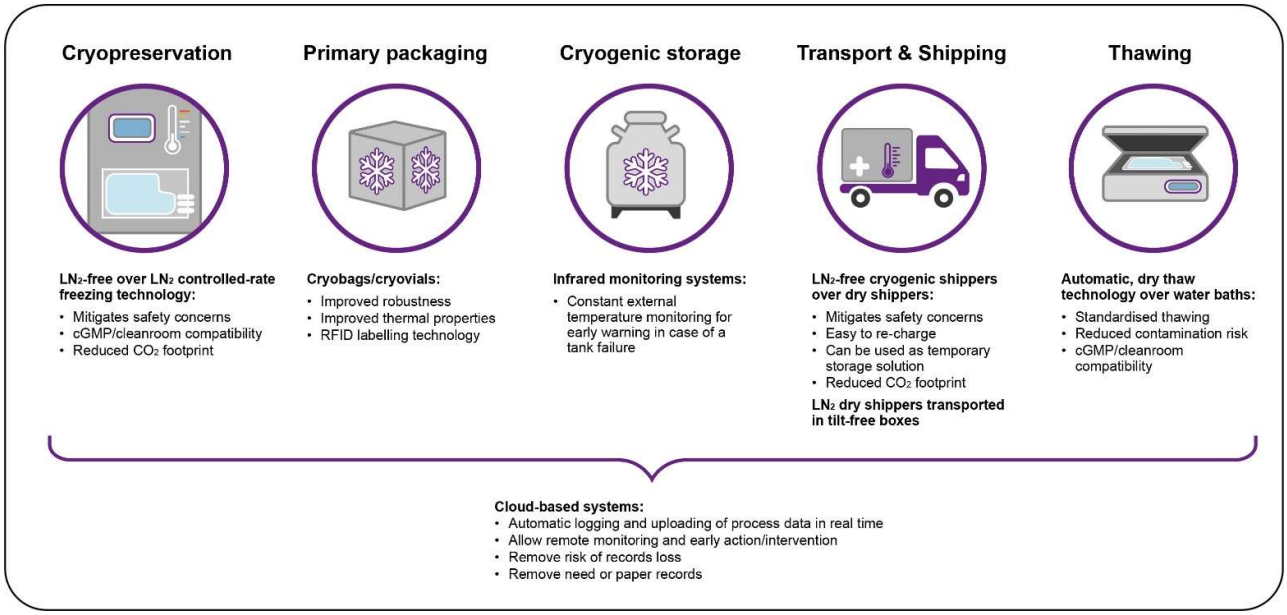

Figure 3. Recent developments at key steps of the cryogenic cold-chain. Major developments in cryopreservation, primary packaging, cryogenic storage, transport & shipping, thawing and cloud-based systems are listed.

4.2 Sample collection and cryopreservation considerations

Designing an effective cryopreservation strategy begins with the ability to effectively procure and stabilise the starting material.

If cells lose viability after collection, the ATMP manufacturer might have difficulties developing the therapeutic product or potentially lose the sample altogether, requiring a new sample that is not always available. Treatment efficacy is also at risk if the viability of the therapeutic product is compromised between manufacturer and patient. In either case, there are patient health implications and substantial financial implications for the manufacturer, given these therapies are costly to produce.

4.2.1 Upstream process considerations: from starting material to cryopreservation factors

Once collected from the patient, a sample might be kept in cold storage at 4°C, within preservation solutions that maintain tissue/cell viability prior to processing, as a short-term measure at the

collection site. If cryopreservation is deemed necessary, key elements, such as ischemic time, size, and composition of the starting material, cryopreservation protocols, and use of cryoprotectants need to be observed to maximise cell viability. These elements apply to cells in suspension, tumour or pieces of tumour, as well as starting material or finished products.

Reducing the time taken to efficiently process and cryopreserve a sample (i.e., reducing the cold ischemic time) is the first key element[62–64]. If the collection centre is not equipped with cryopreservation capability, starting material should be rapidly transferred to the cell therapy lab where cryopreservation should take place within 24 hours of collection but certainly within 72 hours.

The size and composition of the starting material will affect the freezing process and, therefore, the protocols that need to be developed. Of particular importance is the cooling rate. Cryopreservation cooling rates are defined as either very slow (<1˚C/min), slow (1-2˚C/min), or fast (>10˚C/min). Optimising cooling rates can be a complex task since it depends on several factors (such as cell type and level of organisation – cell suspensions versus tumours, cryoprotectants used, and sample volume) but is underscored by a theory developed in 1972 by Peter Mazur that is still considered valid today. The so called 'two-factor hypothesis' states that too slow cooling rates result in 'solute effects' injury; the accumulation of toxic solutes due to dehydration. Conversely, when cooling too fast for a particular cell type, intracellular dehydration may not have sufficient time to take place before ice forms in the intracellular space ('Intracellular Ice Formation', or IIF).

The importance of the 'solute effect' injury component should perhaps be reconsidered in light of a recent study where -1 and -0.1˚C/min appeared equally good at cryopreserving T cells[65]. As such, optimising a cooling rate for the cryopreservation of mammalian somatic cells boils down to avoiding IIF by using slow-enough cooling rates that enable cells to dehydrate sufficiently while extracellular ice crystals develop. Consequently, the importance of slow, controlled rates of cooling does not apply prior to the formation of ice in a sample, where the rate applied can, in theory, be faster. As an easy rule of thumb, for most cells in suspension, a cooling rate of up to approximately 1-2˚C/min is deemed optimal, while for samples of higher cellular organisation (e.g., pieces of tumour), cooling rates would need to be slower (<1˚C/min).

During freezing, cells will be subjected to some degree of supercooling, also known as undercooling. Supercooling is the state of a solution during cooling, in the temperature interval between its equilibrium freezing point (which is 0˚C for pure water, and a few degrees below for cryoprotected cell suspensions) and ice nucleation (when it effectively starts to freeze). In other words, it is the process of lowering the temperature of a solution below its freezing point until ice starts to form. The lower the ice nucleation temperature, the more extensive the supercooling. Therefore, the degree of supercooling is dependent on the temperature at which ice nucleation occurs within a specific sample. Ice nucleation is normally a stochastic process that is

progressively more likely to occur as the degree of supercooling of the sample increases, unless this is specifically controlled[66].

The sensitivity of cells to supercooling is cell-type dependent and can cause a substantial decrease in cell viability in small volume samples (typically <1 mL)[67]. Viability loss is much less of a concern for large volume samples such as those acquired as starting material for ATMP manufacture (typically around 100 mL per sample)[8]. This is due to the higher amounts of impurities and other structures that serve as nucleation sites in large volume samples and, consequently, keep supercooling at safe levels[68].

Another, often overlooked yet important, consideration is determining when to finish controlled rate cooling and transfer the sample to cryogenic storage. Although, in practice, this is typically done when samples have reached temperatures as low as -100˚C, the scientific evidence shows that cells become osmotically unresponsive below approx. -50˚C (after intracellular glass transition has occurred)[24,44,69,70]. Thus, controlling the rate of cooling, and therefore ice crystal formation and cell dehydration, is crucial between ice nucleation and intracellular glass transition (approximately between -10˚C and -50˚C). Consequently, it is possible to finish controlled rate cooling earlier than is typically seen in most protocols without compromising sample integrity. ATMP manufacturers can benefit from shorter cryopreservation protocols that may help facilitate adoption.

However, the endpoint temperature of a cryopreservation protocol differs from the temperature of cryogenic storage and/or shipping. To ensure stability of the cryopreserved sample over time, these temperatures must be maintained below -120°C, the glass transition temperature of cell suspensions containing Dimethyl Sulfoxide (DMSO)[8,24]. This is the point below which molecular mobility within the sample becomes restricted to vibrations of atoms or bonds and reorientation of small groups of atoms[71]. Therefore, the risk of degradation over time is substantially reduced compared to warmer *negative cold* storage (e.g., at -80°C), or *positive cold* storage (at 4°C). In practice, extra precaution appears to be taken by ATMP manufacturers with cryogenic storage and transport temperature requirements, which tend to remain at or below -150°C.

Another final consideration for cryopreservation is the use of cryoprotectants. Added to cell and tissue samples prior to the freezing process, their presence is essential to minimise cell damage[8]. Since they work by a range of mechanisms and exert varying cytotoxicity profiles[72], it is important to consider the details of each cryoprotectant option, including whether they meet regulations and have evidence to show they can be used clinically. For instance, the recommended limit on the gold standard DMSO administration is 1 gram per kg body weight per day.

While cryoprotectants are essential for successful cryopreservation, they also carry two major risks for the sample: their cytotoxicity and the osmotic shock of their addition/removal. For instance, with DMSO, cytotoxicity is temperature dependent, and consequently it must be added to a chilled sample. Furthermore, the time between addition of cryoprotectant and

cryopreservation should be minimised to limit any cytotoxic effects. As such, the physical distance between cleanrooms or processing areas and the place where controlled-rate freezing takes places needs to be considered. For instance, while LN_2 controlled-rate freezers (CRFs) need to be located in a room supplied with LN_2 where health and safety measures are in place (environment monitoring system and alarms) – typically this would be the cryogenic storage room itself – LN_2-free CRFs can be installed and operated directly within cleanrooms/processing areas. The same applies upon thawing as the cryoprotectant's cytotoxicity resumes. Therefore, the sample should be kept chilled until it is rapidly washed/diluted (if starting material prior to manufacturing) or infused (in the case of a final product). Osmotic shock in turn is minimised by progressive or drop-wise addition of the cryoprotective solution to the sample prior to freezing, and its progressive removal or dilution on thaw.

4.2.2 The freezing technology

For the actual cooling process, passive freezing in a -80°C mechanical freezer or LN_2-based controlled-rate freezers have been the default choice for many years. Controlled-rate freezers provide the advantage of ensuring the desired optimal cooling rate is applied and, by using a temperature probe in a mock sample or affixed to a sample's primary packaging, keeping a record of the actual freezing process for COC purposes.

More recently, LN_2-free controlled rate freezers have emerged, which mitigate the health and safety risks associated with LN_2 exposure and reduce the costs of implementing an LN_2 infrastructure, as well as the cost and carbon footprint of LN_2 supply[73]. LN_2-free controlled-rate freezers use electricity to power a Stirling Cycle engine as a cooling source – operating by means of cycles of compression/expansion of an inert gas – to reduce temperatures to cryogenic values. By using a conduction cooling process that pulls heat from the cryochamber evenly, all samples freeze at the same rate, a significant benefit. On the other hand, LN_2 controlled-rate freezers still present a number of advantages over their LN_2-free counterparts, including higher capacity and the ability to achieve faster cooling rates; up to approximately -50˚C/min.

Consequently, cooling protocols appear to vary across sites. For example, while some protocols detail a single linear cooling rate, other protocols outline an incremental increase of cooling rates in discrete steps or the use of a plunge-cooling step at a point where the sample may nucleate. However, the extent to which these variations influence the post-thaw outcomes of the rather robust lymphocytes or haematopoietic stem cells seem to be limited[44,74], provided that the key upstream process considerations, as described in section 4.2.1, are met.

Part of the SAMPLE programme has focused its efforts on cryochain requirements for the starting material for ATMP manufacture. Specifically, LN_2-dependent and LN_2-free controlled-rate freezers were compared and the results suggested equal performance in the cryopreservation of different types of starting material.

4.2.3 Primary packaging

Beyond losing viability during cooling, contamination is also a risk with cryopreservation. As starting material is intended for eventual therapeutic use, primary packaging must be hermetically sealed to prevent contamination from LN_2-borne contaminants or other contaminants in the cryochain[8]. In addition, primary packaging needs to maintain strength at low temperatures to ensure the integrity of the sample during transport. Hermetically sealed cryovials would be a robust option, however, their thermal performance prevents their use for the large volumes typically needed for ATMP starting material. Instead, the leukapheresis procedure, which ends with the samples in a bag format, makes cryobags the natural and practical choice for cryopreservation of starting material. Since air is a very good insulator, it can negatively impact thermal transfers during freezing and thawing. Therefore, air needs to be meticulously removed from cryobags before freezing.

Regulatory requirements recommend using overwrap bags for long term storage in LN_2 tanks, hence cryobags should always be used with an overwrap bag. Ideally, the overwrap bag should be vacuum-sealed to the cryobag to remove air and, in turn, fitted inside metallic canisters or cryocassettes prior to freezing. The benefits of metallic canisters or cryocassettes are two-fold:

- They improve thermal performance due to a greater thermal conductivity compared to cryobags alone and ensure a thin and even thickness of the sample along the surface of the cryobag.

- They provide increased robustness that serves to protect potentially delicate frozen samples and consequently, reduce the risk of damage and cross-contamination.

In addition, small cryovials or segments sealed from the cryobag's tubing can be used alongside the bulk cryobags(s) as QC samples. While QC samples are necessary for evaluating the effect the cryopreservation process has had on the bulk sample without having to sacrifice whole cryobags, studies have questioned their representativity. For instance, Kilbride *et al.* (2019) demonstrated that ice nucleated at significantly lower temperatures in QC samples of umbilical cord blood compared with the bulk samples because of their lower volume. In turn, osmotic stress was exacerbated in the QC samples, which could lead to discarding quality starting material unnecessarily.

The primary packaging also helps identify the sample. The label contains information on identity and ensures the traceability of the sample. A guidelines document produced as part of the SAMPLE project and relating to the procurement of starting material by apheresis for ATMPs covers the requirements for labelling of these samples in great detail[75].

Radio Frequency IDentification (RFID) technology is being examined as a solution to manage cryogenic storage of ATMPs. RFID technology uses high-frequency radio waves to automatically identify and track tags attached to samples. Moreover, RFID tags can store a range of information, making them suitable for identification and traceability of ATMP material. While commonly used at ambient temperatures, a number of companies have now developed highly durable RFID tags that can be used at cryogenic temperatures and for multiple containers and packaging types. Ultimately, the aim of cryogenic RFID tags is to improve tracking and tracing of cryogenically-stored samples, sample retrieval, and the complete audit trail[76].

4.2.4 Cryogenic storage

The design, operation, and contingency of cryogenic storage requires considerable attention to ensure the safety and quality of ATMP starting materials and final products. Reliable cryogenic storage systems that minimise risk of sample damage and catastrophic losses while maximising personnel safety are of paramount importance.

The two main options of cryogenic storage tanks are LN_2 freezers or mechanical ultra-low freezers. The unique advantages and limitations of these two types of freezers need to be carefully considered. Mechanical ultra-low freezers can typically cool down to approximately -150˚C, however, they suffer from heterogeneity of temperature distribution within the freezer and temperature fluctuations when opening the door. Ultimately, uneven temperatures can compromise sample integrity and quality.

LN_2 cryostorage tanks offer the ability to store samples in the liquid phase or the vapour phase of LN_2, however, storing samples in the liquid phase presents a contamination risk. There is a lower risk of crossing the storage temperature requirements with LN_2 cryostorage systems compared to mechanical ultralow freezers. Nevertheless, LN_2-based approaches require a guaranteed supply of LN_2 and stricter health and safety measures – including ventilation requirements, an environment monitoring system coupled with an alarm system, and staff training – due to the oxygen depletion and cold burning risks.

For both personnel safety and to ensure the proper functioning of the cryostorage system, remote monitoring/alarm systems and regular integrity checks are critical. Continuous monitoring of temperature in combination with a remote system activated by out-of-range measurements is a vital tool for ensuring temperatures are maintained within an accepted range. However, since temperature measurements are a poor gauge of LN_2 level – except when critically low – a secondary monitoring system is needed[77]. LN_2 sensors can even be coupled with an autofill system to maintain a consistent level or indicate a refill.

In addition to remote monitoring/alarm systems, regular integrity checks are needed to ensure cryostorage systems are operating safely and to mitigate potential catastrophes. Assessing the evaporation rate of the LN_2 tank as well as physical inspection of welds, seals, and visual and audible anomalies (e.g., ice, frost, condensation, unusual sounds) should form part of a regular routine[77]. New technology, such as the CryoSentinel® early warning system can support these activities[78]. The CryoSentinel® system employs infrared cameras to constantly monitor the external temperature of the cryogenic storage tank fleet for early diagnosis of failing units.

Even with these systems and checks in place, there is still potential for cryogenic storage units to fail. Therefore, a contingency plan needs to be in place. In the event of a cryogenic storage system failure, a back-up cryogenic tank should always be kept available for sample transfer.

4.3 Transport and logistics

Once the starting material has been cryopreserved, it must be suitably packaged for transport. The cryogenic shipping process, just like storage, must maintain temperatures below at least -120˚C. As such, this traditionally means using a dry shipper, containing vaporised LN_2 in porous zeolite sponge internal walls, as tertiary packaging. These dry shippers have large capacities, and are widely used and familiar to most personnel in a cryogenic cold chain.

There are numerous considerations for using LN_2 dry shippers for the transportation (and possibly short-term storage) of ATMP starting material. Pre-conditioned LN_2 dry shippers, shipped empty to the cell therapy lab and then with payload held in the vapour phase to the manufacturer, will gradually vent their vapour phase. A key advantage of these dry shippers is that the slow venting means most can maintain steady cryogenic temperatures for several days depending on the size of the dry shipper and typically between 5 and 10 days under normal conditions. The length of time a shipper can maintain a steady cryogenic temperature is known as the hold time. This is usually sufficient to transport materials between clinical and manufacturing sites, even internationally.

However, suitable packaging and a carefully planned route are still essential for each shipment as the physics of LN_2 vapour presents many challenges and creates constraints throughout the logistics process. Managing dry shippers requires complex preparation, charging, and validation processes, including[21,79]:

- Cleaning of the fibrous zeolite matrix used to hold LN_2, which can degrade with use.
- Pre-charging with LN_2 to a pre-determined fully-charged weight.

- Checks for damage to seals around the neck or denting of the exterior, which can reduce hold time.

- A 24-hour evaporation validation test.

Despite considerable effort, these steps still do not guarantee the shipper's hold time. Dry shippers are sensitive to orientation, and so an incident like tilting during transit increases the rate at which the LN_2 vents, dramatically reducing the hold time and introducing risk of the -120°C threshold being exceeded. However, "tilt-free" transport boxes have been developed to address the tilt risk for dry shippers, such as the Upright Positioner, WAK Chemie.

The journey of a dry shipper might span days, especially in cases requiring air freight across international borders where customs checks and paperwork can result in extensive hold-ups. These delays present a clear risk to the maintenance of cryogenic temperatures as exhausting the supply of stored LN_2 will lead to a rapid rise in temperature within the dry shipper. Data loggers or smart monitors can capture shipping data; including but not limited to inner temperature, tilt, and location of containers throughout transit. With a standard data logger, it might only be possible to check for incidents at key stops *en route*, leading to a potential delay in response. Smart monitors, on the other hand, can provide real-time data, enabling more timely intervention.

Using a specialty courier for cryopreserved samples is crucial. For example, should there be an incident, a speciality courier may be able to intervene by transferring the sample to a pre-charged backup, reducing the risk of damaging temperature excursions or disastrous thawing. Doing so, however, introduces the complication of a break in the sample's COC and COI.

As a result of these inherent risks, there is a need for LN_2 infrastructure throughout the cryogenic cold chain — at the processing facility, the courier's charge-hub, *en route*, and at the manufacturing site — with associated hazard management training and plans. All parties are also under scheduling constraints to coordinate the timing of the clinical, collection, and manufacturing sites' workflow steps with the dry shipper's preparations and rigid logistics timetable.

A potential alternative option to mitigate many of the challenges related to dry shippers is a change to using LN_2-free systems for both cooling and transport, such as the recently released VIA Capsule™ System (Cytiva). This electrically powered system has been validated to provide hold times equivalent to dry shippers. Moreover, it can easily be re-charged, if necessary, without breaching the sample's COC and COI (see section 2.3). It also provides the option of temporary storage at the manufacturing, collection, or clinical site when left on charge, with a smaller physical footprint relative to a dry shipper and improved manoeuvrability.

As digitalised systems, LN_2-free shippers also have smart monitors built in, providing live data on temperature, tilt, and GPS position.

4.4 Thawing

Once the cryopreserved starting material reaches the manufacturing facility, it will need to be thawed. Generally, it is assumed that cryopreserved samples must be thawed rapidly to reduce ice recrystallisation events. Indeed, ice recrystallisation events expose cells to an osmotic imbalance upon thawing and result in cell damage[80]. However, recent study shows that this is not necessarily the case if the sample was cryopreserved at an optimal rate. An optimal rate ensures that ice fully crystallises upon cooling, ruling out the possibility of ice re-crystallisation events.

Fortunately, starting material and final products are generally cryopreserved at -1-2˚C/min, which corresponds to optimised, slow-controlled rates of cooling. Still, the wider downstream process needs to be optimised to reduce any damage to the sample **(Table 5)**. Most importantly, DMSO toxicity that resumes on thaw must be controlled; especially as cells will have become more fragile, having gone through stressful cryopreservation and ischemia processes. This involves washing/diluting the sample quickly but progressively upon thaw and keeping them chilled throughout the process.

It should be noted that although water baths are the most common thawing devices/equipment, they do not align with current GMP requirements and, therefore, alternative thawing approaches will need to be employed. Moreover, water bath thawing requires continual manipulation, which presents a number of risks to the operator and to the sample including potential cryogen outgassing and therefore exposure to extreme temperatures, as well as container leakage, leading to material loss and/or contamination.

An alternative approach is offered by dry thawing systems. Dry-thawing devices are designed to provide a more uniform and documented thawing process with a reduced contamination risk. While water baths remain the most common approach to thawing, the benefits of dry thawing are becoming increasingly recognised. Indeed, a growing number of studies indicate that dry thawing does not significantly affect factors such as cellular recovery, viability, or overall recovery of biological materials[81–90].

Table 5. Thaw considerations according to Baust *et al.* (2017)[82]

CONSIDERATION	DESCRIPTION	OPTIMISATION STRATEGIES
Airtime	Airtime is the time between removal from storage and placement into a thawing device.	Extended airtime can compromise samples as they are exposed to passive warming and, as such, must be minimised. Consider (1) positioning storage and thawing devices close to each other, and (2) implementing a temporary cold storage strategy (e.g., dry ice container or specialised transport device) to move sample between storage and thawing device.
Thaw rate	Thaw rate refers to the speed of thawing at a given temperature.	Generally, studies suggest that sample-specific thawing rates provide improved outcomes. Many dry thawing devices allow for customised protocols.
Sample agitation	The continuous agitation or mixing of samples during the thawing process.	Without agitation/mixing, the thermal gradient that forms within a sample can become very steep, with the warmest temperature on the outside edges and coldest in the centre. This can reduce cell recovery due to over-heating.
Final sample temperature	The temperature of the sample at the end of the thawing process.	Final sample temperature should be cool and not warm (e.g., ~ 0 to 4°C) to prevent sample overwarming and minimise toxicity of cryoprotectants (e.g., DMSO).
Hold time	The time interval between end of thawing process and next stage in workflow.	The time interval should be minimised, and samples should be kept cool to maintain quality.

4.5 Digitalisation of cryochain data

It is extremely important that the complete details across the ATMP manufacturing workflow are recorded, secure, and accessible. Ensuring this COI is particularly important when considering autologous transplants since it is absolutely critical that the patient receives their own material. In the case of cryopreservation, details regarding storage, transport, and thawing must be tracked and documented to ensure that the sample or final product is not compromised during these processes (COC).

Recently, developments in software and computing capabilities have streamlined the process of documentation. Cloud-based approaches can automatically and securely log data in real-time

regarding the full cryochain. Subsequently, information such as controlled-rate freezing and cryogenic transport conditions and thawing, can be accessed from a digital portal.

For instance, the cryochain portfolio from Cytiva is integrated with the in-house cloud-based Chronicle™ automation software for secure logging of real-time cryochain data. Chronicle™ software provides a unified digital platform to monitor cell therapy facility manufacturing operations and supply chain logistics. While real-time access to data enables the possibility of intervention, it may be challenging to determine what efforts are appropriate and actionable if alarms are triggered *en route*. Nevertheless, cloud-based approaches present several major benefits: they remove the risk of potential validation record loss as well as the need to upload data logger read-outs or manage paper records. Moreover, digitalising records helps ensure samples or final products have been effectively cryopreserved and are safe for manufacturing or patient delivery.

5. Are we moving to cryopreservation as standard?

Designing and implementing an effective cryopreservation strategy and efficiently managing the associated cold chain is becoming an increasingly important consideration for ATMP manufacture. Nevertheless, the use of cryopreservation of biological materials intended for clinical use is extremely variable across workflows.

For example, while cryopreservation has been used as standard for autologous and umbilical cord blood transplantation, only 5-8% of products from the National Marrow Donor Program (NMDP)/Be The Match have been cryopreserved annually[91]. That is until the SARS-CoV-2 (Covid-19) pandemic introduced a plethora of logistical challenges that forced a shift in favour of cryopreservation. Indeed, in the UK, Anthony Nolan, NHS Blood and Transplant (NHSBT), and the Welsh Bone Marrow Donor Registry have been advised to follow the recommendations issued by the European Society for Blood and Marrow Transplantation (EBMT) and the British Society of Blood and Marrow Transplantation and Cellular Therapy (BSBMT&CT) since the first wave of the pandemic. These recommendations have been continually evolving in line with new information on the pandemic and are regularly updated[92,93]. They advise the use of cryopreservation since it provides additional time to evaluate whether a donor carries SARS-CoV-2 and increases the flexibility in the logistics and transplantation process[94]. As such, through the first wave of the pandemic, approximately 95% of NMDP samples were scheduled for cryopreservation[91].

Whether the trend toward cryopreservation will continue post-pandemic or translate into the manufacturing of ATMPs is yet to be seen. However, with continued research confirming equivalent outcomes of many fresh and cryopreserved therapies, it is likely that the benefits of cryopreservation to logistics will force a standard in many ATMP workflows.

To ensure that cryopreservation represents a viable option for all involved in ATMP manufacture, infrastructure supporting an effective and simplified end-to-end solution will need to be in place. This includes, for instance, the ability to process and cryopreserve at or closer to the collection site, such that deterioration of cell function is minimised. Emerging technology, such as compact, benchtop LN_2-free controlled-rate freezers, offer an attractive alternative to space demanding liquid nitrogen approaches – and consequently facilitate rapid ‘collection to cryopreservation’ times. In addition, the continued progress in (1) understanding the basics of cryobiology will inevitably translate into more optimal practices and improved sample post-thaw outcomes, and (2) developing efficacious protocols, particularly for solid tumours, should drive forward the adoption of cryopreservation.

Arguably, the most important benefit of cryopreservation is the ability to store starting materials long-term, enabling time for extensive testing and, ultimately, increasing patient safety. A further advantage of LN_2-free systems for both cooling and shipping is a reduction in their carbon footprint relative to LN_2-dependent systems[95,96]. While only a few ATMPs are currently on the market, and

in relatively small quantities compared to other therapeutics, this may seem of secondary concern. However, as more ATMPs are approved and existing therapies are scaled up, the carbon footprint of LN_2 systems may quickly become a significant consideration for clinical sites and manufacturers. Automatic dry thawing instruments are also available to address the issue of poorly controlled and contamination-prone water baths. Recent advances in the cryogenic cold-chain technology are summarised in **Figure 3**.

From a broader perspective, as more ATMPs move from trial to clinic, there will be continued development and consolidation across the cryochain. The introduction of new tools, technology, and services will only serve to streamline the process and help remove barriers to ATMP market authorisation.

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