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CELL THERAPY

OPTIMIZING THE VEIN-TO-VEIN WORKFLOW

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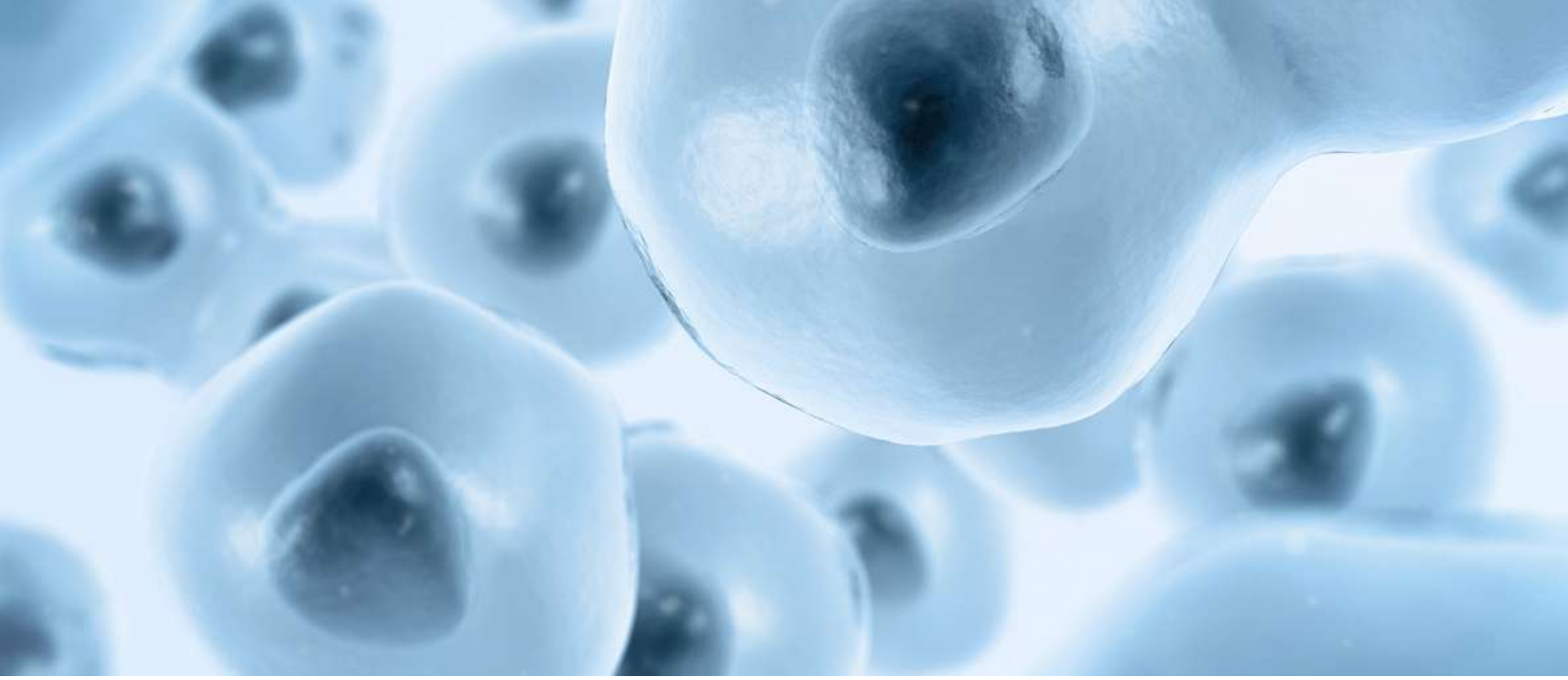

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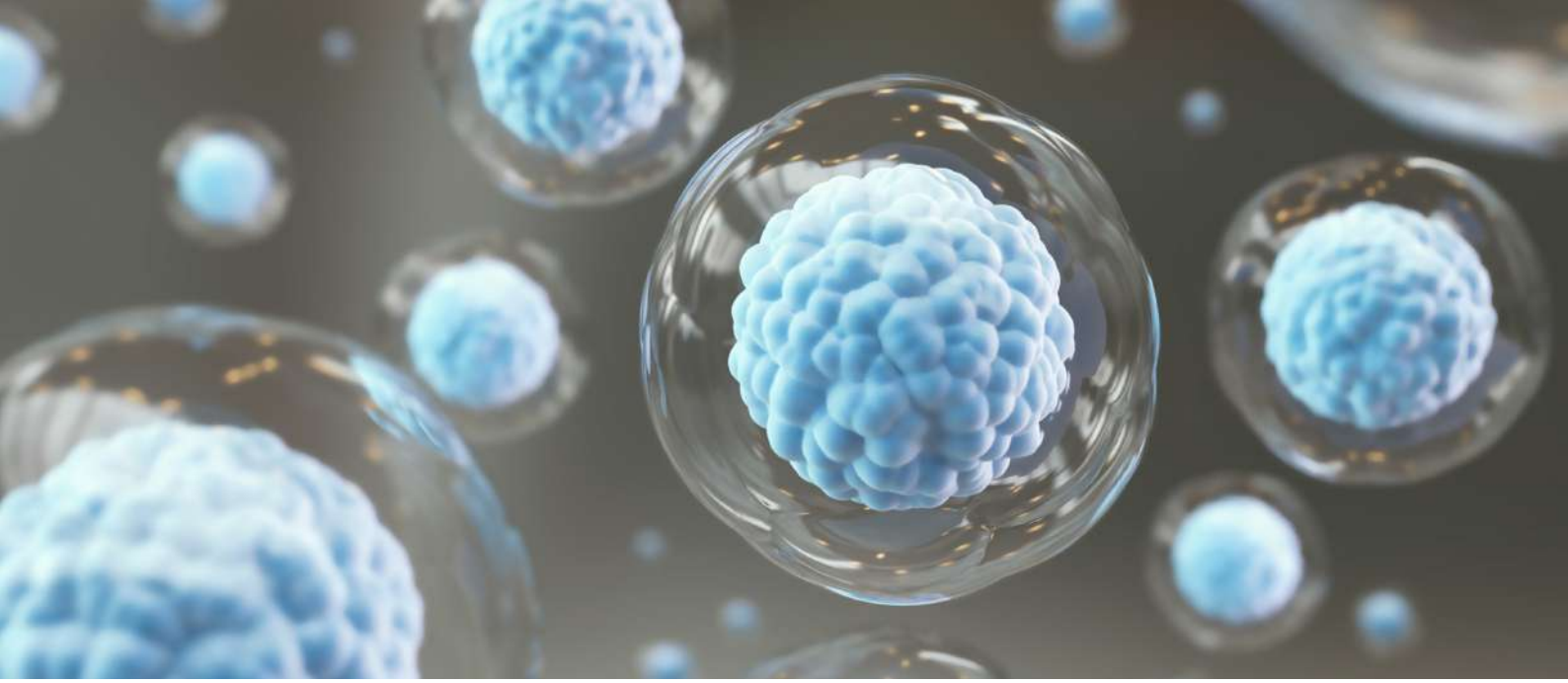

FOREWORD

We are pleased to present you with this eBook on optimizing the vein-to-vein process for CAR T cell therapy, which has been produced by RegMedNet in association with Thermo Fisher Scientific. This eBook aims to bring you the latest developments and leading opinions from key thought leaders in the field.

The cell therapy workflow begins and ends at the patient and requires extensive infrastructure and coordination. At the beginning of the workflow, blood is collected directly from the patient and a tracking system is initiated for traceability. The patient material then undergoes processing to isolate the cell type of choice, engineering of the cell to modify its functionality according to the therapeutic target, expansion of the cell population and then finally characterization and cryopreservation. Throughout this complex process, supply chain and logistical planning is necessary to ensure proper storage and transport of the material from start to finish.

In this eBook, we delve further into the cell therapy workflow and highlight the patient as a focal point throughout this journey. We also explore how a GMP-compliant, semiautomated manufacturing platform can result in consistent, efficacious CAR T cell production.

We hope you enjoy reading about expert insights into the cell therapy workflow with us!

Sharon Salt
Senior Editor, RegMedNet
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From vendor to cell therapy partner: an interview with Xavier de Mollerat du Jeu, Thermo Fisher Scientific

Dr. Xavier de Mollerat du Jeu, Ph.D. is the director of product management in the Cell and Gene Therapy business unit at Thermo Fisher Scientific (CA, USA), developing new products and solutions for cell therapy manufacturing. Prior to this, Xavier was the Director of R&D Cell and Gene Therapy in Carlsbad, California, working on developing new closed modular platforms for the clinical manufacture of T cells. Xavier also identified new DNA delivery approaches for hard to transfect cell lines and primary/stem cells, and he is the inventor of Lipofectamine® 3000 and the author of several patents around nucleic acid delivery. Additionally, his research focused on new delivery solutions for CRISPR delivery, scalable lentiviral production solutions, mechanical delivery approaches for primary T cells and in vivo *delivery of RNAi/mRNA for research and therapeutic application. His team is dedicated to bringing new viral and non-viral delivery solutions for T cell engineering and manufacturing, including automation and closed systems.*

Xavier de Mollerat du Jeu, PhD

1 What makes cell therapies more attractive than other approaches to treat disease?

Small molecule or antibody therapies treat the symptoms of a disease. This alleviates the disease symptoms for a period of time but ultimately the patient continues to live with it. In comparison, cell therapies have the potential to cure the disease and eradicate it from the patient with a single dose. This is revolutionizing the field of cell and gene therapy, specifically the treatment of cancers. So there is a lot of work to be done on the manufacturing side, on the tools, and on the instruments to really make these therapies accessible to all.

2 Tell me about your cell therapy products. What sort of research or trials are currently being supported by Thermo Fisher?

Our products have been used in more than 200 clinical trials and are utilized in drugs that are already approved and on the market, making them a proven choice for clinical stem cell and immunotherapy. The types of commercial products that we currently support include FDA-approved and EMA-approved CAR T therapies, as well as the first FDA-approved therapeutic cancer vaccine.

To ensure our products are produced at the standard needed for use in the clinical market, we offer a line of Gibco™ Cell Therapy Systems (CTS™) products that includes reagents, instrumentation, consumables, and laboratory equipment. CTS products are specifically designed for use in the manufacture of cell and gene therapy products and are cGMP manufactured, follow USP <1043> and Ph Eur 5.2.12 guidelines, and are backed by regulatory documentation.

Our new cell therapy products and instruments are specifically designed to incorporate a high degree of flexibility. This enables our customers to build modular processes; meaning our instruments and products are compatible and adaptable to other parts of the workflow, and therefore can be used or assembled in customizable configurations. More importantly, our instruments, as seen by the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System, are supported by a digital architecture so they can either be used individually, or linked together, which ultimately will facilitate automation.

From vendor to cell therapy partner: an interview with Xavier de Mollerat du Jeu, Thermo Fisher Scientific

We have also taken a different approach to our product development strategy. Our focus is on building complete workflow solutions for commercial use. We have created a collaborative culture and environment where we can directly work with the drug makers, together, not just functioning as a toolmaker of products. We have established collaboration centers that allow us to work side by side with our customers to create complete solutions that are directly translatable to the patient.

3 In the simplest of terms, what do these collaboration centers consist of, and what type of work is carried out there?

The collaboration centers include fully outfitted labs built at the customer sites and staffed with scientists and engineers who specialize in our tools and technologies. The goal of our Thermo Fisher collaboration centers is to partner with customers and directly work with them to then develop their cell therapy manufacturing processes. Through these partnerships, our customers may gain early access to our upcoming new products, we gather feedback to help ensure we are making the right products to meet the customer needs, and we collaborate with them in the earliest stage of their process development, helping them to move their technology to commercial success.

4 How do researchers from biotechs and academia work together with Thermo Fisher at the collaboration center?

We know how great the potential of cell therapies are to cure diseases like cancer, and there is a lot of work to do moving forward, for example with solid tumors. This is not a problem that can be addressed by one person or one company. It is something that needs to be done together – so we bring incredible expertise in manufacturing product development, then by working together with the drug maker, we can work towards achieving their goals. The collaboration center consists of multidisciplinary teams of scientists and engineers from both sides of the collaboration, and is built at the customer site. This enables the customer to focus on the clinical aspects of their therapy, their patients, and their personalized set of logistics, while working with our team on the manufacturing process development optimization – all within one environment. We think it makes the perfect combination.

5 How will your collaboration centers complement Thermo Fisher's existing Cell and Gene Therapy operations?

Currently, there are only five approved cell therapies on the market – and while this is remarkable, it is still a low number. Oftentimes, the tech transfer of manufacturing workflows occurs from R&D environments, and therefore are small scale and primarily "open" lab processes, which are very challenging to navigate during commercialization. Through our collaboration centers, we accelerate this manufacturing process development stage, by working side by side and leveraging each other's expertise. We offer access to our emerging technologies, existing solutions and expertise so that customers can transition to scalable closed system processes, which will speed up the process development phase. If we accelerate the customer's development process, we can get therapies to market faster, and ultimately help create positive change in patients' lives.

To learn more about Thermo Fisher's cell and gene therapy offering, please contact a specialist today.

APPLICATION NOTE

CTS reagents and products

Autologous CAR T cell manufacturing using a semiautomatic, closed, modular workflow

Seamless transition from discovery to clinical manufacturing

Background

Cell-based chimeric antigen receptor (CAR) T cell therapies have rapidly advanced from preclinical research—with a variety of targets in clinical research and several FDA-approved products currently on the market [1]. This success has driven an influx of companies to further develop CAR T cell constructs to make them more effective, safe, and persistent. On the manufacturing side, however, errors, lot-to-lot variation, and contamination can be associated with open processing and manual handling of CAR T products. Overcoming the bioprocessing bottleneck remains a critical challenge in CAR T cell therapy scalability, which can potentially hinder both product development and patient access. It has been reported that about 7–9% of patients have been unable to receive one of the FDA-approved CAR T cell therapies because of manufacturing failures [2].

Autologous CAR T cell therapies are donor-specific, where a donor's own immune cells are used to create therapeutic CAR T (Figure 1A). During the manufacturing process, a Leukopak™ bag from the donor is received by a GMP facility, where the T cells are isolated from peripheral blood mononuclear cells (PBMCs), activated, and genetically engineered by viral transduction to express a CAR. The activated T cells are expanded in a T cell–specific cell culture medium, typically for 7–10 days to reach a therapeutically relevant number, and then they are cryopreserved. The cryopreserved CAR T cell product is then characterized and analyzed before being shipped to the treatment center, where it will be thawed and administered to the donor via infusion. This complicated, labor-intensive process usually involves many open manipulations and manual procedures, potentially

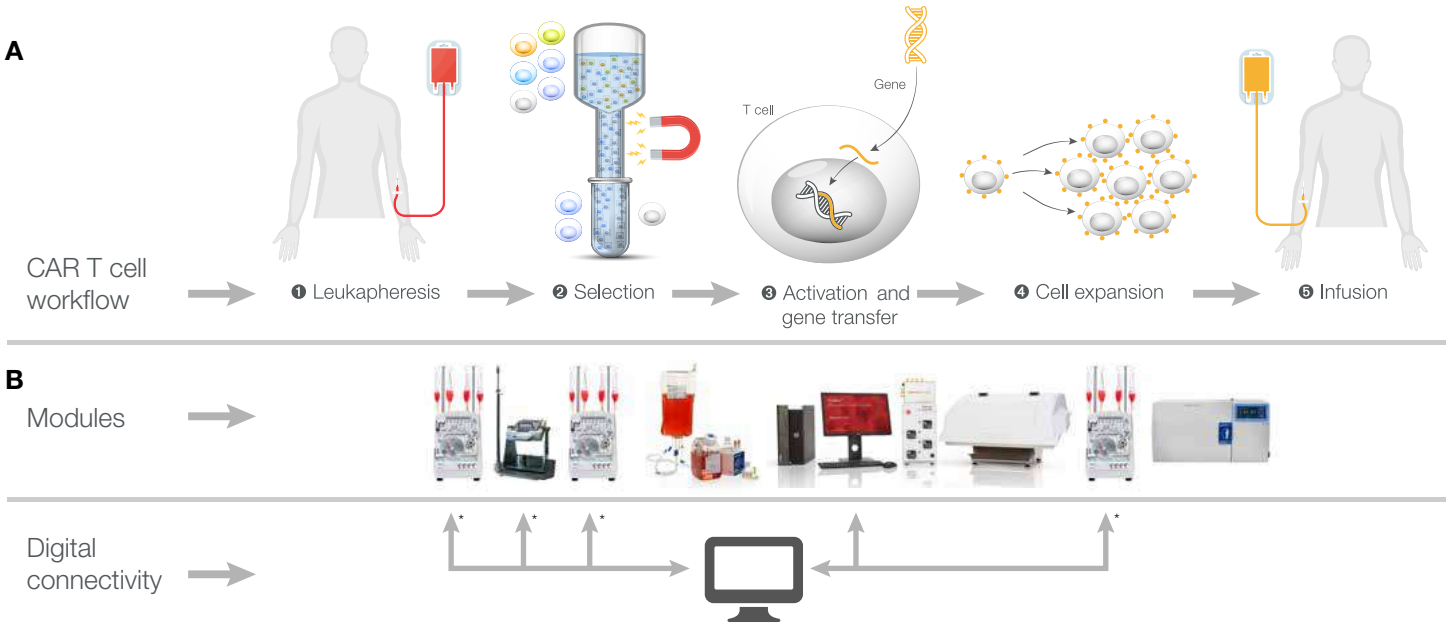

Figure 1. A typical autologous CAR T cell therapy workflow. (A) The typical CAR T cell therapy workflow. **(B)** Workflow solutions from Thermo Fisher Scientific.
* Proof-of-principle prototype solution.

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introducing inconsistencies, errors, and contamination at various steps of the workflow. Currently, no uniform, automated manufacturing process exists to accommodate the wide variety of complex workflows needed—from isolation to cryopreservation—to produce therapeutic CAR T cells. Re-engineering the CAR T cell therapy manufacturing process by integrating the complicated multistep workflow into a closed, modular, benchtop system could enable a smoother transition from the laboratory to clinical research application while improving consistency, purity, and safety of the product. Additionally, as early as possible in the discovery and development phases, the generation of CAR T cells should include safe and effective biomanufacturing processes and trackable workflows, and have the potential for cGMP compatibility.

The closed modular system developed by Thermo Fisher Scientific is a digitally compatible, GMP-compliant, semi-automated manufacturing platform, which when used in combination with Gibco™ Cell Therapy System™ (CTS™) reagents, protocols, and analytics can result in consistent, efficacious CAR T cell production. One important benefit of the closed modular system is the digital connectivity. Here we demonstrate a proof-of-principle digital integration using the DeltaV™ Distributed Control System from Emerson to control and manage the instruments in the workflow, as shown in Figure 1. Taken together with the modularity, digital connectivity, and cGMP compatibility of the various components, the manufacturing of therapeutic CAR T cells with the closed modular system can reduce contamination and production failure, and improve lot-to-lot consistency of products.

Materials and methods

Step 1. PBMC isolation

Starting material was derived from fresh or frozen quarter-size Leukopak bags (n = 7). Peripheral blood mononuclear cells (PBMCs) were isolated using the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System following the predefined isolation protocol (see "Automated PBMC isolation and T cell wash and concentration by the CTS Rotea system"). During the process, red blood cells (RBCs) were eliminated with Gibco™ ACK Lysing Buffer and the PBMCs were further washed with Gibco™ DPBS with 1% human serum albumin (HSA) and 2 mM EDTA. Cells were counted using the Via1-Cassette™ system and NucleoCounter™ measuring chamber (ChemoMetec). Flow cytometry acquisition and analysis were performed using the Invitrogen™ Attune™ NxT Flow Cytometer and FCS Express™ 7 software, respectively.

Step 2. Selection—T cell isolation and activation

PBMCs were incubated with Gibco™ CTS™ Dynabeads™ CD3/CD28 at a 3:1 ratio of beads to T cells for 30 minutes at room temperature. Bound cells were captured with the Gibco™ CTS™ DynaMag™ Magnet, and unbound (nontarget) cells were removed with the supernatant. The bound cells were then washed with 1% HSA, 2 mM EDTA in DPBS, followed by resuspension in complete medium (Gibco™ CTS™ OpTmizer™ T Cell Expansion Serum Free Medium (SFM)), supplemented with Gibco™ CTS™ Immune Cell Serum Replacement (SR), 200 mM L-glutamine, and 100 U/mL IL-2. The cells were then transferred to either a Thermo Scientific™ HyPerforma™ Rocker Bioreactor with Thermo Scientific™ HyPerforma™ G3Lab™ Controller or a G-Rex™ 500M bioreactor (Wilson Wolf) at a seeding density of 1 x 10^6 cells/mL. Cells were cultured overnight and transduced with lentivirus the next day.

Step 3. Gene transfer—lentivirus-CAR transduction

To achieve gene transfer of CD19-targeted CAR into T cells, anti-CD19 CAR lentivirus (CD19 SCFv-CD3z-41BB) was generated using the Gibco™ CTS™ LV-MAX™ Lentiviral Production System (see "Integrated generation and characterization of CAR T cells" [3]). T cells that were isolated and activated for 24 hours were then transduced with CD19 CAR lentivirus at a multiplicity of infection (MOI) of 10. Cell characterization including CAR expression, CD4/CD8 ratio, cell number, and viability was performed on day 6 posttransduction.

Step 4. Expansion—CAR T cells

CAR T cells that were transduced with CD19 lentivirus vectors were then expanded in a HyPerforma Rocker Bioreactor or G-Rex bioreactor in complete medium. Feeding and monitoring was accomplished in a closed automated process controlled by the DeltaV platform in conjuction with Thermo Scientific™ TruBio™ Bioprocess Control Software.

Step 5. Cryopreservation

The CTS Rotea system was used to prepare the CAR T cells for cryopreservation by concentrating, washing, and placing the cells into a cryopreservation medium. Next, the cells were cryopreserved using the Thermo Scientific™ CryoMed™ Controlled-Rate Freezer.

Results

This section focuses on the robustness of the closed modular system in each step of the workflow as presented in Figure 1. In the manufacturing of autologous CD19 CAR T cells for these studies, the lentivirus encodes a second-generation CAR construct.

Step 1. Processing of leukapheresis product for PBMCs with the CTS Rotea system, controlled by the DeltaV platform

The generation of autologous CAR T cells for therapeutic use began by taking blood from a donor's vein through a process called leukapheresis. The white blood cells were then separated from the rest of the blood products, e.g., RBCs, plasma, and platelets. The CTS Rotea system was used to perform this step in a single-use consumable where the RBCs were lysed and eliminated, the platelets were removed in the washing steps of the process, and the PBMCs were optimally concentrated for isolation of T cells. To test the robustness of this critical first step, seven fresh or frozen quarter-size Leukopak bags were used to isolate PBMCs from seven donors. Across seven different Leukopak bags processed in the CTS Rotea closed system, RBCs were lysed efficiently and PBMCs were recovered consistently (Figure 2A). Importantly, the viability (Figure 2B) was only minimally affected by the CTS Rotea system.

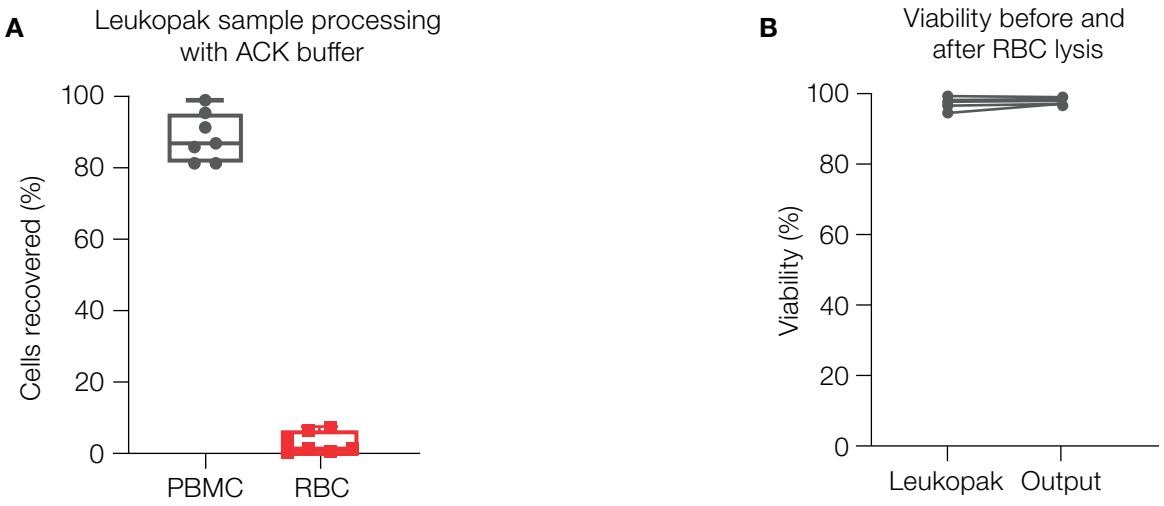

Figure 2. PBMC isolation using the Rotea system. PBMCs are efficiently recovered with no change in viability. Cells from Leukopak quarter packs were collected in the Rotea system and lysed with ACK buffer (n = 7). **(A)** 90% of PBMCs were recovered, versus 3% of red blood cells (CD235z$^+$). **(B)** No significant effect was observed on the viability before and after ACK lysis (P = 0.5).

Step 2. T cell isolation using the CTS DynaMag Magnet

Isolation of T cells from the processed PBMCs, as detailed in step 1, was carried out using CTS Dynabeads CD3/CD28. This is a one-step process to simultaneously isolate and activate T cells, which has been optimized to be used in numerous clinical research settings. As shown in Figure 3, a high percentage of the T cells were initially enriched during the PBMC isolation (step 1) using the CTS Rotea system. The cells were further enriched to 93% after step 2, using CTS Dynabeads CD3/CD28 and the CTS DynaMag Magnet.

Step 3. The generation of CD19 CAR T cells

In the context of manufacturing autologous CAR T cells, the gene transfer step used here is a well-published procedure and involves transduction of the isolated and activated T cells with a lentivirus vector made using the Gibco LV-MAX Lentiviral Production System [4,5]. T cells isolated from 3 of the 7 donors were used for the generation of CD19 CAR T cells. Transduction was performed with a lentivirus-encoded CD19 CAR at an MOI of 10, one day after T cell isolation and activation. CD19 CAR expression was assessed on day 3 or 4 and again on day 6. As shown in Table 1, CD19 expression on CAR T cells ranged from 20% to 60% among the three independent runs.

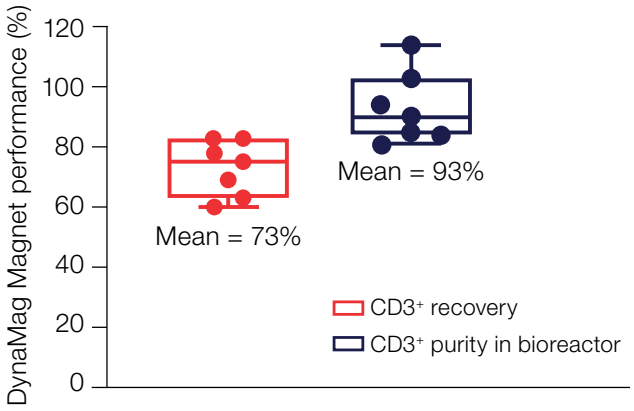

Figure 3. T cell isolation using CTS Dynabeads magnetic beads and CTS DynaMag Magnet controlled by DeltaV platform. T cells ($CD3^+$ $CD56^-$) were enriched to 73% post PBMC isolation using the Rotea system, and then were enriched to 93% post T cell isolation using CTS Dynabeads CD3/CD28.

Step 4. Expansion of CD19 CAR T cells

Culturing and expanding the CAR T cell product in CTS OpTmizer T Cell Expansion SFM is one of the most crucial steps in the manufacture of autologous CAR T cells. All aspects of this process, including every component in the autologous CAR T cell infusion bag for each donor, can be subject to evaluation by the regulatory agency for continuous compliance with quality control. The chemistry, manufacturing, and controls (CMC) process has to be tightly controlled and consistent.

The closed, semiautomated CAR T cell expansion step was carried out using the 10 L Thermo Scientific™ HyPerforma™ Rocker Bioreactor or G-Rex system, either of which can be controlled by the HyPerforma G3Lab platform. The scale can be expanded to 50 L bags if needed. If the HyPerforma Rocker Bioreactor is chosen, this process can be controlled and automated by TruBio software powered by the DeltaV system, which conforms to regulatory requirements for use in cGMP-compliant processes. As shown in Figure 4A, in 6 days there was a 20-fold expansion of T cells grown in either type of bioreactor. These CAR T cells showed significant potency in killing cancer cells, when challenged with a $CD19^+$ leukemia cell line such as Nalm6 (Figure 4B).

As summarized in Table 1, in 6 days, consistent generation of high-quality autologous CD19 CAR T cells for research or clinical use was seen in all three runs. In addition, these CD19 CAR T cells were especially enriched for cell naive/central memory–like phenotypes; these cells are known to show better persistence and function in *in vivo* assays (Figure 5).

Table 1. Characterization of CD19 CAR T cultures on day 6 of the autologous CD19 CAR T cell manufacturing process.

	Day 6 analysis of CD19 CAR T cultures from 3 runs (donors)			
	% CD19 CAR T cells	CD4/CD8 ratio	Total cell number	Viability
Range	20–60%	1.4–3.5	5,230–16,950 x 10^6	91–96%

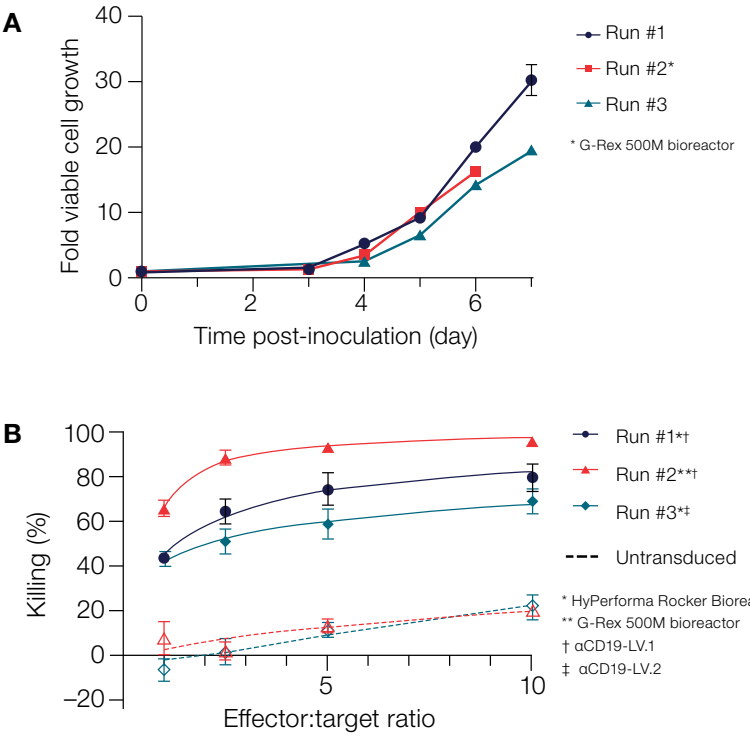

Figure 4. CD19 CAR T cell culture expansion and function. CD19 CAR T cells expanded robustly and were found to have high potency in killing cancer cells. **(A)** CD19 CAR T cells expanded in various bioreactor formats over 6–7 days—runs #1 and #3 in the 10 L HyPerforma Rocker BPC, and run #2 in the G-Rex 500M system. **(B)** On day 6, effector CD19 CAR T cells were challenged with Nalm 6 target cells (CD19⁺ leukemia cell line) at various effector:target ratios.

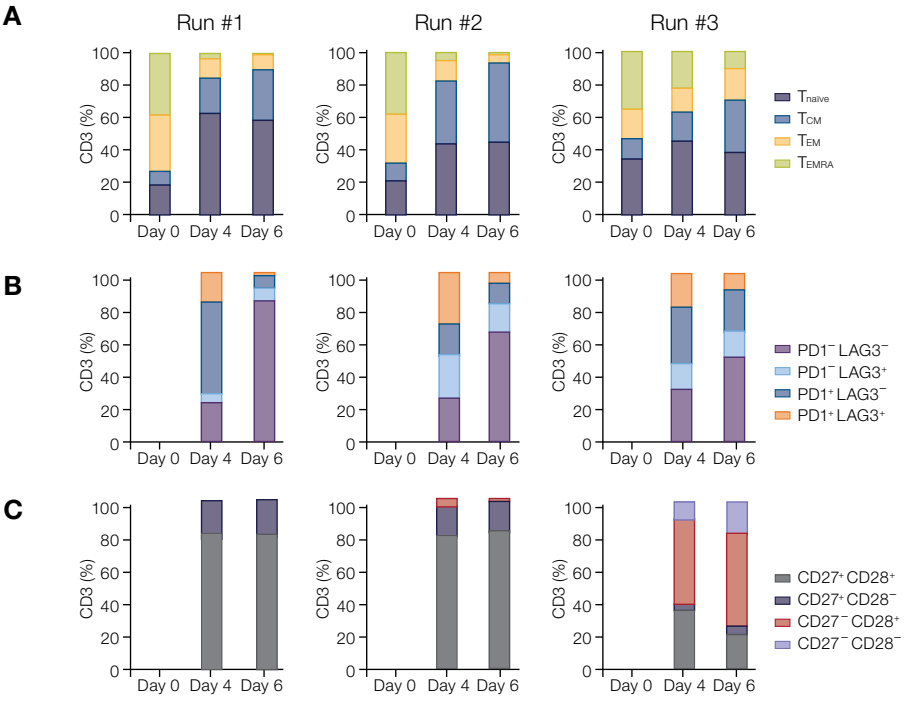

Figure 5. Analysis of CD19 CAR T cell cultures. Cell expansion condition favored both naive and central memory cell subsets over 6 days of expansion. Bioreactors used: HyPerforma Rocker Bioreactor 10 L for runs #1 and #3, and G-Rex 500M bioreactor for run #2. **(A)** T cell subsets markers: T_{naive} CD45RA⁺ CD62L⁺, T_{CM} CD45RA⁻ CD62L⁺, T_{EM} CD45RA⁻ CD62L⁻, T_{EMRA} CD45RA⁺ CD62L⁻. **(B)** Exhaustion T cell markers: PD-1, LAG3. **(C)** Naive-like markers shown to correlate with *in vivo* potency: CD27, CD28. CM: central memory; EM: effector memory; EMRA: effector memory cells re-expressing CD45RA.

Step 5. Cryopreservation of autologous CAR T cells.

The existing complex process for manufacturing autologous CAR T cells makes it challenging for the donors in clinical trials to receive freshly manufactured CAR T cells for therapy. Therefore, currently, they generally receive frozen CAR T cells transported from the manufacturing sites to the clinics, where the cells are thawed before infusion. For this reason, the cryopreservation of the CAR T cells needs to be as stringent and consistent as the manufacturing process, and requires strict regulatory compliance for clinical application if needed. Following preparation for cryopreservation with the CTS Rotea system, the cells were cryopreserved using the CryoMed Controlled-Rate Freezer, which has been routinely used in the cell therapy industry [6]. The controlled-rate freezing process maintained the integrity of the CAR T cells—the T cells were frozen, thawed, and monitored for 2 days for cell recovery, growth, and viability (Figure 6). The CD19 CAR T cells recovered and expanded well after they were placed in the CTS Rotea system and frozen using the CryoMed freezer.

Conclusions

Every component of the modular cell therapy manufacturing system described here is a GMP-compliant solution for manufacturing of CAR T cells. The "fit-for-purpose", semiautomated manufacturing platform for autologous CAR T cells includes modular instruments, proven CTS reagents, and a digital control system for the generation of a consistent CAR T cell product. The system described here is flexible and can deliver standardization, compatibility, and scalability in CAR T cell manufacturing. The modules in this system are compatible with most laboratory benchtops. The modular, GMP-compliant system allows laboratories to eliminate manual cell processing in manufacturing workflows. It is compatible with sterile consumables requiring only a class A laboratory environment, and it can be used to produce consistent CAR T cell therapy products.

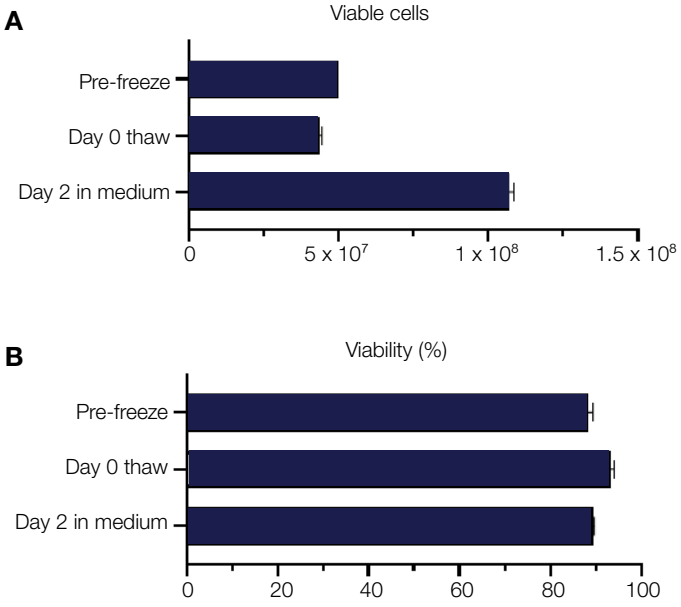

Figure 6. Characterization of cryopreserved CD19 CAR T cells. CD19 CAR T cell cultures showed consistent recovery after they were placed in the Rotea system and frozen in the CryoMed freezer. **(A)** Cell counts and **(B)** cell viability of cryopreserved and thawed CAR T cells.

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Find out more at **thermofisher.com/celltherapy**

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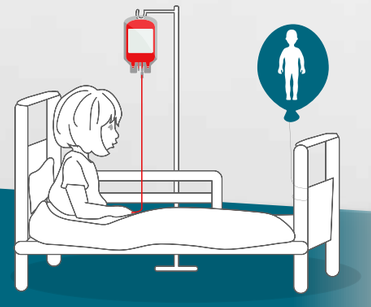
CELL THERAPY WORKFLOW

INTEGRATED SOLUTIONS FROM COLLECTION TO DELIVERY

The journey of the patient from vein-to-vein is a complex process that requires a multitude of products, instrumentation, equipment and regulatory compliant infrastructure. The CAR T cell therapy journey begins with harvesting blood from a sick patient, isolating and activating the appropriate T cell population, and then modifying these cells to express a chimeric antigen receptor.

When the cell engineering and processing is complete, a rigorous characterization and quality control process begins to ensure patient safety and efficacy. This expansive process requires a sample tracking system mechanism for traceability and logistical infrastructure to maintain functional integrity of the living drug product. The final step in the process is delivery of this most valuable product directly to the patient.



Beginning of patient journey

Collection & tracking

- Apheresis
- Supply/cold chain logistics

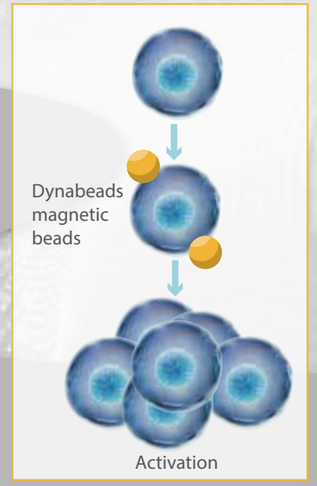
1

2

Cell isolation & engineering

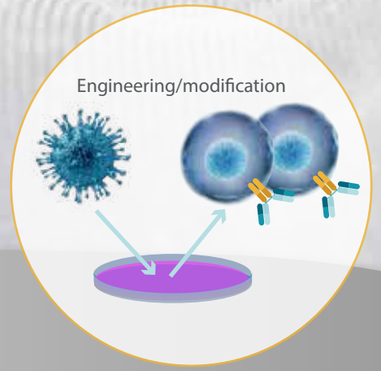
- Closed, modular cell processing systems
- Bead-based cell isolation and activation
- Single-use platforms
- Viral and nonviral gene delivery solutions
- CRISPR and TALEN genome editing technologies

Isolation

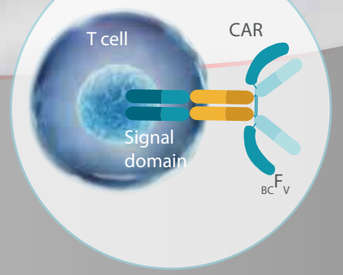


Dynabeads magnetic beads

Activation



Engineering/modification



CAR T cell

T cell

CAR

Signal domain

$_{BC}F_V$

3

Cell expansion

- Customizable media, reagents and supplements
- Closed modular cell processing systems
- Scalable single use platforms
- Specialized laboratory equipment
- Bioprocess automation and control platforms
- cGMP chemicals and process liquids

4

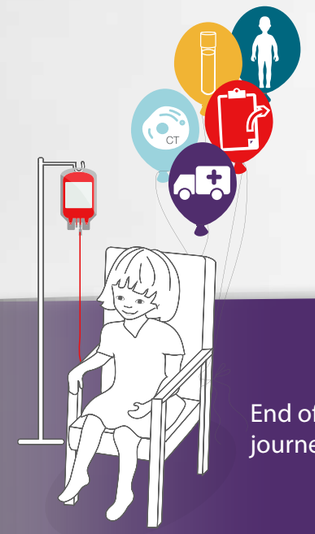
Characterization & cryopreservation

- Identity, purity and potency assays
- Contamination and process-related impurity detection
- Genomic, proteomic and cellular analytical tools
- Cryopreservation solutions

5

Supply chain & logistics

- Supply/cold chain logistics
- Clinical trial support
- Global distribution



End of patient journey

This infographic was created for the eBook on 'Cell therapy: optimizing the vein-to-vein workflow', produced by RegMedNet in association with Thermo Fisher Scientific.

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Cell Therapy Handbook

Considerations for cell therapy development and manufacturing

Whether you're new to cell therapy manufacturing or looking to expand your existing processes and knowledge base, this educational handbook will provide you with the major considerations for successful cell therapy manufacturing. The Cell Therapy Handbook reviews the latest methodologies, common practices, resources, applications and more, to support every step of your cell therapy manufacturing workflow.

The following pages showcase our two introductory chapters focusing on 'Raw material considerations for cell therapy manufacturing' and 'Cell therapy vendor qualification process', respectively. If you are interested in reading further, please visit our web page for full access to the remaining chapters in the handbook.

Intended use of the products mentioned in these documents vary. For specific intended use statements, please refer to the Instructions for Use (IFU).

Raw material considerations for cell therapy manufacturing

Introduction

Successful clinical translation of a cell therapy product hinges on early process- and material-selection decisions that impact the manufacturing process. Not only do materials present in the final approved drug (i.e., excipients) need to meet certain specifications, but raw materials (or ancillary materials) used in the manufacturing process must meet stringent quality standards. If the raw materials chosen early in the product development do not satisfy the necessary regulatory criteria at clinical trials and commercialization stages, they will need to be replaced with materials that do. Those substitutions can result in significant increases in costs and time.

The best practice to support successful clinical trials and commercialization of a cell therapy requires a raw material strategy with the end goal in mind. This longer-term view focuses on the use of higher grades of raw materials earlier in cell therapy product development to meet the necessary regulatory qualifications for clinical trials, and ultimately commercial manufacturing of the final approved therapeutic. This strategy can increase the probability of success and head off costly surprises that could cause an untimely demise for a promising cell therapy candidate.

In this section, we will provide a high-level overview of the considerations used to select raw materials that mitigate risk and align with current regulatory guidelines. For deeper discussion on this topic, please see Additional resources at the end of this section.

What are raw materials?

Raw materials, also referred to as ancillary materials in US regulations*, are components that come in contact with the cell therapy product during manufacturing, but are not intended to remain in the final therapeutic. Cell culture media and growth factors would be examples of raw materials employed in manufacturing a cell therapy. While not present in the final product, raw materials are still important because of their potential impact on the safety, purity, and potency of the final cell therapy product. Generally speaking, raw materials are not regulated products.

However, regulatory documents suggest that developers use therapeutic-grade raw materials whenever possible because of their potential influence on the characteristics and safety of the final cell therapy product. Unfortunately, therapeutic-grade versions will not exist for every type of raw material used. In these cases, the best option would be to choose raw materials manufactured under the appropriate current good manufacturing practices (cGMP).

While the same materials developed for research use only (RUO) and *in vitro* diagnostics (IVD) uses might be available, they will lack some of the necessary traceability and testing that will be required, particularly as a therapeutic moves further into clinical trials and hopefully commercialization. Raw materials designed for RUO or IVD use should be avoided in a long-term cell therapeutic manufacturing strategy.

No specific cGMP guidance exists for raw material manufacturing, unlike that found for medicines and medical devices. Regulatory guidance (e.g., USP <1043> and ISO276) only recommends choosing raw materials made under an appropriate quality management system, a rather vague term. Suppliers may say their products are manufactured under cGMP conditions, with claims ranging from declarations of cGMP based on following particular cGMP guidelines; independent quality management system certification (e.g., ISO9001); or even regulatory agency inspection if the site is manufacturing regulated products. However, there is no such thing as a defined "GMP-grade" material.

The first place to begin to understand what is meant by cGMP manufacturing of a raw material is the published regulatory guidance documents. Table 1 presents some of the main guidelines across different regions. Japan, Europe, and the United States have the most detailed raw material guidance documents, with Japan having some of the strictest.

**There can be some confusion around the terms "raw materials" and "ancillary materials" across regions – Europe uses the term "raw materials", whereas the US uses the term "ancillary materials". Ancillary materials are also synonymous with "processing materials", as defined in 21 CFR Part 1271 and "components" in Pharma cGMP Part 211.*

Table 1. Raw material regulatory guidance from major jurisdictions.

Region	Raw material regulatory guidance
International 	• WHO GMP for Biological Products • Various ISO standards (ISO 9001, 13485 and TC276) • Various ICH guidelines (ICH Q5A, ICH Q5D, ICH Q3, ICH Q2)
Australia 	• Australian regulatory guidelines for biologicals (ARGB) – critical raw materials used in manufacturing

Table 1. Raw material regulatory guidance from major jurisdictions (cont.).

Region	Raw material regulatory guidance
Japan 	• PMDA MHLW Public Notice No. 210 – Standard for Biological Ingredients • Raw material certification process available
Europe 	• ATMP Regulation (EC) No 1394/2007 • Ph. Eur. 5.2.12 Raw Materials of Biological Origin for the Production of Cell-Based and Gene Therapy Medicinal Products • EudraLex Volume 4 GMP guidelines (May 2018)
United States 	• USP <1043> – Ancillary Materials for Cell, Gene and Tissue-Engineered Products • USP <92> – Growth Factors and Cytokines Used in Cell Therapy Manufacturing (limited to rh-IL4) • FDA chemistry, manufacturing and controls (CMC) guidance • 21 CFR 1271.210 – GTPs • Part 211 CFR Part 11 subpart E – GMPs • Master File process available

One of the most globally recognized raw material guidance documents is USP <1043>. USP <1043> presents a risk-based model based on 4 risk categories that are used to assess each raw material (Table 2). These risk categories are defined by specific activities required of the manufacturer [1]. The required activities of each risk level are phased, with a subset required of all products (e.g., Certificates of Analysis and lot-to-lot testing). As the risks associated with the raw material increase, different activities are also required (e.g., safety testing for residual materials containing animal products). Risk also increases as the product moves into later phases of clinical testing. Product developers should aim to source tier 1 and 2 raw materials; tier 3 is less favorable; and tier 4 should be avoided for clinical work. Zero risk is unattainable, so developers will strive to maintain the lowest risk possible when selecting raw materials while still maintaining performance.

Table 2. USP <1043> raw material risk categories.

Tier	Risk level	Description	Example
1	Lowest	Highly qualified material suited for CGT manufacturing	Rh-insulin for injection used as a cell culture medium additive
2	Low	Well-characterized, intended for use as raw material, manufactured under a quality management system in compliance with GMP	Gibco CTS media and reagent products
3	Moderate	Not intended to be used as a raw material	RUO or IVD materials such as some cell culture media
4	High	Not produced under a recognized quality management system, not intended for use as a raw, animal-derived or toxic, biologically variable	Animal cells or animal sera, cholera toxin used in cell culture or selection agents for transgene expression

Critical quality attributes of raw materials in cell therapy manufacturing

When choosing raw materials for use in cell therapeutic manufacturing, developers typically focus on four key product characteristics:

- Material identity
- Purity and presence of impurities
- Lot-to-lot consistency
- Storage and stability

Table 3 summarizes some of the important details to consider for each of these characteristics. With the lack of global standards for critical quality attributes, cell therapy manufacturers will choose raw materials that will meet the standards of the region with the most stringent requirements.

Table 3. Considerations for four key raw material characteristics.

Characteristic	Look for
Identity and freedom from microbial or viral contamination	• Any information on the molecular composition or formulation • If material is proprietary, documentation on the activity of the active components • COO, health statement, and pathogen testing for animal-derived materials • Required viral testing and donor eligibility/screening documentation for human-derived materials
Purity and impurity	• Documentation on purity • For multiple component products, purity of active ingredients • Identification of impurities should be documented • Assays to detect residuals
Consistency	• Supplier effort to determine lot-to-lot consistency on Certificates of Analysis • GMP-manufactured materials easier to demonstrate consistency
Storage and stability	• Supplier's recommended storage conditions (e.g., temperature, light, humidity) demonstrating that raw materials maintain consistent performance • Product shelf life backed by stability testing that reflects product use as a raw material

With regards to a material's identity, cell therapy manufacturers should pay close attention to biosafety characteristics to determine any risks a material might bring to the facility, to the operator, and in the final cell therapy product. The preference is to avoid animal origin components when possible. When this is not possible, a risk-based approach to these raw materials will become important, using the following considerations:

- Possible alternatives (e.g., recombinant proteins)
- Viral inactivation process
- Upstream vs downstream use (risk increases the further downstream a raw product is used)
- Grade of material (e.g., cGMP compliance vs RUO)
- Demonstrated product traceability and documentation (from supplier)
- Country of origin (important for CJD, BSE and TSE risk)

It is within this biosafety area where some of the terminology used can become confusing. When selecting affected raw materials, a cell therapy manufacturer should gain a clear understanding of a supplier's definitions at all levels of manufacture for terms such as "animal origin-free", "serum-free", and "xeno-free" to fully understand the potential risks associated with the raw material.

Cell therapy manufacturers need to also consider the performance testing of raw materials in their final material decisions. The supplier should provide performance data that is reflective of a product's intended use as a raw material. For example, a performance test for media using a CHO cell line is of little use if the intended use of the media is to grow T cells. The data should also enable developers to determine the performance consistency of a raw material, with quantitative data being better than pass/fail results. To assess the data accurately and determine its relevancy to the intended use, the supplier should provide the assay methodology used, preferably using reference test methods (e.g., United States Pharmacopeia or USP).

Whenever possible, developers should choose USP/EP grades of raw materials with monographs. Monographs ensure the raw material meets specific quality standards for identity, strength, quality, and purity determined by specific tests, procedures, and acceptance criteria. If monographs are not available, some (if not all) of the important attribute information discussed should be found in a supplier's material documentation (see Key raw material documentation below). However, it is possible that the developers will need to perform additional testing to make final robust material decisions and mitigate risks associated with any raw material. Even though no global quality standards exist, it is best for manufacturers to choose raw materials that are fully characterized in order to ascertain the risks associated with them, including:

- Numerical specifications for test methods on Certificates of Analysis to demonstrate lot-to-lot consistency
- Performance tests focused on intended use in the manufacturing process, with stability tests linked to performance
- Traceability of biological-derived raw materials to primary, secondary, and tertiary levels, provided on Certificates of Origins
- USP test methods or validated in-house methods used and reported on the Certificate of Analysis

Key raw material documentation

Much of the information on raw material quality attributes discussed above can be found in various supplier documentation (Table 4, Figure 1). Besides determining the appropriateness of a raw material for cell therapy manufacturing, some of the information found in these various documents will be necessary for a variety of regulatory filings. In cases where raw materials contain proprietary components or formulations, developers

should look for suppliers who can provide that information through Regulatory Support Files, which are provided under signed confidentiality agreements. Some regions (e.g., the United States) support Master Files provided by suppliers for the sharing of proprietary materials with the appropriate regulatory agencies. Master Files do not require signed confidentiality agreements and can be a faster way to get the necessary information for regulatory filings.

Table 4. Raw material supplier documentation types.

Documentation type	Description
Certificates of Analysis (COA)	COAs contain information on product lot; product shelf life and expiration; identity; quantity; purity and impurities; safety; and biological activity.
Certifications of Origin (COO)	COOs demonstrate supply chain control (traceability), which is particularly important for human- and animal-derived products.
Safety Data Sheets (SDS)	SDSs, provided as applicable, contain information on the properties of each material and their physical, health, and environmental hazards and subsequent protective measures associated with them. They also contain necessary safety precautions for handling, storing, and transporting the material.
Certificate of Compliance (COC)	COCs may be provided to support compliance claims about quality systems or standards.
Regulatory Support Files (RSF)	Under confidentiality agreement, this summary provides product performance, stability, quality control, and analytical testing methods specifically designed to meet cell therapy raw material regulatory requirements. Used when Master Files are unavailable.
Master Files (DMF)	A detailed submission to a regulatory body that provides confidential information about facilities, processes, and raw materials used in the manufacturing, testing, processing, packaging, and storage. Only available in the United States, Canada, and Japan.

- GMP manufacturing (21 CFR part 820 and certified to ISO 13485)
- Detailed Certificate of Analysis (COA) and Certificate of Origin (COO)
- Drug Master File (DMF) or Regulatory Support File (RSF)
- Aseptically sterile product (validated SAL 10)
- Endotoxin and *Mycoplasma* tested
- Performance tested (T cell functional assay)
- Adventitious viral testing of human-derived proteins and access to viral inactivation data
- Proven use in cell therapy manufacturing

Figure 1. Example of important product characteristics that cell therapy manufacturers should look for in various product documentations.

The Gibco CTS Immune Cell Serum Replacement is an example of a reagent specifically designed for use in cell therapy manufacturing that meets documentation requirements. It complies with the raw material guidances in the United States, Europe, and Japan. The reagent is intended to replace the use of human serum when performing *ex vivo* culture of human lymphocytes.

Supplier and developer responsibilities

Ultimately, it is the drug manufacturer's responsibility to assess the risks associated with and suitability of the chosen raw materials, with much of this assessment occurring during the vendor qualification process (read more about Vendor Qualification). Table 5 provides some of the important items to address during this process to mitigate risk associates with the cell therapy.

Table 5. Some key considerations for raw material risk assessment.

Raw material	Considerations
Source	• Is this material human-, animal-, or recombinant-derived? • Is the source a viral concern? • Can the material be replaced with lower risk substitutes?
Manufacturing	• What are the cGMP, aseptic, and cross-contamination concerns in non-dedicated facilities? • Is there possible exposure of the material to other human and animal products during manufacturing? • Has the supplier's manufacturing site been audited by our team?

Table 5. Some key considerations for raw material risk assessment (cont.).

Raw material	Considerations
Testing	• What tests are available that demonstrate the material's identity, purity, safety, and performance? • Has any viral inactivation been performed? Is it validated?
Traceability	• Can the supplier demonstrate material traceability on all risk components and their supply chain?

The raw material supplier(s) also have obligations during the selection process and beyond. It is important that the cell therapy manufacturer and the raw material provider(s) understand their responsibilities throughout the clinical trial and commercialization process and work together to meet these in a timely manner (Table 6).

Table 6. Responsibilities for cell therapeutic manufacturers and their suppliers.

Activity	Manufacturer	Supplier
Qualify the performance of raw material for intended use	✓	
Provide COA, COO, SDS for raw material		✓
Ensure that the raw material is safe with respect to human/animal diseases		✓
Conduct a risk assessment of the raw material for use in cell therapy manufacturing	✓	
Confirm COA tests critical to final cell therapy product	✓	
Characterize the raw material and set specifications		✓
Assess lot-to-lot variation of the raw material on the final cell therapy product	✓	
Determine biocompatibility, cytotoxicity, and additional safety testing (if not available from supplier)	✓	

Table 6. Responsibilities for cell therapeutic manufacturers and their suppliers (cont.).

Activity	Manufacturer	Supplier
Assess residual raw materials in the final cell therapy product	✓	
Assess stability of the raw material		✓
Prepare regulatory support documentation (Master File or RSF)		✓
Execute quality and supply agreements	✓	✓

Summary

Numerous regulatory challenges exist in the selection of quality raw materials for use in cell therapy manufacturing. No global standard is available covering the critical attributes of raw materials used in a cell therapy, making it difficult to cover all regions a drug might be used. There is also no specific cGMP guidance on the manufacturing of raw materials used in cell therapies and confusion over terminology used exists, making identification of an appropriate supplier a more burdensome process. These challenges put more pressure on developers to define a strategy that balances the costs and performance of a raw material, while mitigating risks. This strategy should be formulated with a long-range view so as to avoid the need to substitute raw materials at later stages of clinical development and trials. This might include developing a product to meet the most stringent regulatory requirement of the regions it is intended.

A cell therapy's ingredients are critical to developing a reproducible and robust manufacturing process. Proper sourcing of materials early in development of a cell therapy from reliable suppliers who make products specifically for cell therapeutics can shorten the development timeline, dramatically reduce costs, and improve the likelihood of approval from regulatory authorities.

Additional resources

This article provides an overview of the numerous challenges and considerations that a cell therapy manufacturer must address while selecting appropriate raw materials. For more in-depth discussions on these topics, we recommend Manufacturing pluripotent cell therapeutics [2], a webinar on GMP ancillary materials for cell and gene therapy manufacturing, and numerous publications from a variety of regulatory agencies, including:

- Ph Eur 5.2.12 Raw Materials of Biological Origin for the Production of Cell-Based and Gene Therapy Medicinal Products
- USP <1046> Cell and Tissue Based Products
- USP <1047> Gene Therapy Products
- USP <1043> Ancillary Materials
- USP <1024> Bovine Serum
- USP <90> Fetal Bovine Serum
- USP <89> Enzymes used as Ancillary Materials
- USP <92> Growth Factors and Cytokines
- Japan's Standard for Biological Ingredients+
- ISO Working draft Ancillary Materials present during the production of cellular therapeutic producs

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[2] Thermo Fisher Scientific (2020). Manufacturing pluripotent cell therapeutics. https://www.thermofisher.com/us/en/home/global/forms/life-science/download-cell-therapy-stem-cell-whitepaper.html

Intended use of the products mentioned in these documents vary. For specific intended use statements, please refer to the Instructions for Use (IFU).

Cell therapy vendor qualification process

Introduction

Vendor (or supplier) qualification (VQ) is the process of determining a vendor's capability to fulfill the specified requirements of necessary products or services. For manufacturing of cell therapies, necessary goods and services can cover a broad range including raw material selection, aseptic filling, manufacturing, formulation and cryopreservation services, analytical assays, kitting services, and cold chain distribution. The VQ process informs all involved parties that the products and/or services meet the acceptable criteria for identity, quality, and purity and provides assurance that the product and service consistently meet the specified GMP requirements.

The cell therapy industry is in its nascent stage, currently with minimal standardized regulatory policies or guidelines for VQ. However, most manufacturing entities (sponsors and contract development and manufacturing organizations) adhere to standards established by the FDA, the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH E6 R2 and ICH Q10), and the International Organization for Standardization (ISO). Unfortunately, these agencies provide minimal details on VQ programs specifically for cell therapy manufacturers, requiring these manufacturers to establish a robust VQ program as a first step in monitoring, identifying, and mitigating vendor-associated risks.

A typical VQ process can be divided into 4 steps:

1) Define vendor requirements and develop a vendor questionnaire
2) Compile a list of potential vendors and evaluate capabilities to identify the top candidates
3) Conduct a comprehensive audit and choose appropriate vendors
4) Develop and implement vendor requalification plan

Define vendor requirements

The first step in a vendor evaluation begins with defining vendor requirements and designing a comprehensive questionnaire. Vendor requirements should address several varied attributes (see Table 1 for some topics). The questionnaire should also address other significant vendor policies such as change control management (e.g., changes in internal suppliers and production locations; change notification policy timing and exceptions). In the end, the vendor's responses to the questions should help assign risk levels to several key areas including the vendor's process performance and quality management system, corrective action and preventive action (CAPA) system, and change management system (CMS; see Compile a list of potential vendors and access their capabilities below).

Table 1. Common topics covered in a vendor selection questionnaire.

- Vendor's skills to deliver the materials and services
- Vendor's open, timely, and transparent communication, with a well-defined plan to manage emergencies
- Vendor's control of its policies and procedures to ensure consistent performance
- Vendor's commitment to maintain quality and performance
- Vendor's guarantee in the form of documents that prove the ability to deliver consistent product/services for timely delivery
- Vendor's sustainability policy
- Cost associated with requested goods and services
- Capacity for timely delivery of required products
- Policies and strategies in place to anticipate and mitigate changes related to the internal supply chain, warehouse, raw materials, and manpower
- Financial standing (cash reserves) and resources to cover any future increased commercial manufacturing demand
- Alignment of supplier and customer corporate cultures and core values

Compile a list of potential vendors and assess their capabilities

The next steps include developing a list of relevant vendors of the raw materials and determining each vendor's capabilities and other attributes based on their responses to the questionnaire. While the questionnaires are being completed by potential vendors, the internal team conducts a further internal assessment focusing on a literature review, the vendor's technical capabilities, the vendor's regulatory history (e.g., FDA 483 documents, recalls, warnings, etc.), the vendor's annual reports, and any previous or current client references.

Upon receipt of the completed vendor questionnaire, the Quality Assurance (QA) team of the manufacturing entity reviews the questionnaire for completeness and acceptability within a specified time period from receipt. Any identified concerns arising from the internal research or the questionnaire triggers a written response to the vendor's quality team requesting specific clarifications needed to make a final selection. Once all questions are answered satisfactorily, the initial evaluation process is complete, resulting in a narrowed vendor list. The evaluation also highlights specific items that require close scrutiny in the audit phase.

An important step in this exercise includes evaluation of the vendor's own supply chain strategy. This relates to understanding the quality and origin of the vendor's raw materials, business continuity and contingency plans for uninterrupted supply.

One of the worst case scenarios facing a cell therapy manufacturer is the need to replace or substitute a raw material during clinical trials. Likewise, once a product is commercialized, the manufacturer needs to closely monitor the raw material supplies in order to avoid delays in production. The supply chains for manufacturing specific cell therapies can be quite complex and require overseeing of a large number of suppliers. A risk-based approach to this oversight can simplify that task [1].

A risk-based strategy would evaluate the individual raw materials based on their criticality to the manufacturing process, leading to a framework that allows the manufacturers to assign risk levels to the supplier's capabilities. This approach also allows manufacturers to better allocate time and resources to monitor the materials after commercialization. Table 2 provides an example of some general factors associated with risk, although individual manufacturers would probably have additional issues to add to each level.

Table 2. Risk levels (adapted from Reference 1).

Risk level	Associated factors
High	• Custom product with no alternatives or alternatives that would be hard to qualify • Product used in critical steps (e.g., direct and/or patient contact) • Specified source in license where alternative would require additional testing (e.g., stability testing)
Medium	• Product alternatives available • Product used upstream in process; general usage; used in well-established steps • Alternative product available with agency pre-approval or only moderate testing
Low	• Multiple qualified product alternatives available; safety stock possible • Product used in well-established steps that are common practice in the industry • Alternative product available with minor regulatory concerns requiring only notification or minimal assessment

Conduct audits and choose final vendor(s)

This narrowed group of vendors moves to the next evaluation step. This process, known as an audit, is ideally conducted by a cross-functional team that includes members from QA, process development, manufacturing, and analytical development as well as other technical experts. It is best practice for the manufacturing entity to have a standard

operating procedure (SOP) to assess vendor capabilities and attributes under a variety of audit levels.

The type of audit conducted is based on many criteria including past relationships with the vendor, the longevity of the approval status, whether the vendor supplies critical or non-critical products/services, and risk assessment strategies of the manufacturing entity. Several types of auditing processes exist that are categorized by levels of stringency:

- No audit or check list – minimal impact materials
- Retrospective audit – qualification based on past performance
- Paper audit – qualification by an audit check list
- On-site audit

The types of audits and the frequency of audits required must be defined in the specific VQSOP. It is also common practice to define ongoing audit frequencies in a requalification plan (see Develop a vendor requalification plan below).

Once the audit is complete, the audit team generates an assessment covering the suitability of the supplier's facility and its quality management systems, the supplier's staff and departmental organization (both staff levels and skill sets), a review of the supplier's documentation procedures (e.g., relevant SOPs), and a review of the supplier's supply chain. In some instances, it might be appropriate to ask the supplier to manufacture a test lot of the raw material prior to final selection.

Once the assessment is complete, the QA team along with designated personnel makes the final vendor selection(s). When a vendor is "Approved", QA updates the Approved Vendor/Supplier List and issues a letter of approval to the vendor. If the vendor is deemed "Not Approved", the QA team will collaborate with relevant departments to determine what additional information and/or steps are required to qualify the vendor. Non-approved vendors can be reconsidered if they provide additional information and/or put a Corrective Action and Preventative Action (CAPA) in place. If such vendor responses are satisfactory, the vendor may be "Approved". If the responses are not satisfactory or the vendor is not willing to make appropriate changes, they will remain "Not Approved."

In certain exceptions (e.g., additional information is not available and/or there is no immediate alternative), a risk assessment plan is put in place to determine if the vendor can be used until further required actions are taken to avoid a shutdown of the manufacturing activities. A Quality Agreement is then put in place for all approved vendors.

Develop a vendor requalification plan

After the VQ process and final vendor selection, a plan and SOP is developed for vendor requalification using defined and pre-established intervals. While the initial VQ process

involves a detailed evaluation of a vendor's attributes and capabilities, a clinical and commercial manufacturing program's success relies on VQ as an ongoing process, with regular supplier meetings and audits to maintain the highest quality of products and services. The requalification plan defines the types and frequency of audits and is shared with the vendor. The requalification plan also identifies instances that would trigger additional audits, such as a change in manufacturing location, addition of new plants or warehouses, moving operations to another country and change in raw materials due to global shortage of existing raw materials.

Costs associated with VQ process

The financial impact of vendor qualification is high, potentially adding to the cost of new commercial cell therapies. An estimated $130–150 million (USD) is spent annually for on-site and remote new vendor qualification assessments [2]. Table 3 shares some typical costs for various entities. These costs do not include the cost of periodically requalifying existing vendors or the indirect costs of distributing and evaluating requests for information.

Table 3. Typical costs (in USD) associated with VQ audits (VQA) [2].

	Average cost per VQA ($)	Average yearly VQA cost ($)
Overall	13,259	270,033
Sponsors	12,432	197,940
CROs	18,704	666,883
Small companies	12,607	150,570
Medium companies	17,072	475,445
Large companies	21,839	1,886,308

Summary

The high-level regulatory requirements established by the FDA, ICH, and ISO are useful, but lack standardization and specificity for implementing a VQ program for cell therapy manufacturing. This results in highly variable and labor-intensive VQ programs and processes, which can lead to delays and increased cost burdens to cell therapy products. Until the cell therapy industry establishes standards to streamline the VQ process, a current best practice requires a collaborative relationship based on open and timely conversations, clearly defined expectations during the qualification process, and a plan to manage risk and achieve success for both parties.

– Author: Rupa Pike, PhD (Thermo Fisher Scientific).

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Perspective

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Regenerative Medicine

Improved harmonization of critical characterization assays across cell therapies

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The field of cell therapy has blossomed, providing exciting new options for treating a variety of diseases. While few cell therapy products have US FDA approval, there are thousands of cell treatments at various stages of development, pointing to a potential revolutionary shift in patient care. The expanding number and nature of cellular therapies necessitate greater standardization. Several international organizations are collaborating to pursue some level of global standardization, especially concerning cell banking. However, less harmonization surrounds assays used for critical quality characterization including: identity, purity, safety and potency. Frequently, there is divergence regarding the terms describing the characterization assays across regulatory authorities and guidances. This review summarizes the critical quality assays currently used for different categories of cell therapies. Areas of harmonization and an absence of standardization are highlighted. We propose potential solutions to facilitate harmonization of critical quality characterization assays and the language used to describe them.

First draft submitted: 6 January 2020; Accepted for publication: 3 May 2020; Published online: 26 June 2020

Keywords: cell therapy • embryonic stem cells • hematopoetic stem cells • induced pluripotent stem cells • industry • manufacturing • mesenchymal stromal cells • regulation

Regenerative medicine is an exciting and rapidly developing treatment arena. Yet, only recently has the larger regenerative medicine industry matured to provide cell therapies directed at a variety of indications. In doing so, the field is moving from a clinical manufacturing model, generally occurring in select academic institutions, to an industry model with centralized manufacturing [1]. Currently, the US FDA lists 14 approved cellular therapy products [2]. Half of these are allogeneic umbilical cord blood hematopoietic progenitor cells (HPC). For allogeneic HPC cord blood, the FDA has provided guidance documents setting out the criteria for manufacturing, including the purity, potency and identity criteria [3].

The remaining cellular therapies are indicated for a variety of disorders including lymphoma, prostate cancer, cartilage defects and cosmetic applications. While only 14 products have been approved, there are currently numerous active human clinical trials using cell therapies. In 2020, Clinicaltrials.gov listed over 1800 active/recruiting clinical trials utilizing some type of stem cell collection or therapy [4]. The FDA has provided numerous Guidance for Industry documents with respect to developing therapies based on human cells. However, with the plethora of cell therapies under investigation, the field could benefit from a more defined approach such as that taken by the FDA in the case of allogeneic HPC cord blood [3].

Many reviews have focused on the challenges of cell therapy manufacturing including the need for closed automated systems that can scale and the innate variability of the cellular starting material, which hampers standardization [5–9]. Several international organizations have worked diligently to create standardization and global harmonization in the field of regenerative medicine. Many of those groups have focused on cell banking. Work by the International Stem Cell Banking Initiative resulted in numerous publications on scientific consensus on topics of expansion, storage and characterization of pluripotent stem cells [10]. In addition, an international consortium established the Minimum Information About a Cellular Assay for Regenerative Medicine in 2016 for cell banks and

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Table 1. Examples of discrepancies in critical quality assays for cell therapies.

HPC cell assay categories [5]	Recommended assays	Somatic cell assay categories [14]	Recommended assays
Safety	Infectious diseases	Microbial testing	Adventitious agent testing
	Sterility		Sterility
	Hemoglobin		Mycoplasma
Purity and potency	Total nucleated cells	Purity	Residual contaminants
	Viable nucleated cells		Pyrogenicity/endotoxin
	Viable $CD34^+$ cells		
		Potency	Measure of biological activity
Identity	Human leukocyte antigen typing	Identity	Distinguish the product from others manufactured in the same facility using surface markers or genetic polymorphisms
	Confirmatory HLA typing		
	Blood group and Rh Type		
Other	None	Other	Viability
			Cell number/dose

The table highlights examples of inconsistent classification of critical quality assays depending on the cell type and the US FDA guidance. For HPCs the FDA guidance sterility testing is classified as a safety test, but for somatic cells, it is listed as a microbial test. Potency and purity are a combined category for HPC cells, but separate categories for somatic cell therapies. Viability tests are listed under purity and potency for HPC cells, but under the 'Other' category for somatic cells.
HPC: Hematopoietic pluripotent cell.

registries [11]. It provides and exhaustive list of cell characterizations for cell banking with the goal of standardizing the data collected on cell lines, but it does not provide guidelines for characterization assays for product release.

Less attention has been placed on the assays used to characterize the quality of either the starting materials or the final product [12]. When comparing FDA guidances for somatic cell therapies versus multipotent stromal cell (MSCs), there are discrepancies between the organizational structure and recommended product tests [3,13]. Table 1 illustrates the confusion. The first two columns provide the categories of recommended critical quality assays for HPCs, which are multipotent cells. The last two columns provide the same information provided by the FDA for somatic cell therapies (unipotent cells). While the HPC guidance is more detailed in the recommended assays compared with a general guidance for somatic cells, still it is clear that the same tests are listed in different classifications between the two guidances. For example, viability of HPCs is listed under the category of 'Purity and potency' [3], but the same tests are listed in the 'Other' category for somatic cell therapies [13]. A comparison of information provided in Table 1 uncovers a variety of discrepancies including different titles for the general categories such as safety or microbial testing. The guidance for somatic cell therapies has a separate category for potency tests, while the HPC documents include potency with purity assays. The goal of this review is to compare the current critical quality characterization assays across different categories of cell therapies, highlighting areas of inconsistency and potential for further harmonization. We conclude by suggesting simple alignments across categories for consideration in the context of efforts underway by standard coordinating bodies.

Cell therapy categories

Cell therapies and cell therapy products can be grouped into three distinct categories based on the starting cell source or material: pluripotent stem cells (embryonic or induced), multipotent (somatic or adult) cells and terminally-differentiated unipotent cell products. Figure 1 provides a simplified hierarchical organization of the various cell sources based on the development potential of the cell, illustrating the relationship of these cell categories to each other. Table 2 summarizes the three categories, listing the general characteristics and providing examples of approved applications for each group.

Pluripotent stem cells include both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Embryonic stem cells are derived from blastocysts, are indefinitely self-renewing and can differentiate into unlimited types of cells. iPSCs are artificially derived cells from adult, differentiated somatic cells that start out as nonpluripotent and are reprogrammed into a pluripotent state. Reprogramming protocols convert the differentiated cells back into embryonic-like states (Figure 1, red arrows) from which they can then progress down any specific lineage, similar to the embryonic stem cells. The figure illustrates this reprogramming for the mesodermal lineage with red arrows, but ectodermal and endodermal cells can be similarly reprogrammed. Figure 1 illustrates that both ESC and iPSC have an inherent ability to produce differentiated cells from any of the three germ layers, dependent upon their external cues in the environment.

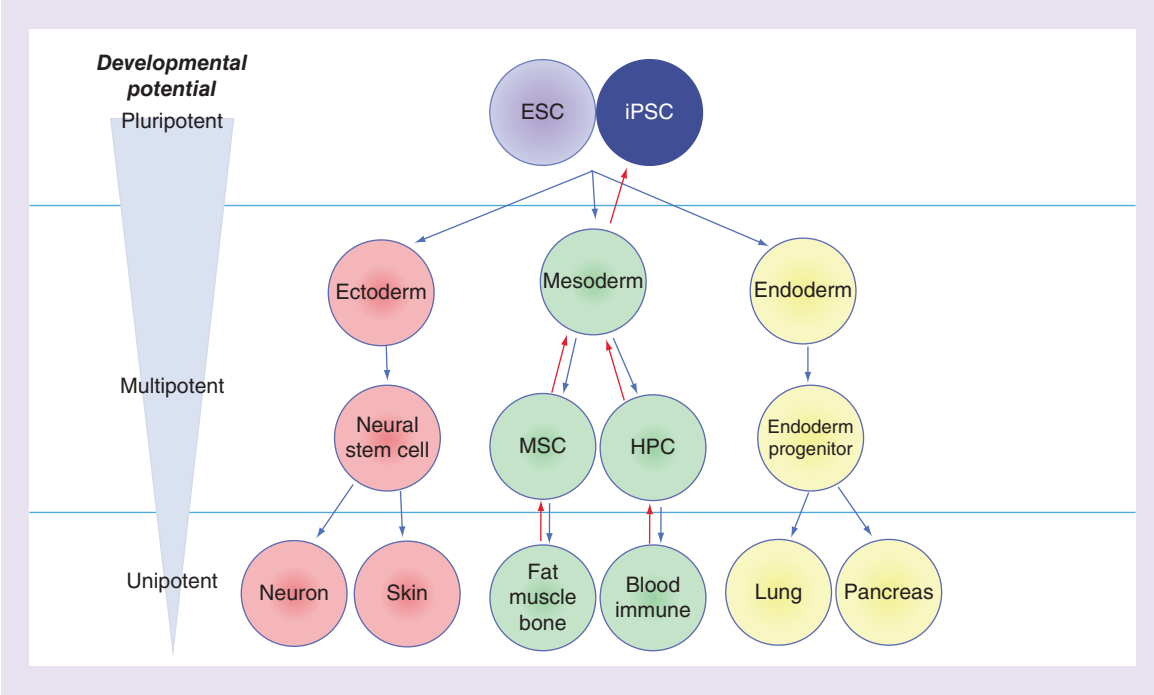

Figure 1. Stem cell differentiation pathways. The capacity of ESCs to differentiate into somatic cells relies on committed pathways via specific somatic lineages (mesoderm, endoderm or ectoderm). Multipotency and replicative potential decline with increased commitment. The blue line represents the ability of ESCs to differentiate into cells of any of the three lineages. The red arrows illustrate a typical pathway for creating an iPSC from somatic tissues.
ESC: Embryonic stem cell; HPC: Hematopoietic progenitor cells; iPSC: Induced pluripotent stem cell; MSC: Multipotent stromal cells.

Table 2. Cell therapy categories.

Category	General characteristics	Starting material	Challenges	Approved therapies	Ref.
Pluripotent stem cells	Self-renewing and pluripotent	iPSC is derived from a somatic cell. ESC from blastocysts	Cells must be differentiated prior to transplant. Potential for tumorgenicity	No current approved therapies	[2,12,15]
Multipotent stem cells	Limited self-renewal, multipotent	Adipose, amniotic tissue, bone marrow, hematopoietic	Limited renewal hampers expansion of cells for transplant. May be transplanted as stem cell or differentiated. Lower potential for tumorgenicity.	Hematopoietic stem cell transplants	[2,5,12,16]
Unipotent cells	Terminally differentiated, not self-renewing or multipotent	Dependent on the cell therapy	Source must have significant volume of cells because of lack of expansion capability, unless genetically modified	CAR-T therapy, autogeneic islet transplants, fibroblasts, chrondrocytes on a membrane	[2,17,18]

The three categories of cell therapies, pluripotent, multipotent and unipotent cells, are described including their general characteristics and the starting material from which they arise. The current major challenges are provided along with approved therapies in each category, when appropriate.
CART-T: Chimeric antigen receptor T-cell therapy; ESC: Embryonic stem cell; iPSC: Induced pluripotent stem cell.

While there are no FDA-approved ESC or iPSC treatments have FDA approval, as of early 2020 there were 43 active human clinical trials summarized on Clinicaltrials.gov using ESCs, which is seven-times more than were listed in 2018 [4]. While the same site lists over 90 clinical trials using iPSCs, the majority are limited to tissue collection with only seven trials appearing to utilize iPSCs for treatment of a disorder [4]. Of the three cell categories (pluripotent, multipotent and unipotent), a significant amount of work has been done to regulate and standardize the characterization of pluripotent stem cells (PSCs) [15].

Multipotent stem cells are another category of stem cells found in most tissues from the body. These cells comprise some stem cell properties but differ from pluripotent cells in that they have a limited ability to renew. Typically, these cells can be differentiated only into cells within their lineage (Figure 1), giving rise to the term

multipotent. Similar to pluripotent cells, differentiation of multipotent stem cells is dependent upon external cues in the environment.

While there are many clinical trials utilizing multipotent cells, currently only HPC transplants are approved by the FDA for patient treatment [3]. As shown in Figure 1, these cells arise from the mesoderm lineage and can produce any of the mature functional hematopoietic cells of the body, including the immune system. Autologous HPC transplants are often used to rescue the hematopoietic toxicity of high dose chemotherapies for patients with blood cancers [16], making them the most common applications for multipotent cells [19].

MSCs, first called mesenchymal stem cells [20,21], are another category of multipotent cells. They are widely popular for research and clinical trials, partly because the tissues from which MSCs are isolated are easily retrieved and are most frequently autologous, with the exception of those derived from amniotic tissues [22]. MSCs have been derived from a long list of tissues including lung, fallopian tube, fetal liver, amniotic tissues, umbilical cord, fat, skin, muscle, liver and dental tissue. By definition, MSCs can be differentiated into three terminal cell types: osteogenic, adipogenic and chondrogenic, but, like all multipotent cells, they have limited self-renewal capabilities. Typically, they lose the ability to differentiate at about 5–12 passages *in vitro* [23]. In 2020, there were over 1000 human clinical trials listed on Clinicaltrials.gov, using the term mesenchymal stem cells or mesenchymal stromal cells, with most therapeutic applications utilizing autologous tissue harvested from fat or bone marrow and more recently allogeneic MSCs isolated from amniotic tissues [4].

Unipotent cells are terminally differentiated cells that have limited renewal properties or developmental potential (Figure 1). A high number of unipotent cell therapies are currently in different stages of development. For regulatory purposes the FDA excludes blood transfusions from their definition of somatic cell therapies [17,24]. Rather, the FDA regulates the safety of blood through current good tissue practice [17].

Other types of unipotent cell therapies include the transplantation of islets, the insulin-producing clusters of cells taken from the pancreas. Islet transplants have been used to treat severe Type 1 diabetes for 20 years. While considered standard of care in other countries, allogeneic islets transplants are still classified as experimental in the US and patients can only receive them by enrolling in a clinical trial [25]. However, autologous islet transplants for the treatment of conditions such as pancreatitis are approved and covered by private insurance in the US (Table 2).

Some unipotent cell therapies involve genetic modification, such as therapies utilizing chimeric antigen receptor T-cells (CAR-T), which are autologous, genetically-modified T cells. The genetic modification optimizes the T cells to actively proliferate and recognize cancer cells. This is an extremely active field of study with two approved CD19-directed CAR-T-cell treatments as of 2020 and over 2500 current clinical trials for genetically modified cellular therapies for a variety of indications [4]. Additional examples of unipotent cell transplants include lymphocyte infusions to treat relapsed leukemia [26] and mature dendritic cells to assist in abating solid organ transplant rejection [27].

Each type of cell therapy has unique challenges, but a consistent hurdle that must be overcome is the reproducible manufacturing and testing of the cell therapy product. Autologously derived cells are inherently variable, reflecting the age, health status and genetic makeup of the donor, making the characterization of the starting material more challenging than traditional biologics [28]. Additionally, cells are not inert materials; rather they change over time and in response to external signals [29]. Given this inherent variation, valid release testing and product characterization is essential to ensure consistency in cell therapy manufacturing.

Critical quality attributes & their assays across cell therapies

The critical quality attributes that allow for the release of a cell therapy are specified in the US Code of Federal Regulations (21CFR610) as sterility, purity, identity and potency. As discussed previously, other regulations do not use the same organizational structure. For example, safety, sterility, purity and potency, but not identity, are defined in 21CFR600.3 [30]. In 21CFR600.3(p) safety is defined as "*relative freedom from harmful effect to persons affected, directly or indirectly, by a product when prudently administered, taking into consideration the character of the product in relation to the condition of the recipient at the time*". Thus, while the HPC Guidance uses the term 'safety' to describe the testing of infectious diseases and sterility, safety should encompass a broader set of tests for quality, nonclinical and clinical testing. In fact, safety is captured in all of the critical quality attributes of the cellular product.

The FDA has issued several guidelines describing how the agency interprets these definitions with respect to critical quality attributes needed for release of cell-based therapies [30–32]. Sponsors developing a cellular therapy must provide data supporting the assays used to accurately and reproducibly confirm: the cellular identification of the final product, the preparation purity, sterility and potency [31]. The schematic in Figure 2 organizes these

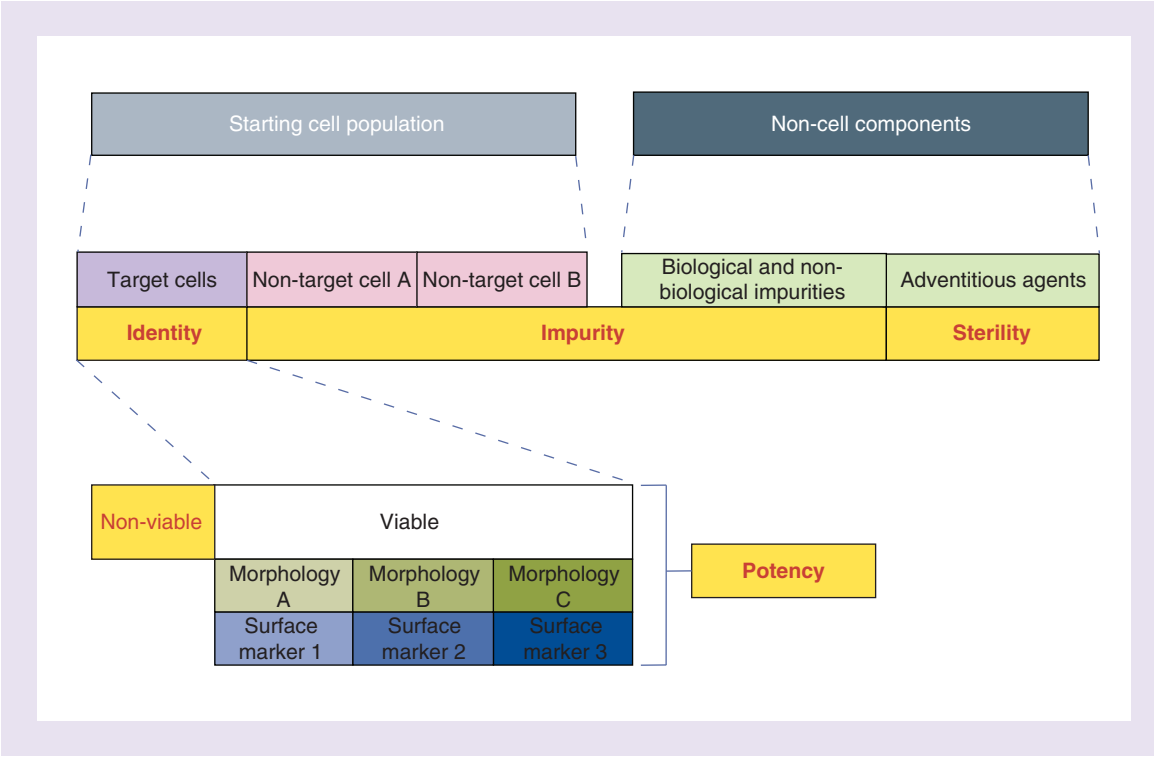

Figure 2. Organization of critical characterization assays. Cell therapies contain noncellular components and a cellular population. Testing of the target cellular component determines the identity and the cellular purity of the preparation. Assays focused on the noncellular components test for impurities. Components that include adventitious agents are considered part of the sterility assessment. Characterization of the target cellular component can define the potency of the product including assays for viability, cell number and the assumed mechanism of action.

four critical quality attributes based on a format suggested by authors from the National Institute of Standards and Technology [33]. We have adapted their framework which describes the composition of a cellular preparation and organized it according to identity, impurity, sterility and potency testing.

The characterization process begins with a starting cell population comprised of the target cells along with other nontarget cells (Figure 2). The 'Identity' of the preparation is determined by quantitative testing (phenotypic or biochemical assay) to confirm that the target cells are present in the product. Characterizing the nontarget cells is required for assessing the 'impurity' attributes of the preparation. This includes identifying and quantifying other extraneous matter, including biological and potential nonbiological impurities such as reagents. In practical terms purity and identity are often provided in a single heading due to their overlapping assays and results [33]. By clarifying identify as the description of the target cells and impurity as identification of nontarget materials and cells, better harmonization across cell therapies can be achieved. Similarly, unwanted microorganisms affect the 'sterility' of the product, resulting in a critical characteristic with respect to safety. Finally, the target cells must be tested for 'potency'. Viability should be considered a critical potency assay for all cell therapies along with the cell number and individual tests based on the assumed mechanism of action. The schematic in Figure 2 creates a common approach and language for all cell therapies.

While the schematic may seem well defined, a lack of clarity and common definitions still creates confusion. For example, several characterization assays are placed under multiple headings of identity, purity, sterility and potency. For instance, an assay focused on the safety concern of endotoxins is listed as a purity attribute in some FDA guidances [32], while bacterial testing is listed under sterility. Given the fact that endotoxins are products of bacteria, it would be more informative and appropriate to include both under the same category.

Development of an assay for assessment of critical quality attributes is a progressive, iterative process. A better understanding of these terms and definitions and their application in the development of cellular therapies will lead to more efficient delineation of critical quality attributes needed for product release and licensure, providing

Table 3. Current cell identity assays.

Characterization	Pluripotent stem cells	Multipotent stem cells	Unipotent cells	Ref.
Molecular markers	STR genotyping			[35–37]
Surface markers (starting material)	SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, Nanog, SOX-2, OCT-4	HPC Lin$^-$, CD34$^+$, CD38$^-$, CD90$^+$, CD45$^-$ MSC CD73$^+$, CD90$^+$ CD105$^+$ CD34$^-$, CD45$^-$, CD11b$^-$, CD14$^-$, CD19$^-$, CD79α^-	Cell-specific, but typically surface markers tested using flow cytometry	[36–43]
Phenotypic markers	Colony formation (number, density and quality) EB formation with detection of trilineage (mesoderm, ectoderm, endoderm)	Colony formation Adherence to plastic Differentiation into adipocytes, chondrocytes and osteogenesis	Microscopic identification	[42–44]
Cellular morphological	Nuclear/cytoplasmic area	General morphology	General morphology	[45]
Teratoma	Negative	Negative	Not required	[46]
Karyotype analysis	46, XX or XY	46, XX or XY	Not required	[47,48]
Other tests	ALP staining Genome-wide gene expression pattern should match the donor	SA-β-Gal SRC assay (*in vivo*) LTC-IC	Not required	[49]

Determining the identity of the different cell categories consists of tests that are specific to each cell type as illustrated. Few tests or biomarkers can be utilized for more than one category.
CD: Cluster of differentiation; EB: Embryoid body; HPC: Hematopoietic pluripotent cell; Lin: Lineage negative; LTC-IC: Long-term culture-initiating cell; MSC: Multipotent stromal cell; SRC: Severe-combined immunodeficiency mouse-repopulating cells; STR: Short tandem repeats.

a clearer approach to the design and implementation of characterization assays. Below, we review the four critical quality attributes in more details for each category of cell therapy.

Identity

Assays to determine identity are used to confirm that the product contains the intended cellular and noncellular components and are a part of the critical quality attribute for the product [12]. The FDA does not define identity, but only specifies that identity must be established. It is up to the sponsor to provide the method of identification, whether it is through morphological features, chemical characteristics or *in vivo* tests [14]. These assays are essential for testing the starting material and equally important for the final product because cells can undergo changes during the manufacturing process. While the concepts of identity and purity are fairly straightforward when the drug is a chemical entity or protein, they become extremely complicated when the 'drug' is composed of cells containing thousands of different proteins, lipids and nucleic acids in addition to multiple cell types [34]. Further, each cell is slightly different, even when the starting material is banked ESCs. Assays for cellular identity are some of the most disparate between the different types of cell therapies and are summarized in Table 3.

Pluripotent stem cells

The identity of the starting material or master cells used for PSC therapies must be established and carefully monitored over time. Significant work in this arena has been undertaken by other organizations regarding identity assessments [15,29]. In fact, across the world there is wide acceptance of the identity criteria for PSCs and they will briefly be summarized here as other reviews provide more detail on the topic [15,50].

Because of the inherent risk of transformation of the cells during expansion and manufacturing, the genetic identity of the cells compared with the starting material is essential. The International Stem Cell Banking Initiative provided critical steps to characterize iPSCs. This group concluded that short tandem repeats (STRs) should be a mandatory assay for identity and that the acceptance criteria should be an identical match of STRs with the original cell source (Table 3) [51]. STRs are 1–6 base pair repeated DNA sequences that have a high mutation rate that can be affected by cell population doubling and the sex and the age of the donor [51]. The exact STRs that should be included in testing has been standardized by the US Federal Bureau of Investigation [35]. The objective of STR genotyping of pluripotent cells is to demonstrate that the STRs for an iPSC line or later passages of ESCs are identical to those of the starting cells [36,37,51].

Chromosomal aberrations for ESCs and iPSCs are a hallmark of human cancer due to potential genetic instability [47]. Thus, part of identity testing involves knowledge of the genetic stability of the cells. Karyotyping via

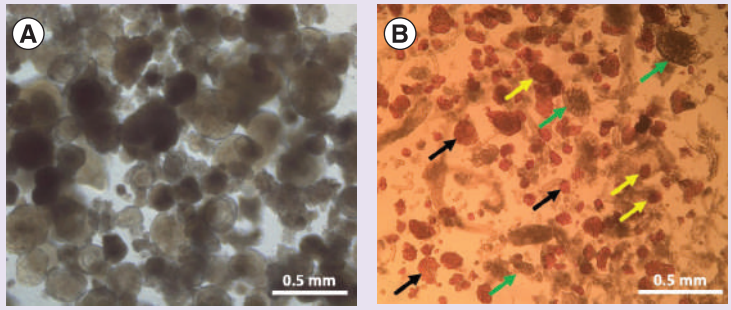

Figure 3. Examples of ambiguity inherent in some morphology assays. Often the quantification of image-based assays can introduce subjectivity into the assay. **(A)** Embryoid body-like cell clusters formed from differentiated human induced pluripotent stem cells develop in a variety of sizes and shapes and often bind together, making the counting and measuring of the embryoid body-like clusters unclear. **(B)** The assay to identify endocrine from exocrine somatic cells for islet transplants uses dithizone to stain islet cells red (black arrows highlight some examples) and nonislet cells are left a brown shade (green arrows). However, there are many cell clusters that are ambiguous in their color. Examples are illustrated with yellow arrows.

G-banding detects gross chromosomal duplications, deletions or translocations [47]. The International Stem Cell Banking Initiative Guidelines for release testing of banked cell lines suggest counting at least 20 metaphase spreads with greater than 95% of the cells determined to be of normal karyotype [48]. As Table 3 shows, identification of 46 chromosomes with either XX or XY is essential.

Identity for the starting ESC or iPSCs also includes assessment of membrane-bound surface markers. The major surface markers are identical for iPSC and ESC and include SSEA-3 and SSEA-4, which are canonical cell surface markers and tumor recognition antigens TRA-1-60 and TRA-1-81 [38] along with self-renewal genes such as Nanog and SOX2 (Table 3) [39,40]. While individual product specifications are different, most manufacturing sites use a 70% cut-off for major surface markers for rejection of a batch, meaning that at least 70% of the cells should be positive for these markers [12,36,37]. Upon differentiation for the end product, the pluripotent markers decrease in expression levels. Thus, the final product will have a very different surface marker profile than the starting material. For example, differentiated cardiomyocytes require the inclusion of muscle-specific genes or proteins like those associated with sarcomeres, gap junctions and ion channels [52]. It is important to remember that even fully differentiated cells may hold some epigenetic memory of their initial source [53] and can dedifferentiate at points in the manufacturing process or postrelease.

The level of ALP is another marker of stem cells because they have high levels and high activity of ALP. As pluripotent stem cells become committed to a lineage, ALP expression downregulates and it only appears in discrete specialized somatic cells [49], for example, in osteoblasts.

Phenotypic or morphological assessments are essential for identifying the master pluripotent cells. Although not automated and somewhat subjective, these manual assessments are still considered standard procedures. Embryoid body formation is commonly used to verify the pluripotency of human ESC and iPSCs (Table 3) [51]. Colonies are graded based on colony number, density and quality [54]. Figure 3A provides another example of the complexity when attempting to characterized EB (embryoid body)-like cell clusters differentiated from pluripotent cells. The figure shows differentiated human iPSCs cultured in suspension that form EB-like clusters. The extreme variability in size and shape makes grading the EB-like clusters based on number, size and quality prone to inaccuracies. Other cellular morphological assessments are quite common [45]. One example consists of measuring the ratio of nuclear area to cytoplasmic area, which is typically high in pluripotent cells [47].

Multipotent stem cells

The identity of the source material for multipotent cells is significantly more complex than for pluripotent cells. By nature, the starting material for multipotent cells is human tissue, which is a heterogeneous mix of cells and support matrices. Those working in the hematopoietic cell field have established unique *in vivo* and *in vitro* assays to test HPC identity and functional activity. One *in vivo* assay first described in 1997 is the severe-combined immunodeficiency mouse-repopulating cells assay. The test identifies cells capable of repopulation in conditioned

immunodeficient mice (Table 3) [46]. Alternatively, the long-term culture-initiating cell assay, is an *in vitro* assay comprised of culturing cells on bone marrow feeder cells to determine the capability of the stem cells to produce mature hematopoietic cells [55]. Unfortunately, both assays are lengthy, making them difficult to implement in real time release testing. For example, the long-term culture-initiating cell test can require 6 weeks or longer.

Surface markers for HPC identity include $CD34^+$, $CD38^-$, $CD90^+$ and $CD45^-$ as defined by the International Society for Cellular Therapy [41,56]. In contrast, multipotent stromal cells have at least 42 different surface proteins that could be used for identification [42]. The International Society for Cellular Therapy suggests that the positive cell markers (CD73, CD90 and CD105) should be expressed in >95% of the MSCs in the sample and the cells should be negative for CD34, CD45, CD11b, CD14, CD19 and CD79α (Table 3) [56]. Other groups have suggested labeling for different proteins including STRO-1, CD271, SSEA-4 and CD146 to identify MSCs [42,43]. This has led some experts in the field to suggest that the surface antigens for MSCs are dependent on the starting cell source and therefore a single set of surface markers cannot be used to identify the category of multipotent cells [42,57].

To highlight the disparity of markers in the multipotent cell category, a review of investigational new drug (IND) applications at the FDA revealed that many companies are not using the International Society for Cellular Therapy Guidance. In 66 different INDs, a lack of CD45 expression was the most commonly used indicative surface marker, followed by $CD105^+$ and $CD90^+$. The remaining surface markers were only utilized in approximately half of the applications, while other markers such as CD29, CD106 and CD80 were used in about a quarter of the applications [58]. The review of FDA INDs provides a window into the great variability in the manufacturing and characterization of cell therapy products.

Further identification by the International Society for Cellular Therapy identifies MSCs by their ability to adhere to plastic. However, only a fraction of the plastic-adhering cells exhibits multipotency [43]. Another test of identity is the trilineage test; the ability to differentiate into chondrocytes, osteocytes and adipocytes [42,43]. Unfortunately, there is great variation in the differentiation protocols used around the world, which can influence the outcome of the test [42]. In addition, it is thought that only a small percentage of cells within the culture can differentiate into specific lineages; cells that are more likely to produce adipocytes are less likely to differentiate into osteocytes and vice versa [59].

Like their pluripotent relatives, multipotent cells should be assessed for phenotypic characterization. Early in the manufacturing process, multipotent cells have the ability to form colonies [44], much like pluripotent stem cells. Colony formation is a very crude estimate of the MSC titer, but a routinely accepted standard for identity [42].

Finally, MSCs have the unique disadvantage in that they cannot be passaged in an unlimited fashion such as ESCs [23] but are still susceptible to chromosomal aberrations with passaging. Thus, karyotyping should be performed, similar to PSCs [6]. While the antigen markers discussed above have been shown to be preserved through multiple rounds of passage in culture, the phenotype of the cells clearly changes. The standard assay for MSC senescence is expression of SA-β-Gal [60].

Unipotent cells

The concept of cell identity for somatic cell therapies is itself complicated. For example, matrix-induced autologous chondrocyte implantation (MACI) is an approved treatment for lesions of the articular cartilage utilizing the patient's own cells in an engineered matrix. Current manufacturing of MACI utilizes genetic markers to identify the chondrocytes in the product. Comparisons with other tissue engineered-products shows great variability between the final products, partly due to the inherent variability in the starting material [61].

When the final product is a heterogeneous cell cluster such as pancreatic islets, additional challenges occur. Identity assessment in these cases tend to be more subjective. For example, the standard test to determine insulin-producing β-cells within islets is a subjective colorimetric assay, based on the Zn-binding probe, dithizone. The insulin producing β-cells have a higher Zn content than surrounding cells and thus stain a red color when exposed to dithizone, as shown in Figure 3B. The image shows a preparation with a high level of dithizone-positive cell clusters (red) compared with the darker brown nonislet tissue. Black arrows identity examples of the dithizone-positive cells and green arrows distinguish the nonislet clusters. However, there are cell clusters that are ambiguous (yellow arrows) and whether they are identified as islet or not, is subjective. Differentiating between the islet and nonislet tissue using dithizone staining is tedious and extremely subjective, leading to high intratechnician reproducibility with a coefficient of variance of up to 16% [62].

Table 4. Current cell purity assays.

Characterization	Pluripotent stem cells	Multipotent stem cells	Unipotent cells	Ref.
Other cells (final product)	Below 5%: $CD34^+$ Low levels of OCT4, NANOG, LIN 28A, TROMA-1 For iPSC: Low levels of $CD13^+$	Below 2%: $CD34^+$, $CD45^+$, $CD11b^+$, $CD14^+$, $CD19^+$, $CD79\alpha^+$	Off-target cells Below 80%: $CD3^+$ (for CAR-T treatments)	[53,54,56,66]
Tumorgenicity	Negative	Negative	Not required	[67]
Serum albumin	<1 ppm	<1 ppm	<1 ppm Exception for blood transfusions	[12,63,64]
Endotoxins	<0.5 EU/ml	<0.5 EU/ml	<0.5 EU/ml	[65]
Competent vectors and DNA	No trace of residual vectors or DNA	Not required unless genetically altered	Not required unless genetically altered	[68]
Other	Reagents including benzonase	Not required	For genetically-modified cells, no trace of benzonase	

Assays that fall under the current heading of purity assays have some areas of agreement, such as the level of serum albumin and endotoxins. Other purity assays are unique to the type of cell being tested.
CD: Cluster of differentiation; CART-T: Chimeric antigen receptor T-cell therapy; EU: Endotoxin units; iPSC: Induced pluripotent stem cells; LIN: Lineage negative; ppm: Parts per million.

Purity

For almost every cell therapy, there will be a purification step in the manufacturing process, utilizing methods such as fluorescently- or magnetically-activated cell sorting, metabolic selection or density gradient separation along with other less common methods [48]. It is important to assess any impurities that may result from these manufacturing steps, such as the potential for introduction of adventitious agents and residual beads. Additionally, it is unlikely that the final product will contain a single pure cell type, meaning that the nontarget cells included in the product should be considered impurities and may represent a potential safety risk (Figure 2) [12]. The 21CFR600.3 defines purity as "*the relative freedom from extraneous matter in the finished product, whether or not harmful to the recipient or deleterious to the product*" [30]. In current regulatory documents, impurities are varied and include endotoxins, residual proteins, vectors or DNA, along with contaminating cells or culture reagents such as fetal bovine serum.

For some of the characterization assays under the heading of purity, there is wide agreement on the assay outcome. Culturing cells with animal serum is still common in cell manufacturing and often is essential for some cells to survive, although new animal serum-free supplements have shown promise [12,63,64]. Federal regulations determine that the animal serum is an impurity and levels must be below 1 ppm in the final product, no matter the type of cell therapy (Table 4). In addition, endotoxin levels must be below 0.5 EU/ml for the final product to be released regardless of the cell category [65].

Pluripotent stem cells

While most surface markers for iPSCs and ESCs are used for positive identification, unwanted cells in the final products can be ruled out using the same surface markers. For the starting material, it is common to set expression of unwanted $CD34^+$ cells at levels below 5% of the cell population to qualify (Table 4) [53]. Other surface markers can be used to identify nontarget cells such as endoderm and trophoblast cells. One of the most common is TROMA-1 which is directed against cytokeratin-like filaments of trophectoderm and endoderm cells [54]. For iPSCs it is important to rule out high numbers of $CD13^+$ cells as this is a marker for fibroblasts, which are often used as the starting cells in the manufacture of iPSCs.

As the manufacturing process progresses and pluripotent cells are differentiated into the final cell product, some of the most important contaminants are the residual undifferentiated stem cells that could eventually proliferate in transplanted hosts to form teratomas [69]. Tumorgenicity is one of the greatest safety concerns for pluripotent and multipotent cells (Table 4). The delivery of cells with an unlimited ability to renew and the capacity to differentiate into other cell types carries a significant risk that must be addressed [67]. The gold standard test for teratoma formation is to inject the cells into immunodeficient rodents and wait for the formation of a teratoma in the animal [67]. Only a few undifferentiated cells are theoretically needed to form a teratoma in mice, although for testing purposes typically 3–5 million cells are injected into immune-deficient mice [70]. However, this test is costly, time consuming and has animal welfare concerns when used on a wide scale, as identified by the International Stem Cell Initiative [71]. Alternative, more quantifiable methods are needed, such as *in vitro* assays, including qRT-PCR [72].

Initial work with iPSCs included reprogramming the cells using retroviral constructs, which were permanently integrated into the cell genome. This method left great uncertainty concerning the long-term effect of the viral DNA. The residual transgenes could affect the function of the cells, but more importantly posed a potential safety risk to the recipient [68]. More recently, safer, nonintegrating viral particles such as Sendai virus or mRNA reprogramming can transfect cells that are used for clinical programs [72,73]. Regardless of the method used, assessment of residual transgenes must be viewed as a potential impurity.

Multipotent stem cells

For HPC cord blood, the FDA has provided purity specifications that must be met with respect to number of total nucleated cells, viable nucleated cells and viable $CD34^+$ cells [3]. It is uncommon for the FDA to set defined purity specifications for a cell therapy, thus for the majority of cellular therapies there is wide variation and uncertainty in which surface marker assays should be employed for multipotent cells. This also hampers the evaluation and quantification of impurities related to 'inactive' or unwanted cell populations. As stated earlier, the International Society for Cellular Therapy considers cellular impurities for MSCs as those cells with surface markers for $CD34^+$, $CD45^+$, $CD11b^+$, $CD14^+$, $CD19^+$, $CD79\alpha^+$. These cells should make up <2% of all the cells in the final preparation (Table 4) [56].

Another consideration with respect to multipotent cells is the lack of stability over time, which affects the identity of the cells. In one study, ten serial culture passages without differentiation caused changes in $CD45^+$, $CD34^+$ and $CD73^+$ levels compared with the unpassaged bone marrow-derived HPCs [66]. In addition, there were changes in expression levels of $CD98^+$, $CD205^+$ and $CD106^+$ cells, all suggesting a decrease in the stemness and thus purity of the HPCs [66].

Concerns of teratoma formation are not as great with MSCs as with pluripotent cells, due to their limited development potential. In fact, decades of use of MSCs and HPCs in the clinic have found few major health concerns around teratoma formation, yet studies have shown that approximately 10% of MSC samples contain chromosomal aberrations after expansion [74]. It should be noted that for many MSC therapies there are few long-term follow-up studies and so the postmarket safety data is incomplete [75].

Unipotent cells

Donor tissue used for unipotent cell transplants will always contain a mixture of cells, often including vascular cells and neurons. The best approach for the manufacturing site is to identify the nontarget cells and determine their percentage in the starting material and the final preparation (Table 4).

Purity is important when manufacturing a CAR-T therapy as it may have an impact on patient outcomes. For CAR-T cell therapies, in addition to the peripheral blood mononuclear cells obtained from the patient during apheresis, the starting sample contains red blood cells, monocytes, platelets and several other blood cells. The T-cell content can be enriched using $CD4^+/CD8^+$ antibodies on bead conjugates [18]. The percentage of the nontarget cells after enhancement has been shown to impact the overall success of the CAR-T manufacturing process [28].

As mentioned previously, endotoxin levels must be below 0.5 EU/ml for the final product to be released regardless of the cell therapy category [65]. Reports of islet transplants with endotoxins show that the frequency and level of endotoxin contamination declined from 1999 to 2010, but still averaged 7.8 EU per patient from 2007 to 2010 with few or no associated adverse events [76],

Sterility

According to the FDA, sterility is defined as freedom from viable contaminating microorganisms, as determined by the test requirements specified under 21CFR610.12 [30]. Typically, testing for sterility includes assays for bacterial, viral, fungal and mycoplasma [12,77].

Obviously the more a cell therapy is manipulated during the manufacturing process, the higher the chances for contamination of the product, but even minimally manipulated products can contain contaminants [78]. A large study of over 4000 HPC products found that over 1% were contaminated, most commonly with *Staphylococcus epidermidis* [78]. Most of these contaminated products were transplanted into the recipients either by choice or necessity, due to the low yield of cells in the manufacturing process and the dire needs of the patient. Few significant adverse clinical outcomes have been reported [79–81]. Contamination can arise from several different sources such as the initial donor sources or from the manufacturing processes and reagents, including incubators, water baths, lab benches, sinks and human error [81].

Table 5. Current sterility assays.

Characterization	Pluripotent stem cells	Multipotent stem cells	Unipotent cells	Ref.
Virus (donor screening)	Negative: HIV types 1 & 2, HBV, HCV, human transmissible spongiform encephalopathy, syphilis	Negative: HIV types 1 & 2, HBV, HCV, human transmissible spongiform encephalopathy, syphilis	Negative: HIV types 1 & 2, HBV, HCV, human transmissible spongiform encephalopathy, syphilis	[30,48,82]
Bacteria/fungus	Negative	Negative	Negative	[12,77]
Mycoplasm	Negative	Negative	Negative	[12,83,84]
Teratoma	Negative	Negative	Not required	
Karyotype analysis	46, XX or XY	46, XX or XY	Not required	

Sterility assays have a high level of harmonization across cell categories and are comprised of viral screening of the donors and testing of the starting material and end-product for bacteria, fungi, mycoplasm, teratomas along with karyotype analysis.
HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Uniform sterility assays

Sterility testing is the single critical quality classification that does not require separate tests for different cell therapy categories and can be reviewed as a general topic. For all allogeneic cells and tissues, the FDA requires screening of the donors in accordance with good tissue practice guidelines. This includes screening donors for: HIV types 1 and 2, hepatitis B and hepatitis C, human transmissible spongiform encephalopathy and syphilis (Table 5) [30]. However, there are cases when additional tests must be conducted for infections such as vaccinia, sepsis, West Nile Virus, SARS and Zika Virus [30,82]. Donors of leukocyte-rich cells or reproductive cells have additional requirements [30]. Other organizations such as International Stem Cell Banking Initiative Guidance suggest additional tests for Epstein–Barr Virus, cytomegalovirus, papillomavirus, herpes simplex virus and herpes viruses [48]. FDA regulations do not require testing for autologous donors for transmissible agents.

For all cell therapies, in-process sterility tests for bacteria and fungus are the only way to test for microbial levels [77]. Fungi infections are rare, but when they occur, they typically include the candidiasis and aspergillosis families. However, most of the time, these infections come from medical treatments during or after the infusion, such as catheters and other breaks in the skin and are not from the donor tissues [85]. Standard sterility tests require some of the longest incubation periods (14 day) and the results are often not known until after the lot has been released and administered to a patient. However, in certain cases the FDA has now cleared several automated systems for rapid sterility testing, such as BacT/ALERT and BACTEC [86].

Studies on allogeneic islet transplants have determined that a clinically significant number of human islet final preparations contain microbial contamination, ranging from 16–66% depending on the manufacturing site [87–89]. Bacterial contamination of hematopoietic stem cell products is estimated at 4.5% for peripheral blood progenitor cells and as high as 26% for bone marrow harvests [90]. Most studies found that microbial contamination had little or no effect on the patient outcome, nor was it associated with local or systemic infections [87–91].

Mycoplasma is a unique member of the bacteria family and must be negative for all cell therapies. Mycoplasma are significantly smaller than other bacteria and can pass through a 0.1 μm filter [83]. Without a rigid cell wall, they are resistant to most common antibiotics. Once the mycoplasma enters the cell, it is difficult to kill even with mycoplasma-effective antibiotics [84]. Mycoplasma contamination is surprisingly common in cell culture systems and can have a serious negative impact on the outcome of the therapy, including causing genetic instability, physiological changes and increased viral susceptibility [84]. Mycoplasma are often introduced into the manufacturing process by personnel. For example, more than half of all mycoplasma infections in cell cultures are correlated with the healthy human oropharyngeal tract [84].

Potency

Potency is a critical attribute of any cell therapy, confirming that the product possesses a biological function that is relevant to treating the clinical indication [31]. The FDA interprets assessment of potency measurements as "*used to demonstrate that only product lots that meet defined specifications or acceptance criteria are administered during all phases of clinical investigation and following market approval*" [31]. Best practices entail choosing potency assays that are relevant to the expected mechanism of action for the specific indication [12], linking it to the *in vivo* functions and to clinical efficacy [92].

Table 6. Examples of potency assays utilized for select cell therapies.

Product	Cellular identity	Current potency assays	Ref.
Cord Blood from multiple organizations	HPCs	Total nucleated cells, $CD34^+$ cells and cell viability	[94]
Yescarta and Kymriah	$CD19^+$ T-cell immunotherapy	Cell viability and CAR expression	[95,96]
MACI	Chondrocytes	Expression of Hyaline1	[97]
Gintuit	Keratinocytes and fibroblasts	No known mechanism of action; cytokine assay	[98]
Laviv	Postauricular fibroblasts	No known mechanism of action; cell number	[99]
Provenge	$CD54^+$ cell immunotherapy	Expression of CD54 on antigen-presenting cells after activation	[14]

Potency assays are unique for each product due to the assumed mechanism of action of the therapy. The table lists the current US FDA-approved cell therapies and their stated potency assays.
CAR: Chimeric antigen receptor; CD: Cluster of differentiation; HPC: Hematopoietic progenitor cells; MACI: Matrix-induced autologous chondrocyte implantation.

Developing reliable assays for cell therapy potency is particularly challenging [93]. Therefore, compared with identity, purity and sterility, potency tests are the least likely to have harmonization or standards set by regulatory agencies. Rather, each product may require a unique potency assay or set of assays [93]. It is not possible to list all of the potential potency assays for the cell therapies currently being tested. However, Table 6 provides a summary of the current potency assays for the FDA-approved cellular therapies. In cases where the mechanism of action for the treatment is unknown, the number of live cells is the surrogate potency assay.

Unfortunately, it is common that *in vitro* assay results fail to predict the *in vivo* efficacy even when the mechanism of action is known. It may be that the *in vivo* microenvironment is essential for the pathway of interest to function correctly. For example, *in vitro*, glucose-stimulated insulin secretion should predict the outcome of the islet transplant *in vivo*, but little correlation has been found [93]. Likewise, the *in vitro* cytotoxicity of T-cells against cancer cells should equate to a relative potency assay that would predict the *in vivo* response. However, *in vitro* cytotoxic activity appears to have very little correlation with the *in vivo* potency for some CAR-T therapies [34].

The FDA has outlined some of the challenges for potency assays for cell therapies in its guidance for industry [31]. These challenges include the inherent variability of the starting materials, the limited lot size for testing, limited stability, a lack of appropriate reference standards, multiple active ingredients and the potential for interference or synergy [31]. In a recent review by the FDA, less than half of the submitted INDs included any assay for bioactivity in their documents [58]. Potency assays are required prior to the start of pivotal registrational clinical trials. While it is suggested that *in vitro* data on potential potency assays be collected early in the development process, this is often difficult when the mechanism of action is not fully understood or easily measured. Typically, potency assays are not validated prior to initiation of the first phase of clinical development. However, for rare diseases, where the first clinical study has the potential to be the registrational study, it is imperative to develop potency assessments early.

Suggested assay harmonization

In reviewing characterization assays across cell therapies, one thing that becomes clear is that various regulatory and professional organizations classify assays under different headings, which confuses the field. For example, for allogeneic umbilical cord blood the FDA combines 'purity and potency' tests into one category measuring the total prep viability versus the viability of the target cells [3]. Other groups have combined purity and Identity assays into a single heading [36]. By standardizing the purpose for the tests through the categories of identity, impurity, sterility and potency, simple changes can help to provide a common framework for future studies and data sharing.

As previously described, safety is defined in the CFR as relative freedom from harmful effects to the patient by a product but is not a codified release requirement (21CFR610) [30]. Within the manufacturing process, safety should encompass aspects related to all of the critical quality attributes: identity, purity, sterility and potency. Control of these product quality attributes is a critical part of demonstrating a product's overall safety. Thus, listing safety as a separate critical quality attribute is confusing and should be avoided.

Table 7 provides a suggested single organizational structure when considering the minimal critical characterization attribute assays across cell therapy products. The structure was designed to minimize confusion so that the same assays are classified consistently across cell therapies and provide an outline of the minimum mandatory assays required for critical quality characterization. Of course, additional release tests will be necessary that are specific to each cell therapy.

Table 7. Suggested organization of characterization assays.

Critical quality assay categories	Recommended assays
Sterility	Adventitious agent
	Bacteria/fungal
	Endotoxins
	Mycoplasma
Identity	Surface markers of target cells
	Molecular markers of target cells
	Phenotypic markers of target cells
	Karyotyping
	Other tests: HLA, ALP levels, genome-wide gene expression patterns
Impurity	Surface markers of nontarget cells
	Molecular markers of nontarget cells
	Phenotypic markers of nontarget cells
	Serum components
	Competent vectors and DNA
	Teratoma formation
	Remaining reagents
Potency	Cell number/dose
	Viability
	Tests of mechanism of action

Changes to the four critical quality characterization categories includes changing purity to impurity to distinguish it from identity tests. Within the four categories we propose a standardized manner of classifying the recommended assays so that they are uniform across cell therapies.

In order to better differentiate identify from purity, we have clarified that identity relates only to characterization of the target cell population, while purity characterizes the nontarget cells and other nonbiological impurities. Thus, we suggest that the category be termed 'Impurity' rather than 'purity' to provide more clarification, a concept graphically reinforced in Figure 2.

One of the most important changes is to classify teratoma formation under the heading of purity of the final product, rather than identity of the starting material. While scientists understand that formation of teratomas are a rigorous test to identify whether cells are pluripotent, the public may question identifying target cells by their ability to form tumors. With respect to the final product, the results of teratoma tests are informative to identify undifferentiated cells, in other words off-target cells. Thus, teratoma tests directly reflect the impurity of the preparation.

Additional changes include the removal of endotoxins from the purity tests. Some groups have omitted endodoxin testing from their required criteria for cell bank release [53], although it is likely done. Others place the testing of endotoxins in the sterility category [100] or in its own category [51], whereas the FDA includes endotoxins as a purity issue or fails to explicitly include endotoxin testing in its guidance [3,32]. We suggest that the measurement of endotoxin levels should be considered a sterility issue, not one of purity. Tests for the presence of bacteria are already described under the sterility category. Since endotoxins are lipopolysaccharides found on the surface of bacteria and are shed when bacteria divide or die, it is reasonable that bacterial and endotoxin assays be placed in the same category. Thus, the new organizational structure places the testing of endotoxins under the sterility heading.

Finally, we have provided a suggestion for the minimal assays necessary to characterize potency. Up to this point, viability assays have not been included in many regulatory documents, because traditionally cell viability has not been listed consistently in any of the four categories. Rather FDA guidance lists viability in an 'other' category, along with cell number [31]. Yet, viability is one of the most important and commonly utilized assays in cell therapy manufacturing. The percentage of live cells certainly is related to the potency of the product and we suggest that viability and cell number or tissue volume be set as minimal potency assays. Additional potency tests would be based on the mechanism of action for the product.

Conclusion

Until the field of cell therapy matures, critical quality characterization assays must be broad, collect as much information as possible with the smallest sample and be agnostic meaning that there are no preconceived ideas

about which outcomes will be most relevant. We have attempted to standardize communication about critical quality attributes with a suggested organizational structure. By first comparing the differences in critical quality assays across cell therapy types, the design of a simplified and uniform structure is possible.

Future perspective

We are in an unprecedented time of growth for an entirely new field of medicine. Not surprisingly, there has been a significant lack of standardization around characterizing the starting material, the manufacturing process and critical criteria for product release of cells. However, the pendulum moved quickly with approval of cancer treatments that combined genetic and cellular therapy technologies. The quick acceptance of anti-cancer immunotherapies required manufacturing procedures that met standards similar to traditional chemical drugs, which brings us to a point where the industry can begin to speak with a more unified vocabulary about common practices. To that end, we offer one approach to facilitate harmonization around critical quality attribute testing. Undoubtedly, the field of cell therapy manufacturing will mature to the quality of chemical drug manufacturing. The length of time required to get to that point, depends on our ability to learn from each other and adapt.

Executive summary

- While the banking and manufacturing processes for cell therapies has been addressed by some international organization, less attention has been placed on the assays used to characterize the quality of either the starting material or the final product.
- The disparities between organizations and regulatory documents when describing critical quality attribute assays are highlighted.
- A clear definition of the three categories of cell therapies based on the starting cells: pluripotent, multipotent and unipotent is presented.
- Critical quality characterization assays are reviewed for each cell category covering identity, purity, sterility and potency.
- Discrepancies and agreements concerning the organization and types of assays utilized are highlighted.
- A simplified organizational structure was proposed to unify the classification and vocabulary around cell therapy critical quality characterization assays is proposed.

Financial & competing interests disclosure

All authors are employees of their designated organizations, which work in the stem cell field. Dr. Stehno-Bittel is a partial owner of Likarda, LLC. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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Commentary

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Regenerative Medicine

Development of a closed and automated bioreactor technology for cell therapy manufacturing – a sharing of our journey

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"In this manuscript, we share our bioreactor technology development journey and several key observations on how to strike a balance between technology development and technology transfer effort from the perspective of a government-funded laboratory"

First draft submitted: 22 September 2020; Accepted for publication: 10 December 2020; Published online: 7 January 2021

Keywords: automated systems • bioreactors • cell therapy • closed manufacturing • closed systems • intellectual property • legal/regulatory • manufacturing • technology platforms

Cell therapy uses live cells to treat diseases. For example, chimeric antigen receptor T cells (CAR-T) are T cells that have been engineered to express CARs to produce a cytotoxic effect against cancerous cells that express the targeted antigen [1]. Epstein–Barr virus-specific T-cell therapies are produced by stimulating naive T cells with Epstein–Barr virus-transformed lymphoblastoid cell lines (LCLs) [2].

As more cell therapies are progressing to clinical trials, with 1052 clinical trials underway worldwide in 2019 [3], several manufacturing challenges have emerged. At the same time, the approval of Kymriah and Yescarta by the US FDA in 2017 initiated many conversations on the bottlenecks in the manufacturing of cell therapy products [4–6]. These bottlenecks include high dependence on skilled labor and lack of automated options for cell processing. Our research lab based in the Agency for Science, Technology and Research (A*STAR), Singapore focuses on technologies for improving manufacturing process and efficiency for applications in cell therapy and regenerative medicine. With 4 years of research and development, we have successfully developed a patent pending bioreactor technology to address some of the bottlenecks of cell therapy manufacturing.

In Singapore and many other countries, the local industry is conservative and unwilling to adopt technologies of low technology readiness level. This is especially the case for emerging industries such as cell therapy. While research laboratories could be aware of the importance of technology derisking, new technologies developed from research laboratories are usually prototypes without the final product functionalities. This is in our opinion largely because, research funding typically does not support productization work and researchers are typically not equipped to tackle the relevant regulatory requirement such as implementing safety features for compliance to GMP. This situation generates a difficult question for noncommercial research laboratories – how might their work remain focused on research, and yet not overlook key considerations that may hinder future technology transfer?

In this manuscript, we share our bioreactor technology development journey and several key observations on how to strike a balance between technology development and technology transfer effort from the perspective of a government-funded laboratory.

Case study: development of a closed & automated bioreactor technology for cell therapy manufacturing

When cell therapy first gained popularity, there were very few GMP-compliant cell manufacturing equipment and cell therapy conferences were more focused on early clinical trial data than the scaling of the manufacturing.

Future Medicine

Figure 1. Novel single-use bioreactor (automation mechanism not shown).

Recognizing that manufacturing would be a bottleneck for this emerging industry, we leveraged our strong foundation in manufacturing engineering to innovate platform technologies for cell manufacturing. Specifically, we focused on scale-out manufacturing for nonadherent autologous cell therapy products, where low-volume high-mix manufacturing was required.

The result of the innovation effort is a novel automated single-use bioreactor platform for autologous cell therapy manufacturing (Figure 1). The bioreactor enables *in vitro* cell expansion within a single vessel, to obtain cell numbers sufficient for a therapeutic dose for individual patients. It has a compartmental design that minimizes disturbance to cells during medium change or sampling. It also allows the cell culture surface area and volume to be adjusted during cell expansion process so that the same vessel can be used as the cell number increases. The current design supports up to about 300 ml of culture volume in total, with the cells residing in a compartment that can be expanded from 12 to 108 ml. The other compartment is a media reservoir that holds 180 ml of media that refreshes the cell culture. The automation module of the bioreactor platform automates the different liquid handling processes and regulates cell culture conditions such as temperature and carbon dioxide concentration. Overall, the bioreactor platform significantly simplifies the handling process, reduces the number of culture vessels, reduces contamination risk and minimizes manual handling for cell expansion.

Figure 2 maps some key considerations we had in developing the bioreactor technology to the stages of the design thinking framework. These considerations are selected and highlighted because they either address requirements unique to cell therapy manufacturing or affect decisions related to technology transfer. This selection of considerations is not meant to be exhaustive, and does not cover the entire design thinking process.

Identify unmet need: identifying the true bottleneck

The development of the bioreactor started in the 'Empathize' stage of the design thinking framework, with the team undergoing an immersion experience in a clinical collaborator's cell processing laboratory. The collaborator was developing an immune cell therapy product for nasopharyngeal cancer, and their protocol involved the stimulation of donor peripheral blood mononuclear cells (PBMCs) to generate cytotoxic T cells. The team observed and mapped the manufacturing process to uncover the primary manufacturing bottlenecks.

From our observations, cell expansion took the most of time in the cell manufacturing process flow. Each expansion step involved the removal of confluent cells from multiple 24-well plates and replating at a lower density into multiple new 24-well plates in order to maintain the cell density within an optimal range. Toward the end of the manufacturing process, the operator was manually handling stacks of 24-well plates. If the same process were used for commercial production, the amount of manual labor and consumables required to produce one therapeutic dose

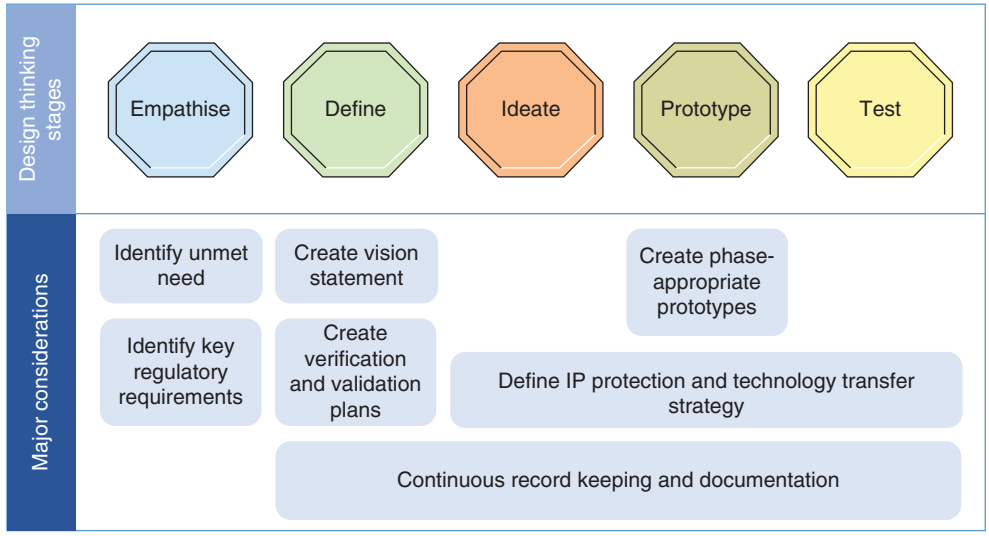

Figure 2. Our major considerations to stages of the design thinking framework.

of cell therapy product under GMP Class A or International Organization for Standardization (ISO) Cleanroom Class 100 environment, would result in massive labor and facility costs that are eventually transferred to patients.

The research team recognized that, in order to reduce treatment cost of cell therapy and increase patient access to the treatment, the number of manual steps and open processes for cell expansion have to be reduced, and this could be achieved through innovation in bioreactor technology. In addition, new bioreactor technology should also drive easy process scaling from research development to commercial production. At last, the bioreactor platform must also accommodate a wide range of cell expansion protocols as cell therapy protocols can vary significantly from each other.

Identify key regulatory requirements: assessing compliance requirements

During the 'Empathize' stage, it was also important to identify the key compliance requirements because cell therapy product is a regulated cell-based medicine. While it was not practical to scrutinize all regulatory requirements in the initial discovery phase, the team attempted to identify relevant regulatory requirements to assess the regulation-related risks and costs of developing solutions for cell therapy manufacturing. The regulatory guidelines for cell therapy products is still under review at the time of writing this manuscript. Nonetheless, from our assessment, cell therapy providers, rather than cell processing system manufacturers, carry the responsibility of ensuring the final cell therapy products meet the relevant compliance and regulatory requirements such as cell viability and potency thresholds, and United States Pharmacopeia (USP) <85> Bacterial Endotoxins Test. In turn, cell therapy providers expect cell processing systems to meet the relevant quality standards and regulatory requirements such as ISO 10993-5 Biological evaluation of medical devices: tests for *in vitro* cytotoxicity, and USP <665> polymeric components and systems used in the manufacturing of pharmaceutical and biopharmaceutical drug products.

Therefore, there is certain flexibility in classifying the developed bioreactor technology for cell therapy manufacturing: the bioreactor can be classified as a manufacturing equipment or a medical device. The key difference between the two classifications is the extent of liability taken up by the manufacturer of the equipment, which is in turn indirectly related to the effort needed from our research team. In the US, a medical device manufacturer is legally required to have a quality management system (QMS) that complies with ISO 13485 and the FDA's Code of Federal Regulations Title 21 Part 820 (21 CFR 820). In contrast, an equipment manufacturer will typically have a less demanding QMS for general quality assurance. From the end user's perspective, the medical device classification indicates high quality and promises greater support from the manufacturer when things go wrong.

The research team decided to classify the bioreactor as a manufacturing equipment due to two main considerations. First, as a generic platform that is able to culture any nonadherent cell therapy products, the bioreactor is accessible by a bigger market. Second, potential licensees that are entering the biotechnology industry may not yet have the required QMS in their facilities. Classifying the bioreactor technology as a generic equipment as opposed to a medical device is thus a more manageable regulatory strategy for technology transfer. With a high-level regulatory

plan in place, the research team were then able to engage the right industries and prioritize the validation plans in preparation for technology transfer.

Create vision statement: defining long term technology goals

In the 'Define' stage, the research team created the vision of a closed and automated bioreactor system that can scale from laboratory development work to commercial production. Closed and automated manufacturing process not only reduce contamination risk and standardize the product quality, but also reduce the manufacturing cost of the cell therapy products in the long run. Scalability was an important consideration because the regulations require process revalidation whenever the manufacturing equipment was changed to increase scale. Revalidation during clinical trials could be very costly and time-consuming. Thus, it is imperative that the same bioreactor and manufacturing processes are used for all scales of manufacturing so that revalidation would not be required even as production scale changes during clinical trials.

With the long term technology goals defined, two versions of the bioreactor were planned: an open version to be used at laboratory scale and handled manually; and a closed version to be used at commercial scale and integrated with automation. In particular, the open version must be easily converted to closed form to save production costs, and equivalent in performance to avoid revalidation.

Create verification & validation plans

Once the vision statement was clear, the team began to create plans for technology verification and validation. Verification is the process of ensuring that the technical specifications are met by the technology [7]. Validation is the process of ensuring that the technology meets the end user's requirement [7]. Verification and validation must be completed to generate data as evidence that the technology performs as intended and to specifications. Both processes involve tests to generate supporting data during the 'Test' stage of the design thinking framework. For bioreactors, both mechanical and biological tests were required. As extensive testing is usually too costly for research laboratories, a testing strategy is necessary to prioritize the tests as early as in the 'Define' stage.

In cell manufacturing, many of the standard tests relate to product safety, and are highly dependent on the manufacturing site. According to regulations, the final manufacturer of cell therapy products will have to requalify the technology and processes at their registered manufacturing sites. Therefore, our research team performed only tests that can generate data to increase confidence in the bioreactor's unique features, and reduce uncertainty in the functionality and performance of the bioreactor. Standard tests, such as tests for Extractables and Leachables, transmissible spongiform encephalopathy, pathogens and endotoxin, were not directly related to the bioreactor technology itself and were deprioritized.

First, verification tests were performed to ensure that the material and assembly method used for making the bioreactor prototypes have no adverse impact on the cell culture. Given the prolonged cell contact with the bioreactors during *in vitro* cell manufacturing, which can be up to 2 months, the standard biocompatibility test ISO 10993 with incubation time of up to 72 h was deemed inadequate. A more stringent screening test was designed to incubate the material directly with Jurkat cell cultures (direct contact) for one week. Indeed, despite the fact that all materials tested were marketed as medical-grade or noncytotoxic, a few of them were found to hinder cell growth.

Second, a key feature of our bioreactor was an adjustable plunger that was pulled outwards to expand the cell culture space inside the bioreactor. As the cells were very small (diameter of 10 μm), the plunger had to be perfectly sealed. However, due to the unique design of the bioreactor, the standard leakage test methods such as ASTM F2096 could not be directly applied. The design team thus worked with the prototyping collaborator to design and implement a custom leakage test. Through repeated design-build-test cycles, the plunger design and the leakage test protocol were optimized to achieve a complete and reliable seal.

At last, validation experiments were planned appropriate to the phase of the bioreactor development. During early phases, the bioreactor performance was assessed using only cell viability and cell number. In addition, relatively robust Jurkat cell were used, as they were able to survive in less polished prototypes. These two test considerations enabled rapid initial iterations as the tests were straightforward to perform and analyze. After most of the bioreactor features were incorporated, further cell characterization assays specific to the cell therapy products, such as biomarker expression, metabolite analysis and function assays, were performed and donor PBMCs were used to better model the actual process. These data elucidated the impact of the bioreactor technology on the quality of the produced cell therapy products in terms of identity, potency and purity. Later, research collaborations

were proactively established and bioreactor prototypes were transferred to collaborators' facilities for testing by the collaborators. These validation experiments performed with donor cells provided real data for technology licensing.

Create phase-appropriate prototypes: picking the right prototyping technique

Regardless of the unmet need, iterative cycles of ideating, prototyping and testing are unavoidable and also desired to reduce the overall project cost. The more uncertainties we can eliminate through prototyping, the less risk remains in the productization process. Commercial cell culture vessels are typically produced by molding techniques such as injection molding and blow molding. These techniques produce excellent surface flatness and tolerances, and are economic options for large-scale production. However, the upfront cost of tooling can be prohibitive and the lead-time is long for prototyping phase.

Rapid prototyping methods such as machining and 3D printing, and bonding methods such as solvent bonding, thermal bonding and adhesives were thus used to create functional prototypes at early prototyping stages of the bioreactor development. There were caveats to the use of each method. For instance, machined surfaces were inherently uneven and machining marks could alter cell growth. Certain adhesives could be structurally weak for part assembly. A combination of different rapid prototyping techniques was carefully selected to circumvent the limitations and exploit the strength for making specific bioreactor components. For example, new extruded plastic sheets were used for the cell culture surface, and complicated surfaces that were not in contact with cells were 3D printed. Components were assembled by silicone adhesives or solvents that were tested for biocompatibility. Assembly interfaces exposed to high stress were reinforced with additional design features. Selective and informed use of rapid prototyping methods enabled faster iteration cycles and feature updates. Undoubtedly, in-house expertise in rapid prototyping methods was vital to maximize the benefits of rapid prototyping.

While the team exploited the advantages of rapid prototyping, they were also mindful in ensuring that the designs could also be manufactured by common mass production methods. Later in the development phase, when the design features were locked down, injection molding and ultrasonic welding were invested in to produce functional prototypes for validation with PBMCs and donor cells.

Define IP protection & technology transfer strategy

During the ideate-prototype-test cycle, the team assessed the IP portfolio and whether the generated IP aligns with the business model of the organization. During regular risk assessment, the team assessed if they should continue the innovation process, exploit other opportunities or even terminate the process to cut losses.

IP assessment may often involve the technology transfer office. In our case, the IP and technology transfer office was consulted on the mode of protection and the avenues of technology transfer. The technology transfer office suggested that the patent application was an effective protection based on two main factors. First, the bioreactor's features were patentable. Second, there were interested potential licensees and the useful lifetime of the bioreactor far exceeded the time needed for the patenting process. On the contrary, if the bioreactor are expected to be relevant for only two years, the product would be irrelevant by the time the patent was granted and enforced. A licensing agreement based on copyrighted technical drawings would be a more reasonable strategy to reduce cost and react more quickly to licensing opportunities.

Engaging the technology transfer office early in the development process helped the research team better understand the potential IP outcome and technology transfer avenue. It also gave the technology transfer officer an idea of how much work and effort went into this technology development process, which could be part of the considerations for the licensing terms.

Continuous record keeping & documentation

Proper record keeping and documentation not only safeguard the interest of the research team, but also ensure a fair and informed deal for the future licensee. This is especially important when it comes to technologies and products with stringent regulatory requirements such as cell therapy related technologies. The team thus put in place appropriate record keeping and documentation to ensure that the technology development process was properly documented, starting from user requirements and idea sketches to verification/validation data. These documents supported the tracking of project progress, project reporting and data referencing. Documentation can also reduce future entanglement or conflicts, and be included as part of the technical documents for technology transfer.

For our bioreactor technology development, drawings for major iterations were saved, design requirements were documented, design specifications were traced back to user requirements and verification and validation data were recorded and related to design specifications.

Conclusion

Our journey in developing a closed and automated bioreactor technology has elucidated the importance of analyzing, defining and addressing requirements specific to the cell therapy industry, as well as the conundrum for noncommercial research laboratories to balance the technology innovation and technology transfer effort during technology development. We hope that by sharing observations from our own journey, we have provided fellow researchers in the same field some useful ideas on how to better plan and manage their technology research and development. Technology innovation for cell therapy industry is challenging, but the chances are improved if the project is strategized and planned for the long-term goal.

Financial & competing interests disclosure

This research is supported by the Agency for Science, Technology and Research (A*STAR), Singapore. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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