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SPOTLIGHT ON
Gene therapy CMC and analytics

Guest Editor
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SPOTLIGHT

FOREWORD

Lauren M Drouin

"The gene therapy field is opening a whole new world for patients and their families, and changing lives..."

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After years of trials and tribulations, uniQure's Glybera (alipogene tiparvovec) became the first gene therapy approved by the EMA in 2012. Several years later, the FDA approved Spark's Luxturna (voretigene neparvovec-rzyl) in 2017, and since then, the gene therapy field has gained momentum and continues to advance at a rapid pace. Regulatory agencies have been pressed to keep up with the increasing number of requests from sponsors as well as to provide updated guidance on these novel and complex biologic drugs. The FDA has responded to this need with increased informational meetings for sponsors on hot topics such as gene therapy Chemistry, Manufacturing, and Controls (CMC) and potency assurance. This year, the agency is expected to publish additional draft guidance documents on these topics, emphasizing the importance of CMC and analytics in gene therapy product development.

Further highlighting the advancement in the field, two new gene therapy treatments were approved by the FDA in the past month alone: BioMarin's Roctavian (valoctocogene roxaparvovec-rvox) for hemophilia A, and Sarepta's Elevidys (delandistrogene moxeparvovec-rokl) for Duchenne muscular dystrophy. In June, Pfizer also filed a biologics license application for fidanacogene

elaparvovec, for the treatment of hemophilia B, which is currently under review. The gene therapy field is opening a whole new world for patients and their families, and changing lives; I look forward to seeing its continued progress in the years to come.

In this months' special Spotlight edition of *Cell & Gene Therapy Insights*, the focus is on the hot topic of Gene Therapy CMC & Analytics. The CGTI team has curated a fantastic lineup of articles on this topic including:

- An interview with Juliette Reviron from Lysogene, in which she discusses the current adeno-associated virus (AAV) analytics toolkit, including key challenges for AAV vector characterization and CMC, as well as the significance of understanding packaged DNA impurities.
- An Expert Insight article in which Aishwarya Shevade from Regeneron Pharmaceuticals describes the optimization of a duplex ddPCR method to characterize AAV vector genome integrity and compares this approach to other orthologous methods.
- A Regulatory Perspective from Stuart Beattie of Biogen where he provides a series of case studies pertaining to common CMC development challenges with AAV vectors, along with direction to available guidance resources and possible approaches to mitigate these regulatory risks throughout the product development lifecycle.
- An interview with Jonathan Appleby and John Churchwell from Cell and Gene Therapy Catapult in which they discuss the application of process analytical technology (PAT) to address challenges in gene therapy development and manufacturing, including how the introduction of AI and machine learning can be harnessed to drive the gene therapy field forward.

To everyone in the gene therapy sphere: keep up the great work, and happy reading!

BIOGRAPHY

LAUREN M DROUIN is Director of the Genomic Medicine Analytical Development group at Alexion, AstraZeneca Rare Disease. Her team supports analytical testing for AAV gene therapy and gene editing products, including initial method development, assay qualification, and product characterization, in addition to assisting CMC operations with plasmid DNA, cell bank, and DS/DP batch release and stability programs. Lauren managed CMC analytical operations for the Methylmalonic Acidemia clinical program, the first *in vivo* gene editing therapy delivered systematically to pediatric patients, and was significantly involved in both the manufacturing campaign and subsequent regulatory submission. Her current research interests include novel AAV capsid characterization and developing a robust understanding of the factors that influence potency of gene therapy products. Previously, she worked at Voyager Therapeutics where she was responsible for analytical method development and overseeing CMC analytical operations for the Parkinson's disease clinical gene therapy program. Lauren received her PhD in Biochemistry and Molecular Biology from the University of Florida where she utilized molecular, biophysical, and structural techniques to characterize the AAV capsid for improved gene delivery applications. She currently serves as Chair of the AAV Gene Therapy Products Expert Panel for the United States Pharmacopeia and is a member of the Global Outreach Committee for the American Society of Gene & Cell Therapy, working to expand access to cell and gene therapies on a global scale.

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY CMC & ANALYTICS

SPOTLIGHT

EXPERT INSIGHT

Optimizing ddPCR assay for characterizing AAV vector genome integrity

Aishwarya Shevade, John S Reeves & Andrew D Tustian

Recombinant adeno-associated virus (rAAV) is a promising gene therapy vector to deliver DNA as a treatment for numerous human diseases. Accurate quantification of the rAAV vector genome titer and characterization of its integrity are critical for determining the clinical dose and ensuring product safety and efficacy. Genome integrity can be defined as the intactness of the vector genome both in relation to its expected size and sequence. The replication and packaging stages of rAAV production can potentially result in the incorporation of truncated or partial genomes compromising genome integrity. Droplet digital PCR (ddPCR) using two sets of primers-probe pairs that target the 5′ beginning and 3′ end of vector genomes allows for estimation of percentage linkage between the targets when a double positive signal is detected. Here we describe ddPCR method optimization for rAAV vector genome characterization and show that omitting a heating step in the ddPCR workflow improves the estimated percentage linkage.

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PARTIAL GENOMES IN rAAV VECTORS

Adeno-associated viruses (AAV) have become a leading vector of choice for human gene therapy due to their simple genome structure, non-pathogenicity, and broad tissue tropism. Currently, five AAV gene therapies are approved by the US FDA, and several are in different stages of clinical trials worldwide [1–3] AAV is a non-enveloped parvo virus and carries a single-stranded DNA (ssDNA) genome of approximately 4.7 kb length packaged inside a capsid comprising of three proteins, VP1, VP2, and VP3, in a ratio of approximately 1:1:10 [4–6]. Wild-type AAV genome contains rep and cap sequences, which are flanked by the left and right inverted terminal repeats (ITRs).

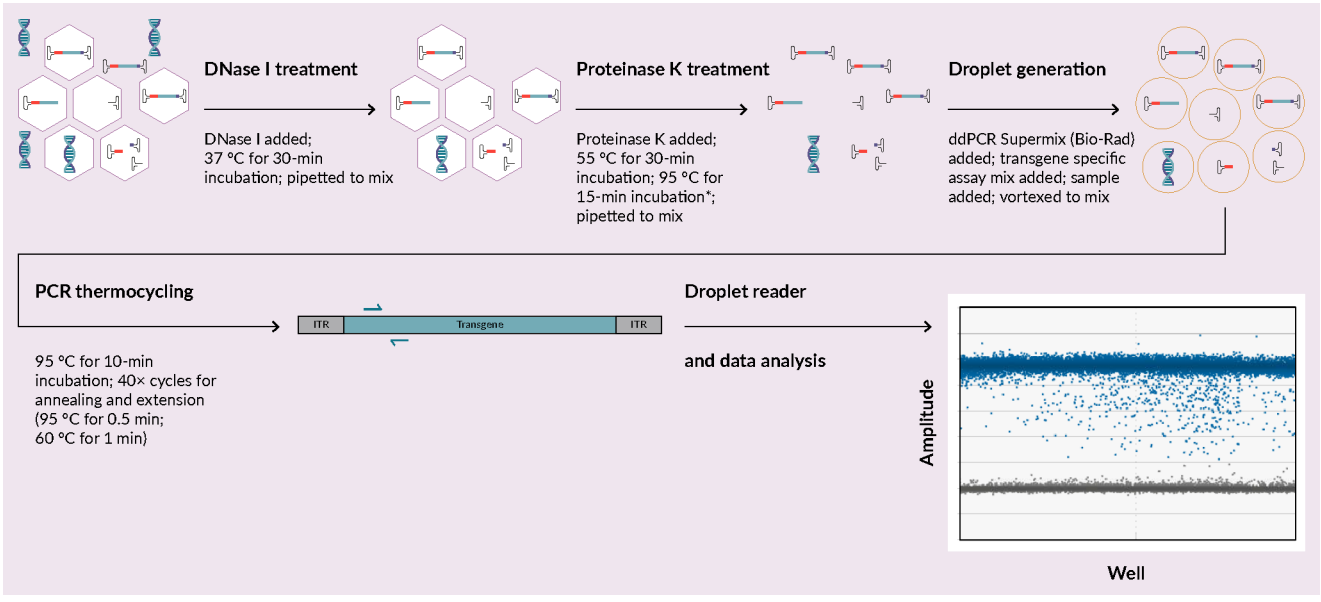
Recombinant AAV (rAAV) viral vectors carry the transgene instead of rep and cap between ITRs. A commonly used platform for manufacturing rAAV vectors involves transient triple transfection of the HEK293 cells with three plasmids: one encoding the transgene, one carrying the rep and cap genes, and one expressing the genes from a helper virus such as Adenovirus [7]. Partial capsids are one of the main product-related impurities generated during the manufacturing of the rAAV vectors due to replication and packaging errors [8,9]. These are capsids with an intermediate density between 'full' and 'empty' capsids as identified by an analytical technique such as analytical ultracentrifugation (AUC) [10]. Partial capsids may contain truncated genome or other residual DNA such as host cell DNA or plasmid DNA [11,12]. Genome integrity can be defined as the intactness of the vector genome both in relation to its expected size and sequence. Determining genome integrity and characterizing the partial rAAV vectors are critical, as the rAAV genome quality impacts the infectivity, safety, and efficacy of the drug product [13]. Droplet digital PCR (ddPCR) technology is commonly available and used for viral genome quantification as a measure of product strength. It is a simple and reliable technique for characterizing the genome integrity of rAAV vectors and can be used orthogonally with other analytical tools [14–16].

SIMPLEX ddPCR FOR VECTOR GENOME QUANTIFICATION

Clinical dose is commonly based on the vector genome titer. ddPCR is a well-established analytical technique and has become an industry gold standard for quantifying vector genome titer [15]. ddPCR eliminates the need of a standard curve and is less susceptible to matrix interference, and hence, is preferred over traditional techniques such as quantitative PCR (qPCR) [14]. This is particularly relevant during analysis of in-process samples. Our in-house sample preparation involves treatment with DNase to digest DNA extraneous to the capsid, followed by Proteinase K digestion of the capsid (Figure 1). Treated samples are diluted such that the expected copies/µL are within the linear range of the assay (5–5000 copies/µL). Diluted samples are then prepared for droplet

FIGURE 1

In-house rAAV vector genome titer by ddPCR workflow. Starred (*) step removed in finalized genome integrity workflow.



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generation by mixing with the ddPCR polymerase and assay mix containing a primers-probe pair, which is specific to the transgene. Droplets are amplified on a thermal cycler and fluorescence from the probe is quantified on a droplet reader. ddPCR follows the Poisson distribution and the fraction of PCR-positive droplets enables the quantification of the target sequence in copies/µL. Depending on the primers-probe pair binding location, titer by ddPCR may vary [16]. In such a case, titer bias evaluation and/or assay optimization, such as changing annealing temperature, may be required. Since titer quantification by ddPCR relies on the amplification of a short sequence (100–200 base pairs) on the vector genome, it could overestimate titers of intact genome if the encapsidated genome is truncated or heavily fragmented [17]. Interestingly, the titer assay can be adapted to serve as a rapid tool for characterizing the genome integrity of a purified rAAV.

DUPLEX ddPCR ANCHOR ASSAY FOR rAAV VECTOR GENOME INTEGRITY

A relatively simple approach for determining integrity of a vector genome is developing a duplex ddPCR assay, such that the two distinct, spatially separated *cis* sequences of individual vector genomes can be concurrently evaluated [14,18]. The size of the amplified sequence is short (100–200 bases pairs), and yet the percentage linkage can be derived for the entire length of the viral genome by designing sequence-specific assay mixes at regular intervals.

Data interpretation for a duplex ddPCR assay may be complicated due to the nature of droplet partitioning. Double positive droplets could mean either of the two scenarios; one where the signal from the two fluorophores is generated from an intact genome or second where droplets containing two separate fragments generate a double-positive signal, due to random co-partitioning. Low sample concentration will minimize the probability of co-partitioning two or more distinct fragments in a droplet [18]. Another approach is to calculate the percentage linkage (Figure 2) based on a modified version of Poisson statistics such that the probability of random co-partitioning of the two fluorophores can be considered [19–21]. In the latter approach, linkage represents the copies/µL of two physically linked target sequences as calculated by QX Manager software [27].

We generated an rAAV8 vector containing single stranded genome using the triple

FIGURE 2

Calculation of percentage linkage of a duplex assay.

A

$$\text{Mean assay concentration}\left(\frac{\text{copies}}{\mu\text{L}}\right) = \frac{\text{FAM assay concentration}\left(\frac{\text{copies}}{\mu\text{L}}\right) + \text{HEX assay concentration}\left(\frac{\text{copies}}{\mu\text{L}}\right)}{2}$$

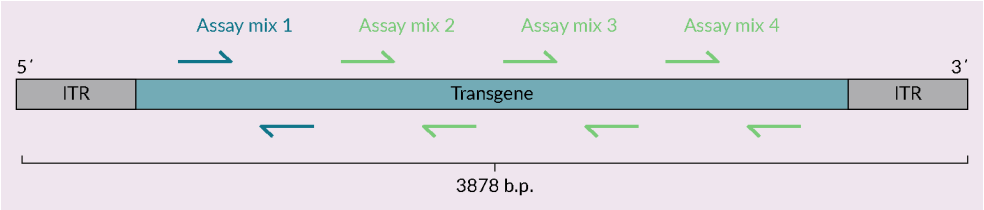
B

$$\%\ \text{linkage} = \frac{\text{Linkage}\left(\frac{\text{copies}}{\mu\text{L}}\right)}{\text{Mean assay concentration}\left(\frac{\text{copies}}{\mu\text{L}}\right)} * 100\%$$

(A) FAM and HEX assay concentrations were provided by QX Manager software. (B) Linkage (copies/µL) was provided by QX Manager software. Refer to the QX Manager user manual reference for details on calculation [27].

FIGURE 3

Schematic of the rAAV vector genome and relative position of ddPCR assay targets.



Assay mix 1 (FAM dye, blue) is proximal to the 5′ ITR and used as the anchor assay in duplex experiments coupled with assay mixes 2, 3, or 4 (HEX dye, green). Amplicon sizes range from 92–109 b.p.

transfection platform. The rAAV8 drug substance was generated at the 500 L scale and purified via depth filtration, tangential flow filtration, affinity and ion exchange chromatography, and ultra-filtration diafiltration. The drug substance comprised rAAV8 in a pH buffered salt solution containing surfactant [21]. As described in Figure 1, the in-house ddPCR workflow involves sequentially performing DNase I and Proteinase K treatments. These enzymes are inactivated by heating the samples to 95 °C. For all steps prior to Proteinase K digestion, rAAV vector containing solutions are mixed by pipette instead of vortex to avoid DNA leakage due to excessive shearing of the capsid proteins. After DNase I and Proteinase K treatment, when the viral DNA has been liberated from the capsids, the treated samples are added to the master mix solution containing the ddPCR polymerase and primers-probe pair, the sealed ddPCR plate gets thoroughly vortexed on four corners. This is followed by droplet generation, primers-probe pair annealing and DNA amplification at an optimized temperature, and lastly, the fluorophore signal detection occurs on a QX-200 droplet reader. To characterize DNA integrity, we established an 'Anchor Assay', wherein four assay mixes spanning the length of the rAAV vector genome were used in a duplex ddPCR assay (Figure 3) [17,21]. Assay mix 1 carrying the FAM dye was the anchor and annealed to the 5′ end of the vector genome. Assay mixes two to four carrying the HEX dye were used in tandem with assay mix one in the duplex assays; assay mix four annealed closest to the 3′ end. We chose non-ITR targets, as it has been demonstrated previously that due to the secondary structures present in ITRs, genome integrity values can be highly variable and sometimes lower than that predicted by orthogonal techniques [21]. The distance from the anchor of each assay mix carrying the HEX dye is included in Table 1. As expected, the double stranded plasmid carrying the transgene demonstrated >96% linkage for each duplex assay. Using the ddPCR workflow described previously for rAAV sample treatment, percentage linkage was evaluated

TABLE 1

Description of ddPCR duplex assays used to evaluate percentage linkage of the vector genome.

Duplex assay	FAM assay	HEX assay	Genomic distance from FAM 'anchor' assay (b.p.)
Duplex 1	Assay mix 1	Assay mix 2	833
Duplex 2	Assay mix 1	Assay mix 3	1906
Duplex 3	Assay mix 1	Assay mix 4	2763

FAM: Fluorescent label carboxyfluorescein; HEX: Hexosaminidase.

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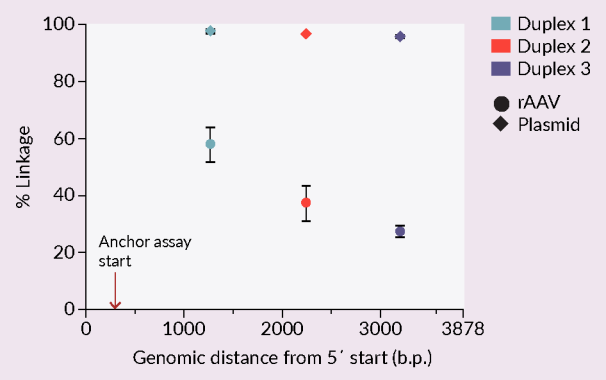
FIGURE 4

Percentage linkage of the vector genome following the ddPCR workflow.

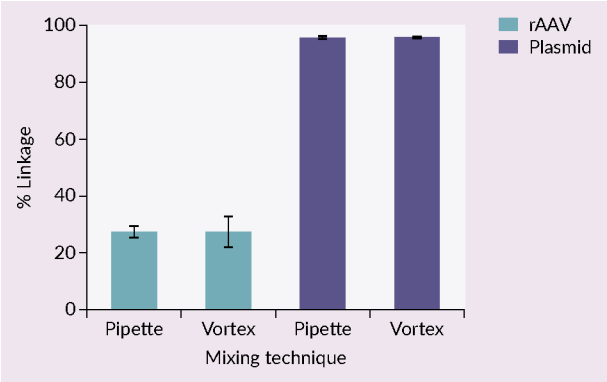


Data from plasmid and rAAV demonstrated percentage linkage of the vector genome decreases as the position from the 5' start increases. N=3 technical replicates for each duplex assay; error bars represent a 95% confidence interval.

for each duplex assay. Surprisingly, duplex one containing assay mixes one and two with the shortest distance from the anchor showed 58% linkage, while duplex 3 containing assay mixes one and four exhibited linkage as low as 28% (Figure 4). The percentage linkage of the vector genome decreased as the distance from the anchor increased, this indicated the presence of high degree of truncated genomes.

FIGURE 5

Evaluation of mixing technique when assembling DNA with master mix reaction for duplex 3.



For each DNA sample, vortex to mix did not result in a statistically significant difference in percentage linkage by ddPCR, P>0.05. N=3 for each condition; error bars represent a 95% confidence interval.

AUC serves as a surrogate measure of genome integrity, where in full vector particles are assessed to be containing mostly, if not all, fully intact vector genomes. Interestingly, AUC analysis for this rAAV vector indicated 72% full, 25% empty, and 3% partial capsids. Since the AUC results differed from initial ddPCR results, we investigated the ddPCR sample treatment further to rule out any artificial fragmentation caused by DNA extraction.

HEATING DURING ddPCR SAMPLE TREATMENT REDUCES LINKAGE

Due to the apparent underestimation of percentage linkage observed for duplex 3, we suspected that sample handling or treatment may have an effect. Three possibilities were hypothesized:

1. DNA fragmentation may occur due to sample handling during mixing of free DNA for steps following Proteinase K digestion;
2. DNase enzyme may not be fully inactivated, resulting in excessive digestion of the extracted genome;
3. Higher temperatures during thermal cycling may cause DNA fragmentation.

For duplex 3 as shown in Figure 5, pipetting to mix, or mixing with a vortex did not result in a statistically significant difference in percentage linkage by ddPCR for rAAV vector genome or the plasmid carrying the transgene.

After ruling out that different mixing techniques did not cause an additional decrease in percentage linkage, we systematically investigated the contributions of DNase activity and high temperature (Table 2). In this experiment a transgene plasmid was included again as a positive control (no treatment other than dilution) and showed 96% linkage. In the absence of DNase and Proteinase K, the rAAV samples were exposed

to different temperatures, i.e., 37 °C, 55 °C, and 95 °C for 30 and 15 minutes, respectively. We observed an increase in the percentage linkage from 26% in the control to 49% in the absence of the two enzymes (treatment 1 in Table 2, Figure 6). When the samples were exposed to 37 °C and 95 °C in absence of enzymes (treatment 2 in Table 2, Figure 6), we observed change in percentage linkage similar to treatment 1. Interestingly, when the samples were exposed to 37 °C and 55 °C in the absence of enzymes and the 95 °C incubation, there was a significant increase in the percentage linkage to 79%. Considering this is similar to the 72% full determined by AUC, these data suggest that 95 °C exposure could be causing increased DNA fragmentation (treatment 3 in Table 2, Figure 6). Finally, we wanted to examine the above-mentioned temperature conditions in the presence of DNase and Proteinase K. DNase is important to ensure that we only measure the integrity of the genome packaged in the capsid, while Proteinase K ensures that all the packaged genome is made available during the assay workflow. Therefore, we treated the samples in the presence of DNase at 37 °C followed by treatment at 55 °C after adding Proteinase K (treatment 4 in Table 2, Figure 6). We observed 81% linkage, which is comparable to the 79% determined without enzyme treatment, which suggests that addition of these enzymes did not further alter the percentage linkage. Our results corroborate the previous finding [21] in that extended period of heat at 95 °C affect percentage linkage, but a notable difference in our sample treatment is the inclusion of the Proteinase K digestion step, which may play a role depending on the serotype.

For all the treatments described here, copies/µL obtained from assay mix three, which targets the middle of the transgene, are included in Figure 6. Data suggests the increase in the percentage linkage in the presence of 95 °C heating in treatments 1 and 2 cannot be attributed to a corresponding increase in copies/µL. While it is conceivable that enzymes (and associated buffers) could have minor contributions, it is clear from the data that exposure to 95 °C is the most significant factor contributing to a decrease in percentage linkage. The mechanism of heat induced DNA degradation is not completely understood and may include hydrolysis of phosphodiester bonds and N-glycosilic bonds [29].

The ubiquitous presence of ddPCR-based approaches in the characterization of rAAV makes it a pragmatic solution to measure vector genome integrity. Moreover, a ddPCR-based approach has the advantage of providing sequence-specific quantitative measurement of genome integrity, which sets it apart from other orthogonal assays such as AUC.

TRANSLATIONAL INSIGHT

rAAV vector genome integrity is an important quality attribute to characterize to ensure

TABLE 2

Description of sample treatment variations.

Treatment	Enzymes added	DNase incubation 37 °C for 30 minutes	Proteinase K incubation	
			55 °C for 30 minutes	95 °C for 15 minutes
Control	DNase and Proteinase K	✓	✓	✓
1	None	✓	✓	✓
2	None	✓	–	✓
3	None	✓	✓	–
4	DNase and Proteinase K	✓	✓	–
Plasmid	None	–	–	–

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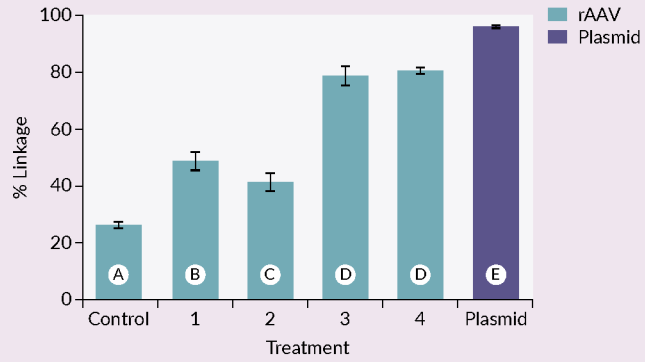
FIGURE 6

Effect of sample treatment variations on percentage linkage by ddPCR using duplex 3.



Treatment	Assay mix 3 concentration (copies/µL)	Duplex 3 % linkage
Control	61.3	26.3
1	63.8	48.8
2	27.5	41.4
3	77.5	78.7
4	75.2	80.4
Plasmid	555.5	96.0

A statistically significant difference in percentage linkage was observed for sample treated with and without the 95 °C for 15 minutes incubation. N=3–6 technical replicates for treatments 1–4, control, and plasmid. Error bars represent a 95% confidence interval. Differences between treatments not sharing a letter were evaluated as statistically significant by Tukey-Kramer test, $P<0.05$. Repeatability for copies/µL and percentage linkage is CV <15%.

product safety, efficacy, and stability. Therefore, the impact of capsids with varied genome integrity should be evaluated for their infectivity and potency. Purified rAAV products are known to carry partial or truncated genomes as an impurity resulting from premature termination of transgene replication, packaging defects in HEK293 cells, and contamination from host cell or plasmid DNA [13]. While historical manufacturing steps such as centrifugation or more scalable methods such as anion exchange strive to enrich full capsids, presence of contaminating partial capsids in the final drug product should be expected [22]. Although orthogonal analytical methods such as AUC, capillary electrophoresis with laser induced fluorescent (CE-LIF), charged detection mass spectrometry (CDMS), and next generation sequencing (NGS) are utilized for the assessment of vector genome integrity [9,10,23,24], ddPCR can serve as a simple, rapid, and powerful complementary tool for quantifying genome integrity. A ddPCR assay is already established in many labs for quantifying genome titer; that assay can be easily adapted for genome integrity analysis. Genome integrity can be monitored with minimal sample treatment optimization during genome extraction and a duplex ddPCR assay utilizing probes targeting different locations of the same rAAV sequence. Presence of a 95 °C heating step in the sample treatment prior to droplet generation, may cause vector genome fragmentation outside of the rAAV manufacturing process, which subsequently results in reporting low values for percentage linkage. Here we have optimized a method, removing the 95 °C heating step, to evaluate the genome integrity of single stranded rAAV using a duplex assay demonstrating 81% linkage. Our duplex assay spans 3103 b.p., Table 1, out of a total vector size of 3878 b.p., representing linkage for 80% of our vector. The impact of the heating step may vary based on the presence of single stranded versus self-complimentary genome as well as the length and sequence of the rAAV genome [21]. Although ddPCR is a robust and reliable technique with reasonable throughput (32 samples per 96-well plate) and can be performed even at low sample concentrations, it is also highly dependent on optimal primer design, and can ultimately provide information on only short target sequences that get amplified and not the sequences in between. Moreover, ddPCR is oblivious to single nucleotide variants, deletions, insertions, or other mutations that may be prevalent. Due to the non-availability of a well-characterized AAV reference standard for

measuring genome integrity, estimating the accuracy of the ddPCR assay is challenging. For comparability we rely on orthogonal approaches in characterizing genome integrity for full size and sequence coverage.

Single molecule real time (SMRT) sequencing can be an alternative technique for vector genome integrity providing full ITR to ITR coverage and can also provide information on truncation events, chimeric species contaminating host cell and plasmid DNA sequences [9,25]. However, such an approach cannot be used as a fully quantitative technique. AUC can orthogonally measure the full, empty, and partial capsids in an rAAV product, but the low throughput and requirement of high sample concentration limits its application in the routine analysis of genome integrity [26]. Newer techniques for empty/full analysis, such as mass photometry, can overcome the limitations of AUC by allowing for real time analysis and short turnaround times [28]. However, like AUC, mass photometry does not provide information as to the location of the truncation for partial AAV genomes. This information can be gleaned from ddPCR. CDMS can be also used to measure the molecular weight distribution of the extracted rAAV vector genome. While this technique can detect the presence of truncated genomes and amount of genome that is missing, it cannot pinpoint to the location of the truncation [23]. A CE-LIF detection method has been developed for evaluating rAAV intact and truncated genomes as well as residual DNA impurities, but this is yet another technique that cannot speak to the sequence of the analyzed genome [24]. In summary, ddPCR integrity analysis can complement orthogonal approaches to provide deeper insight into rAAV vector genome integrity and help inform process development, especially at the anion exchange chromatography step. We hope that our findings on the effects of heat are applicable to sample treatments used in other orthogonal assays for determining genome integrity.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY CMC & ANALYTICS

SPOTLIGHT

REGULATORY PERSPECTIVE

Facing potential chemistry, manufacturing, & control (CMC) development challenges with recombinant adeno-associated viral vectors: available regulatory guidance & recommendations

Stuart G Beattie

These hypothetical 'case studies' and topics illustrate some of the many challenges faced by developers of recombinant adeno-associated virus (rAAV)-based vectors in recent years. Direction to available guidance and resources are provided within this article, along with possible approaches towards mitigating such regulatory risks throughout AAV product development. Whilst the generalized issue topics are not specific to any particular investigator or sponsor, it is intended that they be topical and relevant to challenges that could be faced by developers, including those from smaller enterprises. The breadth of the case studies (or challenges faced by developers) is not exhaustive, and per issue, the approaches below should be modified and developed upon per investigational AAV-based gene therapy medicinal product (GTMP) along with recommendation to seek early endorsement from appropriate health authorities. Most of the references to regional health authority regulatory guidelines are focused to the US Food and Drug Administration (FDA) and European Medicines Agency (EMA).

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EMPTY AAV CAPSIDS

Issue: the risk of health authority non-acceptance of the proposed acceptance criterion for maximum empty capsids.

As per 2020 FDA Guidance CMC Information for Human Gene Therapy Investigational New Drug Applications (IND), "For viral vectors, typical product-related impurities may include defective interfering particles, non-infectious particles, empty capsid particles, or replicating recombinant virus contaminants. These impurities should be measured and may be reported as a ratio, for example, full:empty particles or virus particles:infectious units" [1].

rAAV product-related capsid impurities produced during the manufacture can include the following: empty capsids, which contain no DNA; partially packaged capsids, which contain some DNA but an incomplete genetic payload); overfilled capsids, which contain the therapeutic genetic payload plus additional DNA impurities; and vector aggregates. As these impurities display properties similar to the final product, removal and detection has presented specific challenges.

Per the FDA Cellular, Tissue, and Gene Therapies Advisory Committee (CTGTAC) 2021 Meeting to Toxicity Risks of AAV vectors for Gene Therapy briefing document, AAV empty capsids are "composed of an AAV capsid shell but lacking the vector genome (nucleic acid molecule packaged within)." [2].

The briefing document states how "the presence of empty capsids in clinical formulations is undesirable" and how "elimination of empty capsids can potentially improve the safety margin when high vector doses are administered." The document further states how "reduction of empty capsids to very low levels is achievable, and advances in AAV vector manufacturing designed to optimize downstream purification methods have shown improvements in vector quality with reduced proportion of empty capsids." [2].

If deemed an unacceptable risk to a patient, a health authority may ask the sponsor to introduce manufacturing process steps to remove empty capsids; or, where, as a critical quality attribute (CQA), empty capsids represent an increased risk, this may instigate the need for a bridging toxicity study to support the levels of empty capsids in the product.

Approaches for potential mitigation of risk

Seek health authority endorsement on the proposed release tests, acceptance criteria for empty particles and characterization assays, for example, via a pre-IND meeting with FDA (or EMA or National Scientific Advice) on a case-by-case basis:

- Provide information on all assays used to test product for a First in Human (FIH) clinical trial, whether for release and stability or characterization, their validation or qualification status (if for release and stability) and the intended acceptance criterion for release and stability.
- Since, to date, health agencies have not formalized guidance to specify an upper limit for residual empty particles, it is recommended to continue to adopt a quality by design (QbD), risk-based approach towards setting an acceptance criterion on the ratio of full:empty particles for particular products, based on the indication, route of administration, total capsid input (dose), any immunogenicity concerns and benefit-to-risk ratio considerations.
    - Per the FDA Cellular, Tissue, and Gene Therapies Advisory Committee (CTGTAC) Meeting #70: Toxicity Risks of Adeno-associated Virus (AAV) Vectors for Gene Therapy Sep 2–3, 2021, "For early-phase development, sponsors should, at a minimum, measure the levels of impurities in clinical vector lots and demonstrate

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similar purity between lots used in IND-enabling preclinical studies and clinical lots [...]" [2].

- Utilize reference standards across clinical development that can act as a comparator with regard to levels of empty (and partially packaged) capsids.
- Seek health authority advice early with regard to the specific product in development and the proposed upper limit for the ratio of full:empty particles.
- As previously developed at the 2020 Virtual NIH Workshop on Systemic Immunogenicity Considerations for AAV-Mediated Gene Therapy, there is a recommendation to establish a total capsid titer method as a CQA for safety, especially for indications which require high doses [3,4].
- It is also recommended to correlate levels of empty:full capsids to potency *in vitro* and in animal models that can accurately predict effects of varying % empty:full.

Refer to the following ICH chapters with regard to adoption of a QbD, risk-based, approach towards setting empty capsid specifications:

- ICH Q8 (R2) pharmaceutical development [5].
- ICH Q9 quality risk management [6].
- ICH Q10 pharmaceutical quality system [7].

The British Pharmacopoeia (Medicines and Healthcare products Regulatory Agency) have undertaken a consultation for Guidance on the Characterization of the particle population in AAV products [8]. This closed January 31st, 2023, but, when finalized, will address the use of methods for the characterization of viral particles in AAV-based therapies and will provide current best practices.

In January 2023, Dark Horse Consulting released a white paper regarding empty, full and partial AAV capsids and inherent product heterogeneity, in addition to product-related impurities [9]. This follows a proposed release criterion of no more than 30% empty capsids within guidance submitted to the FDA May 2022 [10]. The criterion was based on other guidance pertaining to percentage unviable human somatic cells [11]. Such a criterion had been dismissed by industry [12], where instead, as above, a QbD, risk-based approach per product, is recommended instead. At the Apr 25th, 2023 FDA Office of Therapeutic Products CMC Town Hall, the FDA stated how they cannot provide a "magic number", nor are they proscriptive with regard to analytical methods [13].

POTENCY TESTING

As quoted in a recent white paper authored by the American Society of Gene and Cell Therapy (ASGCT) together with the Alliance for Regenerative Medicine (ARM), Peter Marks, the Director of the FDA Center for Biologics Evaluation and Research (CBER), has remarked that "there's pretty uniform agreement that one of the key things that has delayed a fair number of approvals over the course of time has been issues related to potency" [14]. Two possible scenarios are presented below.

(Possible) issue scenario 1: that the biological mechanism of action (MoA) is not always fully elucidated prior to clinical studies (e.g., for a structural protein within the retina) despite proof of concept having been demonstrated in laboratory animal model(s). A functional potency assay is absolutely required to correlate the product MoA to clinical efficacy.

Surrogate potency tests can be acceptable for FIH studies, if other unqualified characterization assays are available to demonstrate functional potency of the rAAV GTMP. For example, an *in vivo* potency assay. The draft EMA guidance (EMA/CAT/852602/2018) states how "Surrogate potency markers can be considered for release tests, but appropriate

justification on their relevance in the context of the intended action of the ATIMP is needed." [15].

Regarding the *in vivo* potency example, a clear correlation to functional potency must be provided for across clinical batches.

There should be additional assays to demonstrate other aspects of potency, e.g., infectivity, transduction, mRNA expression (as described below for a matrix of assays) for a FIH submission.

Both the FDA and EMA can accept validated surrogate assays as long as there is a functional assay available for characterization with correlation to the selected assay(s) during early studies.

Selected potency assays should provide an accurate, reliable and consistent demonstration of the biological activity of the product and also be able to detect sub-potent batches. A qualified potency assay can ensure proper activity of the product, help with dose selection extrapolation from non-clinical studies into FIH.

It is advisable to seek health authority endorsement sufficiently early, and to discuss the potency assay matrix, so as to minimize any impact on IND/clinical trial application submissions.

(Possible) issue scenario 2: difficulty developing a validated *in vitro* potency assay prior commencement of a Phase 3 clinical trial.

Despite adoption of a matrix approach to demonstrate infectivity (infectious dose, infectious titer, expression), there can be considerable technical challenges when developing an *in vitro* potency assay. One challenge can be that there are only limited available clonal cell lines that may be permissive to infection with the AAV serotype of the product in development. This may necessitate cell line engineering, for example with stable expression of AAVR (for serotypes where AAVR is a secondary receptor [16]). Such cell line engineering approaches can take a considerable amount of time and should not be underestimated when planning a future submission of a dossier for a pivotal trial.

Potency assays for cell and gene therapy products are expected to be in place for exploratory clinical studies and are expected to be validated prior to the start of confirmatory clinical studies within the EU, so that a correlation between potency and efficacy can be assessed. The draft EMA guidance (EMA/CAT/852602/2018) states how "It is strongly recommended that the development of a suitable potency assay be started as soon as possible. Preferably, a suitable potency assay should already be in place when material for the FIH clinical trial is produced and it should be validated prior to confirmatory clinical trials unless otherwise justified" [15].

Potency assay development is often stepwise with regard to investigation of biological activity and the development of a relevant potency assay [17]. However, as observed by the FDA, historically, assay development and product characterization are often only initiated once a cell or gene therapy product has entered clinical studies, leading to potential delays to approval to commence Phase 3 clinical testing [17].

The potency test for commercial release testing should be qualified before the pivotal clinical trial and be described, justified and validated at the time of the marketing authorization application.

As stated in March 2023 draft guidance for CAR-T products, but applicable to all analytical methods applied to AAV-based therapeutics, including those determining potency, "Validation of analytical procedures is usually not required for IND submissions for Phase 1 studies; however, we recommend providing information that demonstrates appropriate control of the test methods. Each assay should be qualified prior to initiating studies intended to provide primary evidence of effectiveness to support a marketing application, and assays must be validated to support a BLA (21 CFR 211.165[e])." [18].

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Approaches to potentially resolve issue

Seek health authority endorsement of a matrix approach for potency testing of an investigational GTMP. This could be via Scientific Advice/pre-IND, or an end of Phase 1/2 meeting, where there is also the possibility to seek joint advice from EMA [19] and FDA [20] (SOPP 8001.6 Procedures for Parallel Scientific Advice with European Medicines Agency. (For advice on potency testing for a planned Phase 3 investigational GTMP, an end of Phase 2 meeting is generally too late if the health authority disagrees with the proposed approach).

Example, if there is not an available *in vitro* biological potency assay:

Provide information and validation status to (example) assays that constitute the matrix:

(i) Relative Transduction Assay: a measure of the ability of the AAV vector to actively transduce human cells *in vitro*, using a relative comparison to an AAV reference standard;

(ii) Relative mRNA Expression Assay: measures mRNA transcripts, relative to an AAV reference standard;

(iii) Relative Protein Expression Assay: measures expression of the gene of interest, relative to an AAV reference standard;

(iv) (For example) *in vivo* (mouse) potency assay (or tissues maintained *ex vivo*).

Provide information on the correlation between these different assays.

Potency testing should also be performed as part of a product stability program. Further, it is recommended to utilize reference standards throughout development (that have demonstrated comparability and equivalence) and to retain sufficient vials from batches of drug product to bridge data obtained across different potency assays. It is important to ensure that there is a plentiful supply of reference standards to support a variety of analyses.

As before, it is advised to engage with health authorities as early as possible with regard to potency testing strategy that can be endorsed by regulators. Due to the increasing number of rAAV-based products in early development, regulators will often have seen similar issues with other applicants and are generally willing to offer guidance into areas for evaluation.

Refer to health authority guidance and industry recommendations:

- FDA
  - Where there is complementarity between drug substance (DS) and/or drug product (DP) release testing and clinical trial efficacy methods, the Rare Disease Endpoint Advancement Pilot Program (RDEA) [21] is possibly worth considering. As announced Oct 27, 2022, the FDA have established a RDEA Pilot Program for sponsors with an active pre-IND or IND, to support novel endpoint efficacy development for drugs that treat rare diseases, to seek to advance rare disease drug development programs by providing a mechanism for sponsors to collaborate with FDA throughout the efficacy endpoint development process [22];
  - The RDEA Pilot intends to promote innovation and evolving science by sharing learnings on novel endpoint development through FDA presentations, guidance documents, public workshops, and a public-facing website;
  - Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) Guidance for Industry Jan 2020 [23];
  - Bioassays for Potency: An FDA Perspective [Presentation] Price, 2017 [17];
  - Potency Tests for Cellular and Gene Therapy Products, Jan, 2011 [25].

- **EMA**
  - EMA's Guide on Advanced Therapy Medicinal Products Version 1.0 Nov 29, 2021 [26];
  - Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014; Mar 22, 2018) [27];
  - Draft Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (EMA/CAT/852602/2018; Jan 31, 2019) [15].
- **ICH**
  - ICH Q2 (R2) Validation of analytical procedures [28]: The relevance of the potency assay, read-out, and acceptance criteria should be in accordance with ICH Q2 and further supported by (clinical) data to corroborate that the assay will identify sub-potent batches. Step 2b of (R2) closed Jul 31, 2022 and the guideline is currently under revision;
- USP: USP <1033> Biological Assay Validation (2010);
- ASGCT, ARM white paper: Addressing potency-assay related developmental delays for cell and gene therapies. Mar 22, 2023 [14];
- Potency testing of cell and gene therapy products, Salmikangas *et al.*, 2023 [29].

COMPARABILITY

Issue: how different manufacturing process changes (especially upscaling) influence product characteristics determining infectivity, potency and other CQAs for a rAAV GTMP?

The product must be comparable across all studies to be included in the registration dossier. Therefore, the product has to be produced consistently and with reproducible quality, including after upscaling production capacity.

Although not specifically addressed here, the same principles apply for when there could be material changes. Please see the section below for further information relating to raw materials.

To be able to include pre- and post-change clinical data into a registration dossier, product comparability needs to be demonstrated. This may depend on which pivotal clinical data are intended to be presented within a marketing application; some data may be supportive but may not be part of the primary efficacy dataset.

It is generally accepted that for complex biological medicines which cannot currently be considered well-characterized, including rAAV-based products, that making manufacturing process changes can be challenging. Despite the availability of established and emerging methods [30], there are limited analytical capabilities to demonstrate comparability of such complex products. The concern is that it is not possible to fully evaluate quality, and by extension safety and efficacy, through application of simple physicochemical tests. Additional data are required to evaluate the impact of changes in materials or processes. Comparability studies to maintain CQAs are essential to product approval.

According to ICH Q5E, generation of batch comparability data should include results from in-process control testing, extended characterization, and release and stability testing for both pre- and post-change batches [31]. ICH Q5E recommends a stepwise approach, where if there are concerns that analytical comparability has not been demonstrated, then it may be necessary to conduct bridging non-clinical and/or clinical studies.

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Approaches towards & questions to ask when addressing changes to a manufacturing process

- Be aware of general agency expectations for comparability plans:
  - Describe the change in the manufacturing process and the rationale for the change. Determine the best stages for assessing comparability (in-process, DS, DP, etc.);
  - Where possible, all starting materials must be qualified and must undergo extensive characterization during manufacture and as part of any process change. There can be exceptions, for example, following GMP principles, but not necessarily through the use of GMP plasmids as a starting material for *in vivo* gene therapy viral vectors, such as rAAV. This is discussed in further detail below in the section 'GMP requirements for plasmids as a starting material to manufacture rAAV';
  - Describe the risk assessment and the findings from the process;
  - Is it intended to look at stability? What stability-indicating methods and conditions are in your analytical toolbox?
  - Analytical methods should be suitable for purpose and sufficiently sensitive to ensure the detection of differences or modifications.
  - What is the validation status of the assays? For comparability, all analytical methods should be validated and be robust. Methods applied for characterization do not need to be validated but shown to be suitable for use at an early stage;
  - It can be challenging to demonstrate that an analytical method is suitable or sufficiently sensitive to detect differences that may, or may not, be present. Health agencies will suggest taking an orthogonal approach and to provide as much analytical data as possible. However, the applicant has to justify the approach taken and it is suggested to seek endorsement of a comparability protocol.
  - Is there a reference standard, and if so, what is its source?
  - What is the rationale behind your statistical analysis approach? FDA are likely to additionally request a justification for the choice of the statistical approach used for the comparability assessment. A risk-ranking of CQAs can be performed to drive the selection of the preferred statistical methodology;
  - A combination of various methodologies can be used to understand the robustness of the chosen statistical approach. Inclusion of side-by-side analysis of individual values with accompanying descriptive statistics to summarize data (e.g., min-max and 3*sigma ranges) is recommended, particularly when comparing a limited number of samples or batches (e.g., in earlier development phases). Likewise, suitable graphical representations (e.g., individual value scattergrams) could be provided, allowing the identification of possible shifts within acceptance criteria.
  - Describe the comparability study design and explain the underlying assumptions and risk assessment informing the plan;
  - It is recommended to undertake a side-by-side analysis for comparability of CQA's;

- Determine clear methods for determining the impact of a process change. Is it a minor or major change?

- Adopt a risk-based approach for analytical comparability and comparability protocols;
  - The risk-based approach should be used to determine an appropriate amount of comparability data and to select a suitable set of relevant CQAs to be compared;
  - The defined acceptance criteria limits for your comparability protocol should reflect both process and analytical method variability and be justified by clinical batch data.

- It is strongly advised to avoid multiple manufacturing facilities across clinical development, where possible. However, if a rAAV-based product is produced at multiple manufacturing sites, comparability of the product has to be demonstrated with data, even if the same equipment, materials, procedures, quality control tests, etc., are used;

- Changes to the rAAV vector construct should preferably be limited to early development phases as reflected upon by the EMA [32].

Adopt a phase-appropriate approach to demonstrating comparability

- In very early pre-clinical development, changes to the rAAV manufacturing process may be frequent and extensive and comparability is not expected. The main comparability considerations up to Phase 1/2 are to demonstrate that representative product has been used in the non-clinical safety studies, so that the safety profile is predictive for such exploratory FIH / Phase 1/2 studies [33];
  - Comparability may be assessed retrospectively, without a protocol or acceptance criteria: results are used to inform and support Qualified Person release of GMP material for a Phase 1/2 clinical trial.

- In the case of exploratory clinical trials, it is recommended to use investigational product representative of the material used in non-clinical studies. More stringent equivalence is required when toxicity and dose finding studies have been undertaken [15];

- When exploratory studies have already taken place, data filiation program should expand to a full comparability exercise where a higher degree of sameness is expected, and a more comprehensive analytical package should be in place;

- As stated by the office of Gene Therapy in the Apr 25, 2023 FDA OTP Town Hall [13], for developers that are preparing to perform analytical comparability in order to implement a manufacturing change before a pivotal study, it is recommended to qualify non-compendial assays before starting a comparability study, in order to enhance the precision and quality of the data obtained;

- For confirmatory trials, the principles found in ICH Q5E can be applied [31]. During the confirmatory clinical studies, introducing changes to the manufacturing process and the final product should be avoided, because comparability issues may impact the acceptability of the clinical data;
  - Where the relevant information is not sufficient to assess the consequences introduced by the change and if a potential risk to the patients cannot be excluded, a comparability exercise based only on quality considerations most likely will not be sufficient and

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further non-clinical and clinical data will be required through bridging studies.

- In later stages of development, when more product knowledge is available, the manufacturing process evolves (for example, to scale, manufacturing site, formulation, product presentation, storage, etc.) and pivotal studies are performed. A full comparability exercise is required, encompassing a series of in-process tests and parameters, release tests as well as extended characterization assays;
  - As stated by the office of Gene Therapy in the Apr 25, 2023 FDA OTP Town Hall "It's always our advice to think with the end in mind and deal with manufacturing changes and comparability exercises before starting your pivotal study." [13];
  - The introduction of substantial changes to the manufacturing process and the final rAAV-based drug product during, or after, pivotal clinical studies are not recommended due to the complexity of the comparability exercise and the possible impact of its results on the acceptability of the clinical data [33];
  - In cases where late-stage changes in the manufacturing process are unavoidable, it is recommended to seek advice from health authorities;
  - It is particularly important that all stages of development are fully evaluated, justified and tracked within the evolving dossier.

Refer to health authority guidance and information:

- **ICH**—overall, the general principles of ICH Q5E can be applied to ATMPs:
  - ICH Q5E: Comparability of biotechnological/biological products (CPMP/ICH/5721/03; Jun 2005) [31];
  - The comparability exercise should be conducted stepwise, starting with the physio-chemical and biological properties of the product. This will be based on analytical testing, e.g., routine batch analysis, in-process controls, process validation/evaluation data, characterization and stability studies, as applicable.
- **FDA**
  - Manufacturing Changes and Comparability for Human Cellular and Gene Therapy Products. Guidance for Industry Guidance for Industry Draft. Jul 2023 [24];
  - Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) Guidance for Industry Jan 2020 [23];
  - Comparability Protocols for Human Drugs and Biologics: Chemistry, Manufacturing, and Controls Information Guidance for Industry Apr 2016 [34];
- **EMA**
  - EMA'S Guide on Advanced Therapy Medicinal Products Version 1.0 Nov 29, 2021 [25];
  - Reflection paper on statistical methodology for the comparative assessment of quality attributes in drug development (EMA/CHMP/138502/2017; Jul 26, 2021) [35];
  - Draft Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy

medicinal products in clinical trials (EMA/CAT/852602/2018; Jan 31, 2019) [15];

- Questions and answers on comparability considerations for advanced therapy medicinal products (EMA/CAT/499821/2019; Dec 6, 2019) [33];
- Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014; Mar 22, 2018) [27];
- Reflection paper on design modifications of gene therapy medicinal products during development (EMA/CAT/GTWP/44236/2009; Dec 14, 2011) [32];
- Guideline on Comparability of Biotechnology-Derived Medicinal Products After a Change in the Manufacturing Process Non-Clinical and Clinical Issues (EMEA/CHMP/BMWP/101695/2006; Jul 19, 2007) [36].

- **Japan's Ministry of Health, Labor and Welfare**
  - Ensuring the quality and safety of gene therapy products Ensuring the quality and safety of gene therapy products. Notifications and administrative notices. PSEHB/MDED Notification No.0709-2 Jul 9, 2019. Provisional Translation (as of Jul 2020) [37];
  - Guidance is useful for comparing expectations stated for other health authorities.
- **China's Center for Drug Evaluation of the National Medical Products Administration**
  - Technical Guideline for Pharmaceutical Study and Evaluation of Gene Therapy Products:
    - Includes an overview of expectations for comparability assessments, along with expectations for quality studies for gene therapy products.
- **ARM**
  - A-GENE Chapter 8: Comparability, 2021 [38];
  - ARM-USP Workshop Comparability in Cell & Gene Therapies Final Report & Summary; May 31, 2019; Rockville Maryland, USA [39];
- Proving Comparability in Cell and Gene Therapy Development: Untangling FDA Requirements. Burger & Janssen, Mar 2022 [40];
- Demonstrating comparability of AAV gene therapy products during clinical development: managing the link between the product and the process [41];
- Analytical methods for process and product characterization of recombinant adeno-associated virus-based gene therapies [30].

CONTROL OF RAW MATERIALS

As per ICH Q7, the general term material is used to denote (starting materials, reagents, solvents), process aids, intermediates, active pharmaceutical ingredients and packaging and labelling materials [42].

Raw materials are the reagents that are used during the manufacturing process but are not part of the final product. Examples include fetal bovine serum, trypsin, digestion enzymes (e.g., collagenase, DNAse), growth factors, cytokines, resins and media components.

Starting materials (such bacterial cell banks, cell producing banks, virus banks, container closure or further ancillary materials) are not included in this section.

A section regarding GMP Requirements for Plasmids as a Starting Material to Manufacture rAAV is included below.

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Across cell and gene therapeutics, as well as biologics, raw material quality is a key driver of product quality and consistency. Assuring the quality of raw materials is, therefore, an important component of an overall control strategy. A manufacturer's control strategy must ensure the quality of raw materials for producing therapeutic proteins even when supply chains are disrupted, such as during the COVID-19 pandemic [43].

Reference to quality standards (e.g., compendial monographs or manufacturer's in-house specifications) should be made. As per the European Commission Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products [43], "As far as possible, raw materials used in the manufacturing of ATMPs should take into consideration the Ph. Eur. 5.2.12 general chapter on raw materials of biological origin for the production of cell based and gene therapy medicinal products. While raw materials should be of pharmaceutical grade, it is acknowledged that, in some cases, only materials of research grade are available".

For the US, please refer to USP <1043> Ancillary Materials for Cell, Gene, and Tissue-Engineered Products.

One of the challenges that GTMPs continue to face is the high degree of variability resulting from both new raw (/starting) materials and the manufacturing process conditions. Information on the quality and control of non-compendial materials should be provided.

Further, 'Research grade' raw materials that may be used during research are not appropriate during development of an rAAV GTMP, where there may not always be a secondary source. These challenges have necessitated assessments of the risks and testing to ensure that the raw material meets quality standards. Indeed, the EC Guideline for GMP for ATMPs further states that "The risks of using research grade materials should be understood (including the risks to the continuity of supply when larger amounts of product are manufactured). Additionally, the suitability of such raw materials for the intended use should be ensured, including–where appropriate–by means of testing (e.g., functional test, safety test)".

The use of animal and human-derived component-free manufactured raw materials, precured from safe and traceable sources can significantly reduce qualification and validation activities for cell and gene therapy manufacturers. Information demonstrating that materials (including biologically-sourced materials, e.g., media components, enzymes) are suitable for their intended use should be provided.

Steps in a qualification program:

- Identify and select material based on suitability during manufacture and testing.
- Compendial and non-compendial raw materials with in-house testing and qualification, depending on the context of use.
    - Raw materials should comply with compendial monographs, where appropriate (Ph. Eur., USP, JP, ChP).
- Characterization of raw material during process and product development.
- Quality assurance consistent with above.

Source & identity:

- The name of the manufacturer, identity, and quantity of each shipment of each batch of raw materials, intermediates, or labeling and packaging materials; the name of the supplier; the supplier's control number(s), if known, or other identification number; the number allocated on receipt; and the date of receipt.
- The results of any test or examination performed and the conclusions derived from this (including microbial/endotoxin testing).
- Records tracing the use of materials.

- Documentation of the examination and review of labelling and packaging materials for conformity with established specifications.
- The final decision regarding rejected raw materials, intermediates, or labeling and packaging materials.

Safety considerations specific to raw materials:

- Sterility, Bioburden
- Endotoxin
- Mycoplasma
- Leachables from containers, transport and delivery devices
- Source species-specific host cell proteins from animal or plant-derived raw materials
- Source species-specific viral and non-viral adventitious agents from human, animal or plant-derived raw materials
- Transmissible spongiform encephalopathy agents (TSE)
- Context of use, stability and interaction of residual material, formulation or container closure components
- Sensitivity of target patient population, including immune reactions to raw materials of biological origin

It is recommended to discuss the adequacy of raw material qualification protocols, including risk assessments, with health authorities before manufacture of toxicology and initial clinical batch(es) when using non-GMP raw materials.

It is possible for developers to utilize the Master File system for certain ancillary materials to support IND and BLA submissions to the FDA. Draft 2019 CDER and CBER guidance Drug Master Files Guidance for Industry [45] provides FDA's current thinking. However, a master file for such a raw or ancillary material is not helpful if applying to multiple countries since this necessitates the preparation of regionalized sections containing comprehensive information for submissions outside of the US. No such system currently exists in the EU and all raw materials must be disclosed and data provided as part of CTA and MAA.

Furthermore, the use of a master file for such materials removes some of the sponsor's quality oversight over the suppliers of such materials.

In an IND, provide a list of all materials used in manufacturing and a description of the quality of these materials (21 CFR 312.23(a)(7)(iv)(b)) [24]. Place raw material qualification information into Section 3.2.S.2.3., Control of Materials, of an eCTD regulatory dossier Viral safety.

Use a combination of measures to ensure viral safety of AAV-based GTMPs, including viral clearance studies to determine reduction factors of relevant steps, where appropriate.

Consult the (current) draft ICH Q5A (R2) [46] consensus guideline to viral safety evaluation of biotechnology products derived from cell lines of human or animal origin.

TSE agents:

All raw materials consisting of animal tissue or fluids or containing product of animal origin or materials which have come in contact during production with materials of human or animal origin should comply with the relevant guidance, including that from the European Commission [47].

It is advised to liaise with the supplier of the raw material to ensure that the vendor provide a valid and up to date Certificate of Suitability for a material likely to present a TSE risk.

Per the 2020 FDA CMC information for human INDs guidance [24] it is recommended to provide information on any bovine material used in manufacturing, including the source of the material; information on the location where the herd was born, raised, and slaughtered; And any other information

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relevant to the risk of TSE. If serum is used, it is recommended that it be γ-irradiated to reduce the risk of adventitious agents. This information may be included on the Certificate of Analyses and Certificate of Origin (COO) provided from the supplier.

While the risk of TSE propagation by mammalian cells is low [48], measures to eliminate must be enacted to mitigate potential exposure. If this is not possible, a comprehensive risk assessment based on species, including bovine [49], tissue, country of origin, and manufacturing process used to produce the raw material or component should demonstrate that there is no residual risk from TSE agents.

Refer to pharmacopoeial health authority guidance and information:

- **Ph. Eur.**
  - 5.2.12 Raw Materials of Biological Origin for the Production of Cell-based and Gene Therapy Medicinal Products.
  - 5.14 'Gene transfer medicinal products for human use' and 5.1.7 'Viral safety'.
- **USP**
  - The United States Pharmacopeia-National Formulary (USP-NF) contains several general chapters and monographs that describe quality attributes, tests, and acceptance criteria for raw materials and excipients.
    - For example, USP <89> Enzymes Used as Ancillary Materials in Pharmaceutical Manufacturing includes quality attributes and associated tests and acceptance criteria for recombinant trypsin and other enzymes that may be used during production of rAAV.
    - USP <1043> Ancillary Materials for Cell, Gene, and Tissue-Engineered Products.
- **FDA**
  - Human Gene Therapy for Neurodegenerative Diseases, Oct 2022 (Final) [50].
  - Regulatory Considerations for Raw Material Qualification and Related Controls for Biotechnology Drugs USP Workshop on Raw Materials for Manufacturing of Biologics: Best Practices and Quality Standards. Rao. Apr 2021 [51].
  - Chemistry, Manufacturing, and Controls Changes to an Approved Application: Certain Biological Products Jun 2021 [52].
  - Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) Guidance for Industry Jan 2020 [24].
- **EMA**
  - Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014); Mar 22, 2018 [27].
  - Questions and answers on the principles of GMP for the manufacturing of starting materials of biological origin used to transfer genetic material for the manufacturing of ATMPs (EMA/246400/2021) Feb 24, 2021) [53].
- **ICH**
  - ICH Q5A (R2) [46] [Draft] Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin Step 2b. EMA/CHMP/ICH/804363/2022. Oct 10, 2022.
  - ICH Q7A (R1) Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients (2016) [54].
- **European Commission**
  - EudraLex. The Rules Governing Medicinal Products in the European Union. Volume 4. Good Manufacturing Practice. Guidelines on Good

Manufacturing Practice specific to Advanced Therapy Medicinal Products Nov 22, 2017 [44].

- Japan: Guideline to Assure Quality and Safety of Medicines Manufactured Using Human Cells and Tissues Components. Notification: PFSB 0208003. Feb 8, 2008. [see other relevant local guidance].
- **BioPhorum**
  - Raw Materials Cell and Gene Therapy Critical Starting Material–Establishing Release Specifications for Plasmids. Clements. Sep 2022 [55].
  - Raw materials strategy Jul 2022 [56].
  - Includes: Appendix A–Raw Materials Program publications.
- Raw materials for cell & gene therapy: exploring regulatory and supply issues [57].

GMP REQUIREMENTS FOR PLASMIDS AS A STARTING MATERIAL TO MANUFACTURE rAAV:

An EMA questions and answers [53] document clarified the principles of GMP manufacturing of starting materials of biological origin, including those for plasmid DNA used for manufacture of vectors such as rAAV. GMP principles should be applied when manufacturing plasmid, where rAAV manufacturers must use a risk-based approach risk-based [44] approach for the supply of plasmid DNA as a starting material; Whilst the subsequent steps should be compliant with GMP:

- Establishment of a cell bank (MCB, WCB) and virus seeds, when applicable.
- Vector manufacturing and purification.
- Formulation and filling.

The Sep 29, 2022 OTAT CMC Town Hall [58] transcript states that "The US regulations do not require that plasmid starting material be made under strict GMPs, nor that the level of detail for these materials is the same as a drug substance". The transcript provides further context. However, FDA recommend that DNA plasmid intermediates be derived from qualified banks and that in an IND [24] that information be provided on the plasmid manufacturing procedures, reagents, and plasmid specifications for use, regardless of whether they were made by the IND sponsor, or a contract manufacturer.

FDA also recommend DNA plasmid testing include sterility, endotoxin, purity (including percent of supercoiled form and residual cell DNA, RNA, and protein levels) and identity testing (restriction digest and sequencing if sequencing was not performed on the bacterial bank). A Certificate of analysis documenting plasmid quality testing should be included.

Finalized FDA guidance to Human Gene Therapy for Neurodegenerative Diseases [50] replaced the previous statement to how plasmids should be of the "highest purity", to now advise to plasmids meeting "acceptable limits for purity".

Further in this finalized guidance, instead of an absolute requirement to test for the presence of other contaminating plasmids (used to generate AAV-based products), an alternative is provided, where "a risk assessment may be conducted to provide assurance of freedom from other contaminating plasmids that may have been co-purified".

Per draft Ph. Eur. 35.2 (5.3.4) it is advisable to report the percentage *E.coli* cells retaining the plasmid for master and working cell banks and end of production cells.

As yet, there are no specific comparability requirements for plasmids between process changes. However, it is advised that plasmid quality be maintained across such changes, or if there were to be a change in vendor. In such cases, it is recommended that a comparability assessment be undertaken on a case-by-case basis, for which this may involve a paper-based

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exercise, dependent on justification of impact to the rAAV product.

Refer to pharmacopoeial health authority guidance and information, as, above with reference to the below EMA guidance:

- Questions and answers on the principles of GMP for the manufacturing of starting materials of biological origin used to transfer genetic material for the manufacturing of ATMPs (EMA/246400/2021); Feb 24, 2021 [54].
- There is also Ph. Eur. 35.2 (5.3.4) Additional information on gene therapy medicinal products for human use. This draft monograph includes revised text for plasmid vectors for human use. This also includes tests required at each stage of production of the bacterial cell banks.
- **BioPhorum**
- Case study: plasmids release specifications: aligning industry in a bid to reduce costs, timelines, and testing. Dec 2022 [59].
- Further discussion on plasmids to establish release specifications using a risk-based approach to manage supply. Jun 2022 [60].
- Raw materials: Cell and gene therapy critical starting material: a discussion to help establish release specifications for plasmids and the bacterial master cell banks used to produce them. Nov 2020 [61].

LIMIT TO RESIDUAL HOST CELL-DNA LEVELS

Issue: draft FDA [50] guidance recommended that gene therapy "vectors used to treat neurodegenerative diseases not be grown in tumorigenic cell lines and the residual host cell-DNA levels be set to less than 10 ng/dose, if possible".

During production of rAAV, whether in mammalian or insect cells, host cell DNA are encapsidated, representing total residual host cell DNA. During purification of rAAV, non-encapsidated residual host cell line DNA are effectively cleared. As such, total host cell DNA is comprised of encapsidated DNA. The 10 ng/dose limit was considered by industry to be presently unattainable for AAV.

The 2018 EMA guideline [27] states that "If tumorigenic / immortalized cell lines are used during production the total residual DNA level should be strictly controlled and kept at a minimum unless otherwise justified".

In the draft FDA guidance for neurodegenerative diseases, it was noted by industry to how the proposed residual host-DNA limit stated is for "tumorigenic cell lines". There was some inconsistency to the definition of the cells, as the 2020 FDA CMC guidance [24] states "continuous non-tumorigenic cells". Previously, the 1997 EMA CPMP Position Statement [62] on DNA and Host Cell Proteins (HCP) Impurities, Routine Testing versus Validation Studies stated that continuous cell line "DNA poses much less of a risk than previously thought and accordingly should be considered as a general impurity (WHO Expert Committee on Biological Standardization)."

REVISED FINAL FDA GUIDANCE FOR NEURODEGENERATIVE DISEASES

The final guidance does not restrict the use of cell lines and recommends that sponsors "carefully consider characteristics of the cell lines used in the manufacture of viral vectors that may impact the safety of the final product (such as presence of tumorigenic sequences)" and generally "limit residual host cell-DNA levels and DNA size".

Refer to health authority guidance:

- **FDA**
  - Human Gene Therapy for Neurodegenerative Diseases Oct 2022 (Final) [50].

- Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) Guidance for Industry Jan 2020 [24].

- EMA

  - Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014); Mar 22, 2018 [27].

USE OF Sf9 INSECT CELLS WITH COMMON RHABDOVIRUS CONTAMINANT FOR PRODUCTION OF rAAV

Issue: potential future risk of requirement for 'rhabdovirus-free' Sf9 insect cell banks

Spodoptera frugiperda (Sf) rhabdovirus (Sf-RhV or RhV) persistently infects most commercially available Sf insect cell lines [63,64] without causing any overt cytopathic effects [65].

In 2014, investigators at the U.S. Food and Drug Administration Center for Biologics Evaluation and Research reported Sf21 cells obtained from Invitrogen (Carlsbad, CA) and Sf9 cells obtained from Invitrogen and ATCC (Manassas, VA; CRL-1711 lot 58078522) are contaminated with an adventitious viral agent [63]. Based on its negative stranded RNA genome, genome organization, and sequence, this agent was classified as a novel rhabdovirus, which is now known as Sf-rhabdovirus (Sf-RhV; [63]).

Like all other rhabdoviruses, Sf-rhabdovirus encodes five conserved proteins, including nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G), and polymerase (L). Like many other rhabdoviruses, the original Sf-rhabdovirus harbored by Sf9 cells also encodes a non-conserved sixth protein (X) of unknown function [64].

Since the original discovery of this agent, other investigators have confirmed the presence of Sf-rhabdoviral RNA sequences in Sf9 [66] and Sf9^{L5814} [67] cells. Thus, Sf-rhabdovirus is now widely recognized as a common contaminant of at least some Sf cell lines used as hosts for baculovirus-mediated recombinant protein production. This is important because Sf cells are being used not only for research purposes, but also to manufacture products approved for human clinical applications. These include Dendreon's Sipuleucel T (PROVENGE®) and Protein Science Corporation's Flublok® [68].

Maghodia & Jarvis further assessed Sf-RhV infectivity, demonstrating that a productive persistent infection was not established in Sf9 cells, nor in mammalian cell lines [64] confirming previous results indicating that Sf-RhV have a narrow host range [63]. As such, there is no evidence that mammalian cells are permissive for RhV infection.

Given that Sf9 is derived from a moth whose larvae feed on human-edible foods, Schroeder and colleagues explored the prevalence of Sf-RhV in its wild and lab-grown populations, as well as its ability to be deposited on food items during feeding [65]. The authors suggest that environmental exposure of humans to Sf-RhV is likely to be commonplace and frequent, but its inability to replicate in plant or human cells suggests that there is no substantial risk to human health [65].

Possible approaches to mitigate risks

- Implement several physico-chemical orthogonal unit operations to inactivate or remove rhabdovirus from the bulk drug substance.

- For viral clearance studies, a highly concentrated virus stock is required for spiking the test articles. In 2017, it was first demonstrated that Sf-RhV can establish productive persistent infections in Sf cells and that Sf-RhV exhibits low levels of infectivity, existing at low titers, with a limited host range [63,64]. Due to the challenges in generating

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high concentration Sf-RhV stocks, it is recommended to utilize vesicular stomatitis virus (VSV) as a model virus for Sf-RhV. VSV is a commonly used model virus representing the rhabdovirus family (see Table A-1 ("Examples of viruses which have been used in virus clearance evaluation studies" in ICH Q5A(R1)). VSV is approximately the same size as Sf-RhV, with the same genetic and structural configuration.

- It is recommended that sponsors seek health authority early endorsement to the use of the RhV+ Sf9 cells. It could be a matter of time that regulators request utilization of 'rhabdo-free' Sf9 cell banks.

Refer to existing guidance:

- ICH Q5A (R2) [46] [Draft] Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin Step 2b. EMA/CHMP/ICH/804363/2022. Oct 10, 2022.
- ICH Q5A (R1) [69] ICH Q5A (R1) Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin Step 4. Sep 23, 1999.

ALTERNATIVE MEETINGS TO FORMAL FDA MEETINGS & EMA OR EU MEMBER STATE NATIONAL SCIENTIFIC ADVICE:

FDA: An alternative to a specific formal meeting with the FDA is also to ask a general, non-product-specific question (ahead of time or live) in an Office of Therapeutic Products (OTP) Town Hall meeting. A transcript [58] is available for the first FDA CBER (OTAT) Town Hall to Gene Therapy Chemistry, Manufacturing, and Controls (held Sep 29, 2022). Another CMC Town Hall was held April 25, 2023 [13].

EMA: The Innovation Task Force (ITF) [70] is not exclusive to micro, small and medium-sized enterprises or academics, and may facilitate early discussion to some of the common challenges to developers of rAAV-based medicinal products (as above). As noted on the website, topics discussed with the ITF include innovative manufacturing methods.

United Kingdom Medicines and Healthcare products Regulatory Agency (MHRA) Innovation Office [71] is part of the MHRA's Innovation Accelerator and is open to queries about innovative medicines (including ATMPs), medical devices and manufacturing processes. It can provide free and confidential expert regulatory information, advice and guidance to organizations of all backgrounds and sizes based nationally or internationally (academics, not for profit organizations, patient groups involved in research and industry).

Germany Paul-Erlich Institut (PEI) Innovation Office: The PEI Innovation Office [72] was established in 2009 with focus on ATMPs to support their developmental process from drug discovery to marketing authorization in Germany as early as possible.

BIOGRAPHY

STUART G BEATTIE has over 25 years' experience in the field of gene and cell therapy. Following a post-doc and fellowship with Dr Terry Flotte at the University of Florida and UMass Medical School, as the field of gene therapy has developed, Stuart has taken positions within industry, moving from preclinical research to clinical development of AAV-based programs for metabolic, ophthalmological, and neurodegenerative disorders. Stuart is a Regulatory CMC Gene Therapy Clinical Lead for early phase programs at Biogen. Stuart is active across cell and gene therapy industry trade associations with regard to global regulatory intelligence and policy.

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FASTFACTS

Technology transfer process excellence with an AAV comparability study

Isabel Guerrero Montero, MSAT Scientist, Viralgen Vector Core

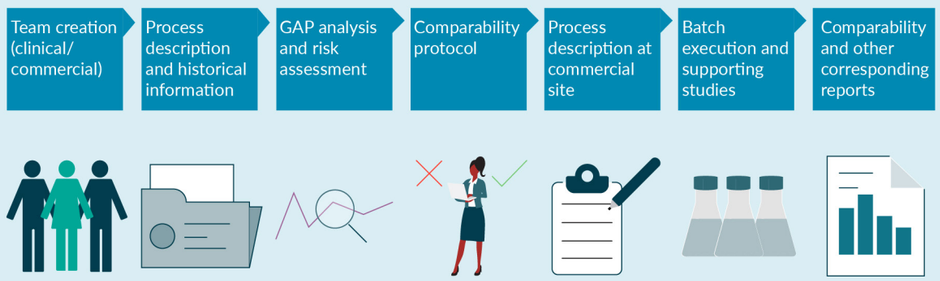
Successful technology transfer of gene therapy manufacturing processes is critical to ensuring that a CDMO has a complete understanding of the implementation of the process. This FastFacts poster provides details of a proven process for technology transfer and shows an example of a recent comparability study from the transfer of a customer's program from a clinical to a commercial facility.

TECHNOLOGY TRANSFER PROCESS

Growing numbers of gene therapy companies are experiencing preclinical and clinical successes and are turning to contract service providers like contract development manufacturing organizations (CDMOs) to support them with development and large-scale manufacturing. Larger indications may need multiple suppliers to reach the scale needed for treatable populations. The technology transfer of gene therapy processes across laboratories and companies is a complex undertaking that requires subject matter expertise, alongside tried-and-true methodologies that limit risk to the therapeutic developer, their manufactured product, and ultimately the patients.

The technology transfer process requires information flow between three key players: client, clinical, and commercial. At ViralGen, the client can approach the clinical facility for clinical manufacturing, or the commercial facility for clinical and commercial manufacturing. If the clinical unit is approached first, a transition to the commercial unit is possible. An overview of the transition phases is outlined in **Figure 1**.

Figure 1. Transition phases.



GAP ANALYSIS & RISK ASSESSMENT

To bridge any differences between clinical and commercial units, gap analysis and risk assessments are performed comparing facility design, equipment, auxiliary services, documentation, raw material, personnel training, etc. A sample gap analysis and risk assessment is shown in **Table 1**.

Table 1. Example gap analysis and risk assessment.

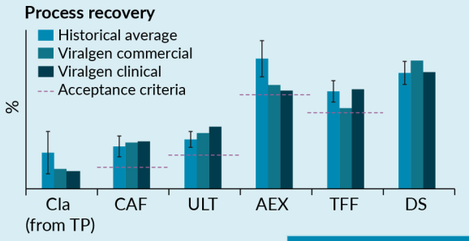
Parameter	Sending unit		Receiving unit		Cause	Impact of failure	Severity	Occurence	Detectability	RPN score	Mitigation comments
	50 L scale		500 L scale								
	value	unit	value	unit							
Temperature	A	°C	A	°C	N/A	N/A					
Volume	A	mL	B	mL	Difference in measurement equipment	Incorrect final volume added during process can affect yield	1	1	5	5	Additional step added in batch record to ensure operators measure correctly

Gap analysis is based on process description, an in-depth comparison of production process parameters, and an equipment and sampling plan for each process step. This compares the sending unit and the receiving unit at different scales. A formal risk assessment and corresponding risk mitigation strategy to control for risks identified based on the information obtained in the gap analysis will be developed. This risk assessment involves assessing the probability of occurrence for each identified risk and classifying risk severity and associated impact.

COMPARABILITY CASE STUDY

Figure 2 shows the results from an example comparability study. This comparison focused on two main objectives: process performance and drug substance critical quality attributes. The acceptance criteria for the comparability assessment are based on historical ranges, in this case from a range of batches at 50, 250, and 500 L. The clinical and commercial batches were compared at the same scale. The process recovery and removal purity met all acceptance criteria established using the historical average.

Figure 2. Comparability study results.



Comparability case study

- Historical average: range of batches at 50, 250 and 500 L
- Clinical batch: 500 L
- Commerical batch: 500 L

Batch	HCP clearance by step (Log10 reduction)					
	Acceptable criteria	CAF	Acceptable criteria	ULT	Acceptable criteria	AEX
Historical average		3.98		1.56		1.12
Viralgen clinical	≥3.83	3.85	≥1.48	1.51	≥1.00	1.15
Viralgen commercial		4.20		1.55		1.02

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SPOTLIGHT

INTERVIEW

Navigating the complexities of biodistribution, transgene expression & vector shedding in gene therapy development

In this episode **Róisín McGuigan,** Editor, BioInsights, speaks to **Paul Byrne,** Senior Director, Genomics, ProtaGene, about the evolution of the gene therapy field, with a specific focus on the complexities posed by biodistribution, transgene expression & vector shedding in gene therapy development.

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Q You've been working in gene therapy for a long time now. What do you see as the key learnings of the industry related to bioanalysis of *in vivo* therapeutic approaches?

PB: Around 25 years ago when I first started supporting these studies, we were receiving cardboard boxes with room-temperature tissues wrapped in kitchen foil and leaking over each other, which obviously wasn't ideal! There have been

a lot of advancements since then. One really key learning is from the regulator's perspective. We now see them being a bit stricter in terms of chemistry, manufacturing and control (CMC) and clinical considerations, but looking at preclinical development, they've become much more pragmatic and practical and very open to science-driven justifications for how these in-life or analytical packages are designed.

A good example is for preclinical biodistributions, which are a key part of investigational new drug (IND)-enabling preclinical studies. Historically, we would test about 40 tissues per animal. Now, while it does depend on the route of administration and the mechanism of action and the tropism, we're generally testing about 15 tissues on average. Obviously, this differs depending on the molecule we're working with, but it's a much more pragmatic approach to how we are supporting this work. The tissue lists are different depending on how the molecule is being dosed—here we're really thinking about adeno-associated viruses (AAVs). An ocular gene therapy would be very different from something that was dosed systemically. But historically, we would just do the same tissues. Now you essentially select the tissues that are relevant for how you are dosing material and what the tropism of your vector is.

Another example is in terms of the analysis for transgene expression. This is something we didn't really do when gene therapies were starting to come onto the market, but then we started doing it for everything no matter what. Now we do a qPCR to look for the vector, and where we see the presence of that vector, we would then look for the transgene. Again, this is very different and much more science-driven.

We are also seeing that potentially there's no need to do these IND-enabling studies if a developer has already done it with a similar molecule. So this is if you've got an AAV capsid with the same capsid and the same vector backbone, and all you are changing is that therapeutic gene. If you've got data for the previous version of that molecule to show that it was successful and moved into the clinic, then you've got good justifications for skipping most of that preclinical development and going straight into clinical development. Again, it's still very much on a case-by-case basis. I'm working with a few companies now who are waiting to hear from the regulators on this. It highlights how practical the regulators have become, which I think is a good thing. Although, there are also places where they've become a bit stricter around what we're doing in development.

One of the other things that's changed is the validation requirements, and this is very much focused on the molecular biology. Back in the day, we were just starting to understand how to validate these assays and what to consider—which parameters, to what level we should do the qPCR, and whether we should also do the extraction. This is quite stringent now, especially for molecules that are in later stage clinical development, and you have to make sure you've got a very precise and accurate assay to give you the confidence you need in the data that's being generated.

Finally, there is the equipment, which has been one of the big advancements. From a science perspective, the principles of what we do are pretty much the same, and this hasn't changed in all those decades. The equipment we have now just makes it a lot easier to analyze samples as quickly as possible, there's better connectivity, and it allows us to apply more high-throughput

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work streams. A very good example is the automated extraction platforms we have now—we can extract nucleic acids from up to 96 samples in a single run.

Q What would you identify as the key commonalities—and the important differences—that exist in approaching bioanalysis of *ex vivo* gene-modified cell therapies?

PB: When we compare gene therapies such as AAV to things like CAR-T there are probably more differences than similarities. The initial difference to note is the scope of the preclinical work. If you look at the regulatory approval documents for CAR-T molecules like Kymriah and Yescarta, you'll see that not a huge amount of work is actually performed during that preclinical phase. They might do some *in vitro*, *in vivo*, or off-target tumor activity, and then usually just a biodistribution.

As a rule of thumb, when we're doing preclinical development for cell therapies, the main analytical tool would be flow cytometry. There are some exceptions to that where we have used molecular biology tools, and then the way that we would support it would be quite similar, albeit a little bit more complicated because we're usually looking for multiple targets for cell therapies.

Looking at the clinical aspect, the bioanalysis focus is again a bit different when considering biodistribution, shedding, and transgene expression. There are many other bioanalytical endpoints to these very complicated clinical trials, but we're focusing on a small part of that. For AAV gene therapy the focus is on shedding primarily, but we can also use the same workflows, expertise, and equipment to also look for things like replication-competent viruses and maybe any other adventitious agents as well. We can do the same thing for chimeric antigen receptor (CAR)-Ts.

For CAR-T, it's more about monitoring that CAR-T, so looking at concentration and persistence over time. The main analytical tool here is qPCR, and the workflows and approaches are quite similar between the two, but the focus is different. For AAV there is more of a safety endpoint, whereas for the CAR-T, it's all about monitoring that product. The take home

message is that it is very important to understand the molecule that you're working with, because the analytical requirements will all be quite different.

Q How is the field of gene therapy evolving, and what new therapies and delivery methods do you see on the horizon?

PB: It seems that every day there's a new or improved way to modify and deliver gene therapies, or a new generation of these adoptive cell therapies. Starting with gene therapy and AAV, I think there are almost two factions that are split geographically. In Europe, the sentiment is very much that AAV is still the future for gene therapy. Whereas in America, there seems to be a moving away, with people thinking that maybe AAV has had its day. There are issues with immunogenicity with AAV-type molecules; potential side effects from people getting very high concentrations of modified viruses. So, there is a push to move away from that and look for other delivery mechanisms—things like plasmids and lipid nanoparticles, and more non-viral delivery. I think perhaps the future lies somewhere in the middle. We'll continue to see classical AAV-based therapies being developed and coming onto the market, and we will also see these non-viral delivery mechanisms coming onto the market at some point in the future.

The next couple of molecules are not generally new technologies, but we are seeing a lot more of these mainly in preclinical, and some in clinical development as well. The first is gene editing, and we are seeing a huge amount of interest in that from a preclinical perspective. Again, that's something we're going to be seeing and hearing a lot of in the next couple of years. For the other types of molecules, it's debatable whether these are actually classed as cell and gene therapy. But for things like oligos, silencing and micro RNAs, and locked nucleic acids, if we do class them as cell and gene therapy then they make up about 25% of the molecules that are in mainly preclinical development.

Again, I think we're going to see these small molecules playing a big part in preclinical and clinical, and then potentially coming onto the market in the future. To make things even more complicated, we're also seeing a combination of therapies—not only AAVs that are being modified to deliver these therapeutic genes, but also delivering gene editing tools as well. That can create a bit of a bioanalytical headache because we're looking at different tests. We need molecular tools for looking at the AAV and the transgene expression, whereas for gene editing that's all about next generation sequencing (NGS), so that can make the analytical work quite complicated. However, it's very exciting to see people combining multiple therapies to try and make even better gene therapies.

Additionally, we're constantly seeing novel approaches to how adoptive cell therapies like CAR-Ts are being developed. We're now—rather terrifyingly!—seeing artificial intelligence and big data being used to modify those molecules at a genetic level to improve the safety and the efficacy. That gives us some headaches from an analytical perspective, but it's really interesting to see where that's going.

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"In the case of AAV we also need to understand the very high levels of gene expression not only in target but also non-target tissues. That data then needs to be correlated back to any toxicology findings before we can move into clinical development."

When the patient's own T cells have been modified, usually that's performed using an integrating virus, which has some safety concerns. I'm hearing that electroporation is getting some traction, so that's something that may be on the horizon. Ultimately, under what I call the cell and gene therapy umbrella, there are a lot of very complex and diverse molecules. As those molecules evolve, the analytical methods need to evolve with them. It's a very challenging space—but at the same time extremely exciting.

Q Why are biodistribution, vector shedding, and transgene expression such critical factors for ensuring the safety of cell and gene therapies?

PB: For the preclinical phase of development, it's very important to understand the distribution and persistence of these molecules. In the case of AAV we also need to understand the very high levels of gene expression not only in target but also non-target tissues. That data then needs to be correlated back to any toxicology findings before we can move into clinical development.

Shedding can play a big part during preclinical development, but some companies are not doing it. For the last two or three AAVs to come onto the market, there wasn't any shedding assessment during the preclinical phase even though it's a regulatory requirement. They instead did it in the clinical phase—this is another good example of how the regulators are being a bit more pragmatic and open to those science-based justifications for taking a different approach.

Within clinical development there are multiple other analytical endpoints we won't touch on today, but shedding is a very important analytical safety endpoint. It's crucial to understand what you are seeing, where, and for how long, as you move through clinical development and ultimately onto the market.

Q Can you outline the key challenges associated with assessing and monitoring biodistribution and vector shedding?

PB: The first will be no surprise to anyone who has worked in this industry and been exposed to the molecular biology tests, and it's the lack of regulatory guidance. Currently there is no guidance for how these assays should be developed or validated. A few years back people were trying to get us to follow the guideline on Bioanalytical Method Validation (BMV), but that's written specifically for things like ligand binding and chromatography-type assays. It's really not applicable to qPCR. I'm quite glad that we moved away from that conversation, but it still leaves us with nothing—so what do we do instead? There's a lot of information out there. We have a number of academic publications, and the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) is a very good example. It is very research-focused and provides guidance for research scientists to make sure that the data they're generating is of the highest quality, but there's also a lot to take from that outside of academic institutions.

We've got many white papers, position papers, webinars, and podcasts in Europe and America. We've got consortiums of CRO all generating opinion papers. There are lot of common themes but also some slight differences on how the work should be approached. I'm not seeing any assays that are being developed or validated badly. It all depends on the context of use and making sure it's fit for purpose. However, I am concerned that as the regulators see more molecules being developed, and if they start seeing more of these IND-enabling studies, there may be a need for more consistency on how these assays are validated and the data presented.

It seems like every other week we're seeing cell and gene therapies going on clinical hold. They're all usually focusing on the CMC and usually related to the analytical tests. Potentially, moving forward, they might start looking at the preclinical and clinical. My concern is that that lack of consistency in how people are validating these methods might become an issue.

The other issue is timelines and potential lead times, as there is high demand for this type of work. We're seeing long lead times across the industry for supporting the analytical requirements for the preclinical and clinical development of these products. Companies aren't really considering the time needed to develop and validate these assays. They are coming to us with samples for testing, and they want results next month. Obviously, we have to say no. It takes two to four months to develop and validate these methods. It's very important that that's factored into lead times. You don't want to approach a vendor, go to the back of the queue, and potentially wait many months to have a validated assay.

The next challenge is what I see as a bit of a battle at the moment between the two main molecular biology tools. The first is quantitative PCR (qPCR): this is a very well-established, robust, and sensitive molecular biology tool that has been used to support the development of cell and gene therapy for decades now. Then we have the new kid on the

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"If you start with CMC, then dPCR is a perfect fit. If you are manufacturing batches or changing your manufacturing process, you want as much confidence as you can get before dosing this material—you want an accurate, precise assay."

block: digital PCR (dPCR). It has been about for roughly a decade now. In the last couple of years there has been an increase in demand to use this during the development of cell and gene therapy.

Q What new approaches are being developed to address these challenges? And what impact is the introduction of digital PCR having?

PB: I think the introduction of dPCR has muddied the water a little bit. We are seeing it being used more and more during the preclinical stage, supporting the manufacture of these products and also in the clinical development stage. It is being included in a lot of literature, white papers and webinars. Most of that is focusing on the development and the validation of that method. What I'm not seeing too much of is where it should be used, and more importantly, where it should not be used.

If you start with CMC, then dPCR is a perfect fit. If you are manufacturing batches or changing your manufacturing process, you want as much confidence as you can get before dosing this material—you want an accurate, precise assay. That's exactly what dPCR brings to the party: much higher precision and accuracy. It's an extremely good fit for those CMC-type applications.

During clinical development it makes some sense to use dPCR. It's potentially better at overcoming potential inhibitors. Although if you've got a good extraction method, then that will get rid of any inhibitors that will be present in those samples. It may also increase the chance of detecting rare events due to the multiple reactions that are set up. But what we've seen when detecting signals close to or below the limit of quantitation but above the limit of detection is really high levels of variability, making it quite difficult to validate. You really need to consider the pros and cons of these methods and what you're trying to achieve before selecting the most appropriate test. If you're looking to get very precise readings of something you're going to get a good concentration of, then it may be a good fit. If you're trying to quantify material that's down near the limits of your assay, then it's potentially not a good fit.

Preclinical is the phase of development where to me it makes the least amount of sense to use dPCR. It has very small dynamic range compared to qPCR, which in contrast has quite a large dynamic range. If you think about an AAV preclinical biodistribution for something that's dosed systemically, you're going to get very high concentrations of your vector in target and non-target organs. You're going to get variability within different animals and different groups. It's very difficult to dilute that material consistently down to the sweet spot of that small dynamic range, and that's going to result in multiple repeats.

When I'm talking to potential sponsors and clients, the take-home message for me is that for many applications, for molecules that are well-designed and validated, qPCR should be sufficient. There are obviously exceptions to that rule. If you're trying to multiplex an assay, then dPCR may be a good option. If you don't have positive controls and you don't have PCR bias, it's going to be potentially easier to develop and validate. For most other molecules and applications, qPCR will give you all the results that you need.

For preclinical studies you could be looking at thousands of samples. For clinical, it depends on how the molecule would be delivered and maybe what kind shedding you're seeing. Some dPCR platforms are very expensive to run compared to qPCR. The reagents are expensive, the plasticware is more expensive, and it takes a lot longer to run, which is factored into the cost as well. That additional cost and duration can be prohibitive for running these types of studies using dPCR. When we factor in that small dynamic range and the fact that you're going to see more repeats, again, that's going to increase your cost and the duration of the study, and potentially make it prohibitive.

However, to go against everything I've just said, we are continuing to see an increase in demand to use dPCR, in both preclinical and clinical, including with clients who we're engaging with. Some are early adopters of the technology and understand dPCR very well. They understand the pros and the cons, and more importantly, they know where their product is going and what concentration it's going to be at. They can therefore put in place the necessary dilution scheme to get it into that sweet spot of the dynamic range and make it as efficient as possible. On the other hand, there are clients who just see it as the new shiny piece of equipment in the lab and they want to use it no matter what, even if it's going to take longer, cost more, and not give them any more data than a qPCR.

It's quite an interesting, and sometimes frustrating, space to be in at the moment. We have lots of conversations about dPCR. Ultimately, if you are developing a molecule and you're trying to decide what the best molecular tool would be, I would advise people to do some research and perhaps try and engage with a subject matter expert. It's important to try and understand what the best tool for you would be to provide the data to move your product through the different phases of either preclinical or clinical development.

Q How can the insights and best practices we've discussed today be applied to specific R&D efforts? What would your key advice be for readers working in this space?

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PB: We touched on the complexity and diversity of molecules that are currently captured under the cell and gene therapy umbrella. It's important to understand the molecule that you're working with and choose the best analytical tool. To summarize, for anything cellular-based flow spectrometry is a very good fit. For anything gene therapy, then you're looking at the molecular biology tools, but care should be taken around what one you use.

For these small molecules, oligos, silencing RNAs, and locked nucleic acids, mass spectrometry is very good. Finally, for anything where you're modifying the genome, NGS can be a good tool. And if you are combining multiple therapies, then you're looking at maybe two, sometimes three analytical endpoints, and they are not easy to perform. This is something to be aware of.

We also touched on the lack of regulatory guidance, and this is more specific to the molecular biology tools. If you are developing a method, then just ensure that you are developing it and validating it as best you can. Take into consideration what phase of development you're currently at, but think about future-proofing. There is a minimal amount of additional work that you could do within the preclinical phase in order to future-proof these assays so they can be used in CMC—albeit the validations will be a little bit different—and also in clinical development. For example, a qPCR to look for the biodistribution of an AAV preclinically can also be used to look for shedding in the clinical environment, just by including appropriate matrices into your validation.

In the absence of any kind of regulatory guidance, we need to utilize the resources we have, and there's a lot of them. Don't be scared to make decisions that are based on the context of use and your own expertise. You will know the molecule you're working with better than anyone and you might know the technology better than anyone, so don't be afraid to make some decisions that are a little bit different to what you've seen out there. As long as you're taking a very science-driven approach to it, then I don't think you can go wrong.

Finally, these products are expensive to develop and bring to the market, and we really want to minimize any delays. Planning is crucial—make sure you've got all your assays ready to go, so you can support whatever phase of development you're at.

BIOGRAPHY

PAUL BYRNE is senior director of genomics at protagene. He has 25 years of industry experience and can frequently be found speaking at symposia on topics such as: analytical development challenges for ATMPs, biodistribution and safety assessment considerations for cell and gene therapies and more. Paul received his BSc (Hons) in biology from the University of Stirling (UK) and his MSc in research from the University of Glasgow (UK).

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We hope you enjoyed reading this interview.
You can also listen to the recorded podcast here:

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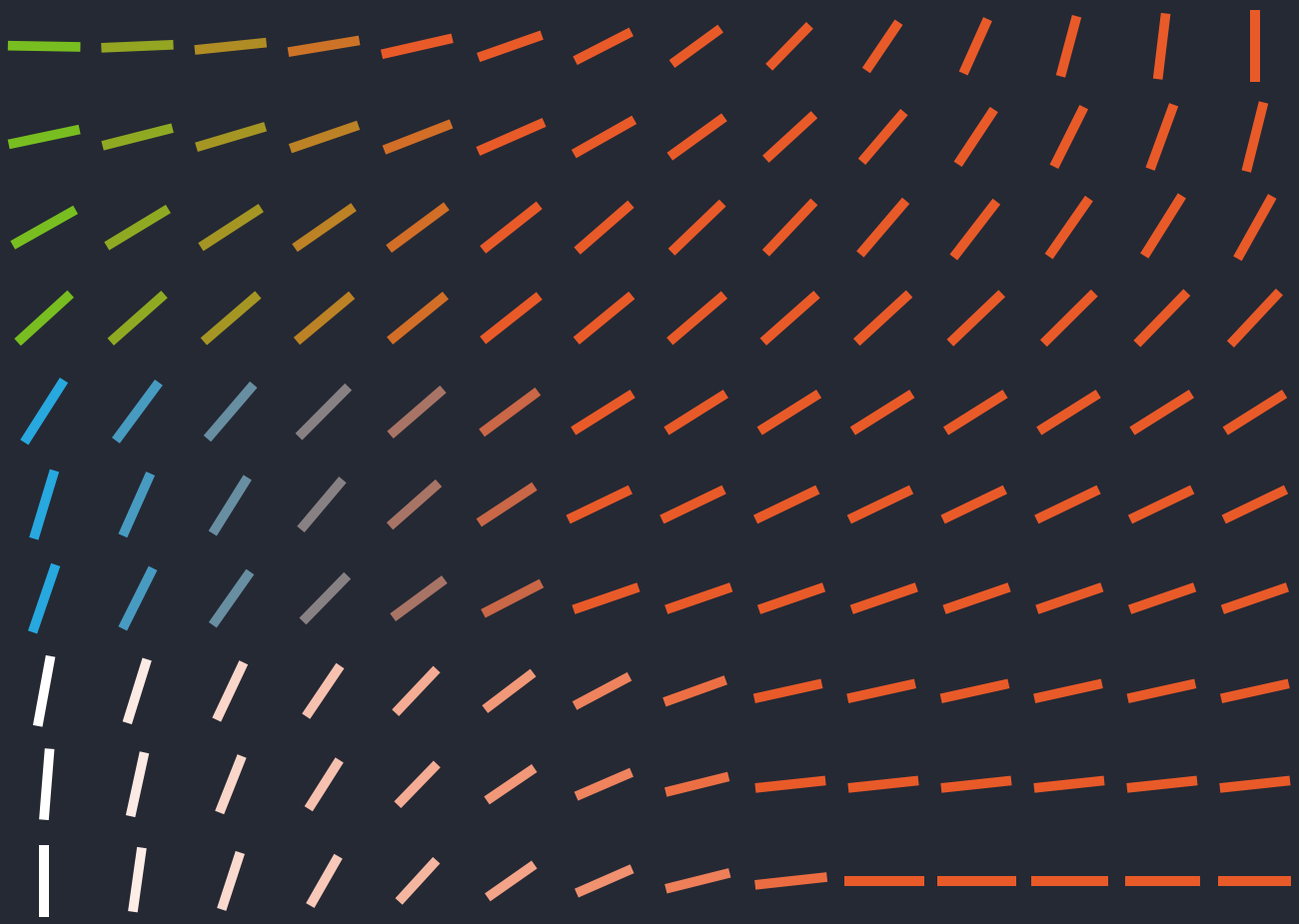

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SPOTLIGHT

INTERVIEW

Assessing the AAV analytic toolkit: considerations for application & regulatory compliance

David McCall, Senior Editor, BioInsights, speaks to (pictured) **Juliette Reviron,** CMC Project Manager, Lysogene

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Q How would you describe the current field of AAV vector characterization and CMC – what are the key challenges and questions the field faces at the moment, in your view?

JR: Alongside the gene therapy field in general, adeno-associated virus (AAV)-based gene therapy has seen tremendous recent progress, with three AAV-based gene therapy drugs already approved by the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA). AAV had become a vector of choice

due to its' numerous advantages. Furthermore, AAV-based therapy is no longer limited to the treatment of rare disease – the field has extended to more widespread diseases for which a greater number of patients are eligible, which requires an increase in the number of doses manufactured and an improvement in production yield. Over the past few years, AAV process have been extensively optimized to help manufacturers achieve higher doses, notably through the shift to suspension-based processes. Moreover, a broad panel of analytical methods have been developed to thoroughly characterize AAV vectors, ensuring their quality and safety.

However, despite these extensive industry efforts, there are still major challenges to be addressed. AAV-based gene therapy remains a heterogeneous product that is complex to characterize.

From a chemistry manufacturing control (CMC) perspective, switching to suspension is not a 'bed of roses': it requires lots of changes that need to be anticipated upfront if one is to establish a robust CMC strategy whilst also attempting to lower the cost impact of this change for the company. If a suspension-based process is not developed and utilized from the beginning, the process will require extensive redevelopment, and a comparability study must be performed, which could be challenging. It is well known in the industry that switching to suspension will involve a change in the full/empty capsid ratio (tending to be lower), because of the charge changes on the surface of the capsid – a considerable issue given that the regulatory agencies are becoming more and more demanding when it comes to increasing the percentage of full particles. This is due both to empty AAV capsids carrying the potential to exacerbate adaptive immune response directed to the viral capsid antigen, and the fact that a large excess of empty capsids may reduce the transduction of target cells by competing for vector binding sites. This leads in turn to increased vector doses and consequently, a higher risk of toxicity, underlining the importance of capsid optimization early in development to maximize the transduction efficiency of the vector.

Maximizing the percentage of full particles whilst ensuring a good process yield represents a second major challenge. Depending on the AAV serotype used, this will require conscientious, sometimes tedious development of both the upstream (notably, improvement of the transfection steps) and downstream (with the implementation of a polishing step) processes. Furthermore, even if a broad range of empty/full characterization tools are available to monitor this attribute, there are limited techniques available that are capable of quantifying capsid content, and even fewer that are amenable to validation and implementation as registered release assays in a regulated environment.

It should be noted that the more empty particles you have, the more likely they will contain undesirable encapsidated DNA. Those undesirable DNA contaminants in rAAV preparations remain a major safety concern, and the characterization of their localization, size, and quantity could be a long journey that will require to a methodological study combining various different analytical tools.

Q Let's explore some specific areas more in-depth – firstly, understanding packaged DNA impurities: how significant are they,

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what does the regulatory guidance tell us, and what is the best technique for looking at them?

JR: Regarding host cell DNA, when using cells that are tumor-derived (e.g. HeLa) or that have tumorigenic phenotypes (e.g. HEK293), in order to limit the oncogenic and infectious risk, the FDA recommendations since October 2020 are that the level of residual cell-substrate DNA should be below 10 ng per dose and a median DNA size of 200 bp or lower.

Since AAV can package large amounts of plasmid DNA (used during transfection) as well as cellular DNA inside the viral capsid, the manufacturing process should be optimized to reduce that kind of contamination. You should consider implementing some steps to reduce the size of the DNA fragment to below the size of a functional gene, and decrease the quantity of residual DNA by performing an endonuclease treatment. The best practice will be to improve the downstream purification steps, characterizing which ones are the most efficient to polish the residual DNA. The implication of this is that DNA impurity will need to be monitored all along the process, from lysis to the final drug substance, using robust analytical tools.

However, it is difficult to define if encapsidated DNA is usually more significant than free-floating residual DNA. This will depend on many factors – in particular, the starting materials (mainly the cell bank and plasmids), the upstream production process utilized, and the downstream purification steps. I don't think I'm incorrect in saying that every AAV vector manufacturer has struggled with the presence of unwanted DNA, whether it is encapsidated or not, since most of the time the DNA is co-purified with the vector.

The topic of encapsidated DNA is not an easy one to address – as the DNA is packaged, it might be difficult to identify or remove it. In that case, the best practice will be a combination of different approaches, including a risk-based approach.

Firstly, it will be necessary to quantify and determine the size of the DNA that is encapsidated. For this purpose, I would advise treating your samples using an endonuclease in order to remove the free-floating DNA, and to compare the quantification results with and without this treatment. There is a broad range of analytical tools that will help you to characterize the DNA content of the capsid in terms of size and quantity. A technique that is commonly used to determine the size of the DNA fragments is capillary electrophoresis (CE). With regard to quantification, the gold standard usually recommended is next-generation sequencing (NGS). While you could use qPCR, this method suffers from a number of limitation – notably, the need to determine and develop the target of each contaminating sequence, variability (both between different labs and between the target regions), and the limited coverage of DNA contaminants it offers, particularly for the genomic DNA of packaging cells. Beyond its application in quantification, NGS is very convenient. If your study is designed correctly, NGS will provide much data on the physical form (single strand or double strand), origin (residual host cell DNA or plasmidic DNA), and with the help of metagenomics, it will be possible to identify the origin of numerous unwanted DNA sequences.

Secondly, depending on the cell type you are using, the regulation recommends controlling the level of relevant infectious or oncogenic sequences that are cell type dependent. For instance, if using HEK293, you will need to quantify the E1A and E1B genes by either developing a specific assay able to quantify them utilizing qPCR or ddPCR, or by using a commercially-available kit.

Last but not least, to mitigate the risk of having hazardous DNA sequences, it is key to pay attention to the way your cell lines are produced and the QC control strategy you have in place. This can be handled using a risk assessment approach.

Q Can you expand on the relative pros and cons of the various tools available for empty/full/partially full capsid ratio measurement and separation? Which solution, or combination of solutions, might be the most optimal and feasible for release testing purposes?

JR: Many innovative analytical tools have come to light over the past few years to measure the empty/full ratio of AAV-based vector product, but currently there is no consensus on an accepted gold standard technique, and understanding how the results compare between different orthogonal technologies can be tricky. However, this issue is well-known by the US FDA and they have formed a working group with the industry in order to come up with a recommendation.

A wide panel of methods are available in the market. It can be difficult to choose the most appropriate between:

- Cryo-electron microscopy (CryoEM;
- Transmission electron microscopy (TEM);
- Analytical ultracentrifugation (AUC);
- Charge detection mass spectrometry (CDMS);
- UV absorbance and Size-Exclusion Chromatography (SEC) ($A_{260}/A_{280,}$ Multi-Angle Light Scattering (MALS);
- Even titer ratio

Among these methods, only AUC, CryoEM, and CDMS can provide a relative quantification of the capsid content; SEC-UV and titer ratio provide an indirect measurement of the capsid content, but they are easy to implement and require a lower quantity of vector material.

For me, the most appropriate approach will be to utilize a combination of different methods. During development, titer ratio could be used in order to have a quick estimate of the capsid content. Then, the results may be confirmed using two orthogonal methods: CryoEM and AUC. If you had to choose one, I would recommend using AUC – notably because

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"We are seeing more and more analytics developers focusing on analytical tool automation. If they succeed in combining all the expected quality attributes of an analytical method with high-throughput screening, it will truly become a game-changer in reducing both cost and time."

from my own experience, this method seems to be more readily accepted by the regulatory agencies for use in release testing. (Quite rightly, considering it is the only method able to accurately measure the absolute value of empty/full ratio as well as intermediates). The only drawback of AUC is its implementation in a good manufacturing practice (GMP) environment; indeed, the equipment software is not covered by an audit trail system, meaning that the software cannot be validated. However, there is some mitigation in place that seems to be appropriate in the eyes of the regulatory agencies.

Regarding the separation technique: depending on the serotype, cell type, and process you are using, separation of the full from empty capsids could require more or less process development. The separation techniques at the downstream process level are quite limited, and the enrichment ratio rarely goes beyond two-fold. It is important to take this into consideration, and ensure as high a percentage of full capsids as possible are entering downstream processing by working to optimize the upstream process in this regard.

In downstream processing, anion-exchange chromatography (AEC) has been widely explored due to its scalability and high throughput, but often, it is necessary to make compromises between having a good yield and an acceptable percentage of full particles. I am also aware that some companies are developing membrane-based separation tools currently.

Q Which particular analytical innovations stand out for you as being capable of making a real difference to the quality, cost, and manufacturing timeframes for AAV-based gene therapy products?

JR: AAV products are significatively more complex to characterize than other types of biologic product, such as monoclonal antibodies. From an analytical point of view, several methods have seen the light of day in recent years in opposition to the widely-used traditional methods such as Elisa, SDS-PAGE, qPCR, Western blot, and TEM. These novel methods aim to provide a deeper understanding of the critical quality attributes (CQAs) of AAV-based products while increasing precision, specificity, sensitivity, and reproducibility of results. We are seeing more and more analytics developers focusing on analytical tool

automation. If they succeed in combining all the expected quality attributes of an analytical method with high-throughput screening, it will truly become a game-changer in reducing both cost and time.

Chief among the methods that could be automated is ddPCR for viral genome or DNA impurity quantification and mycoplasma detection. It is important to note that switching to ddPCR could engender a significant additional cost, but this said, the high quality of the data and having a tight coefficient of variation would most often drive my decision in this regard. ddPCR is becoming the new gold standard particularly for viral titer, which determines the patient dose and is therefore of paramount importance.

Liquid Chromatography Mass Spectrometry (LC-MS) for identification determination, as an alternative to Western blot, is one of the innovations that has affected the AAV analytical characterization field the most. This is especially because the FDA now recommends that viral capsids and encapsulated DNA of all AAV therapeutics should be unambiguously identified before release. The recent optimization of the LC-MS method has resulted in the ability to design efficient liquid chromatography separation. It offers the ideal combination of speed and specificity for viral capsid protein analysis because direct measurement of protein masses avoids the need to generate antibodies for each type of AAV. Along the same lines, SDS-PAGE coupled to CE seems to offer high resolution data and excellent quantitation and reproducibility, combined with automated operation which reduces both the time of analysis and error rate.

Finally, the emergence of artificial intelligence for AAV manufacturing development is of great help in capsid optimization and promoters and transgene selection.

Q What is your view of the feasibility of, and considerations for, AAV-based gene therapy platform processes and the related CMC requirements?

JR: The last few years have seen many plug-and-play approaches being proposed by CDMOs for the manufacturing of AAV. It is a hot topic because while this approach could accelerate patient access to novel therapies by reducing the development timeline and cost to some extent, it becomes a risky choice when moving towards commercialization. The CMC package should be rigorously designed in order to ensure a robust, scalable, and de-risked manufacturing process. Both the process and the product should be well characterized with a reliable analytical approach using pre-qualified methods. The starting materials such as plasmid and cell bank should demonstrate a good reproducibility among different serotypes and transgenes – they also need to be well characterized and understood to meet the regulatory standard for commercialization.

The production model choice (packaging cell line cell bank vs. transfection) requires a holistic approach weighing up the pros and cons of each model. Considering the high

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batch-to-batch variability encountered with adherent-based processes, the suspension model would seem to be the more appropriate to me.

In addition, in the context of process validation, the analysis of critical process parameters (CPPs) and CQA for a plug-and-play platform will need be thought through from the beginning in order to ensure all quality and regulatory standards for commercialization are met.

Raw and starting material supply should be carefully monitored and include testing of different equivalent raw materials/equipment during development to deliver a contingency plan, thus avoiding any potential delays in the development timeline.

Regarding regulatory support to the sponsor, a good balance between IP-sensitivity and transparency with regards to the CMC product specificity information needs to be found to ease the investigational new drug (IND) filling to the FDA. A Drug Master File may be used on occasion – however, it should be noted that for EMA filling, such a model would not be accepted – in this case, the manufacturer/contract development and manufacturing organization (CDMO) would need to provide the appropriate level of information.

The name 'plug-and-play platform' is little bold, for me, because there is not yet a truly ready-for-use AAV process available. The ideal model will be a customizable approach in which there will be space for further development and optimization beforehand, in order to adapt to different sponsors' projects. Finally, the manufacturer/CDMO should demonstrate some flexibility with regards to the control strategy in order for the sponsor to oversee the product quality – without this, a high level of risk is assumed when entering the clinic or interacting with regulators.

Regulatory guidance and standardization are required on the analytics side to drive the vision of an AAV gene therapy platform approach. This guidance should address the CQAs that must be monitored to ensure the safety and efficacy of AAV vector products. Standardization of analytical methods and acceptance criteria would also facilitate comparability assessments between different AAV vector products, allowing for easier product development and expedited regulatory approval.

Q Moving forward, what will be some key steps to tackle the ongoing problem of high batch-to-batch variability in gene therapy products?

JR: With regards to gene therapy investigational medicinal products (IMP), tackling this issue will begin with the definition and understanding of the quality attributes that are critical to product quality and patient safety. Then, a quality by design (QbD) approach can be employed to further define the quality target product profile. That being said, I recommend taking the time to appropriately develop the starting materials, and demonstrate their ability to produce the appropriate yield in a reproducible manner.

Regarding the cell bank production, whether it is the master or working cell bank, the post-production quality control must be exhaustive in order to target all the elements that could hinder the reproducibility of the bank during production. Thorough testing must be

"...in order to avoid any unpleasant surprises during the transition to GMP vector production, it will be necessary to ensure the reproducibility of the data obtained during development in a GMP environment."

established to control the quality of the cell culture parameters post-freezing. On top of that, the cell banks need to be tested to ensure that all the sterility aspects are respected. At the same time, all of the raw materials used in the production process – and in particular, the media – must be sourced in such a way as to cover the production of GMP batches, thus avoiding the use of media the equivalence of which has not been demonstrated with your production process.

The choice of production model is essential. We have seen an important drift within industry towards the suspension production system, and this has not happened for no reason. The production constraints of the adherent model, such as the multiplicity of handling steps, the difficulty to handle vessels in the laboratory, and the batch-to-batch variability of the fetal bovine serum (FBS) required, combined to create a high batch-to-batch variability that is difficult to overcome. The controlled environment provided by suspension bioreactors has greatly helped to improve reproducibility from the upstream processing perspective.

Moreover, it is also important to characterize as much as possible, using reliable analytical methods, the importance of each process step in relation to final product purity as well as activity. This should happen during small-scale as well as large-scale development (when a minimum of two batches produced under the same conditions will be required).

From both upstream and downstream processing perspectives, it is important to have an idea of the critical process and raw material parameters that can influence the quality attributes of the product. With further experience, these parameters can then be defined and set within acceptable ranges to ensure the minimum possible amount of variability for commercial production.

Finally, in order to avoid any unpleasant surprises during the transition to GMP vector production, it will be necessary to ensure the reproducibility of the data obtained during development in a GMP environment.

BIOGRAPHY

JULIETTE REVIRON graduated with an engineering degree in biotechnology, cellular and molecular bioengineering for human health. She is now working as a CMC Project Manager at Lysogene, a clinical-stage AAV based company that has developed two innovative gene therapy-based platform approaches to treat, and cure, life threatening pediatric diseases with a

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central nervous system focus that currently have few or no treatment options. Thanks to her 4 years 'experience in development manufacturing, quality control and vector development she oversee the production of LYS-GM101, an rAAV serotype rh.10 carrying the gene expressing the β-galactosidase enzyme for the treatment of Landing disease (Ganglioside GM1).

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

LATEST ARTICLES:

Driving the expansion of mRNA into the therapeutic sphere

Alejandro Becerra, Thermo Fisher Scienitific, **Andreas Kuhn**, BioNTech, and **Metin Kurtoglu**, Cartesian Therapeutics

The advanced therapies industry is heavily engaged in capitalizing upon the success of mRNA-based COVID-19 vaccines. Novel therapeutic applications in major disease areas, including oncology, continue to show promise in preclinical and early clinical studies, yet challenges remain. *Cell & Gene Therapy Insights* brought together a panel of industry experts to discuss the expanding reach of mRNA technology, exploring how and where it will impact the advanced therapies space moving forward.

Here are some of the highlights…

Cell & Gene Therapy Insights 2023; 9(6), 783. DOI: 10.18609/cgti.2023.098

What are some of the major challenges that face the field as it migrates from infectious disease vaccines into therapeutic drug applications?

"Using mRNA to vaccinate against infectious diseases works really well. The mRNA itself is very immunogenic and the body will immediately react to it. However, when you go after a disease that needs a long-term therapeutic effect, it will be challenging to produce the right type of RNA in a formulation that results in sustained therapeutic activity."

—Mertin Kurtoglu

"From the development perspective, the purity of the mRNA is critical, and closely associated with purity are the analytical challenges. A purification process is only going to be as robust as the analytics that are available to develop it. It will be critical to establish better methods in order to characterize the product-related impurities."

—Alejandro Becarra

"With vaccines, only relatively small amounts of protein are needed in order to obtain a huge amplification by the immune system. On the other hand, using mRNA for the expression of functional protein requires several orders of magnitude higher expression of that protein. Therefore, looking into improved expression of the mRNA is key—for example, through improved sequence design."

—Andreas Kuhn

Looking at mRNA therapeutic manufacturing, what are the main limitations with the current processing tools and technologies?

"The design of the mRNA is the biggest challenge in mRNA manufacturing. How much mRNA is needed to make enough protein in order to achieve the therapeutic function? The answer is that the amount of mRNA required depends greatly on the design of the mRNA. If you can design an mRNA where you only need a microgram to give the desired therapeutic effect, then manufacturing is no longer going to be a challenge. The second challenge relates to the delivery system: whether you are using a LNP or a cell, the limitation and bottleneck right now is in scaling up."

—Mertin Kurtoglu

What will be the key technological and platform developments and innovations required to address mRNA downstream processing challenges?

"There are ongoing efforts to improve the purification toolkit for the mRNA field. More specifically, when we are looking at eliminating double-stranded RNA (dsRNA) from the final product, current efforts focus both on the *in vitro* transcription (IVT) reaction and the downstream process."

—Alejandro Becarra

"There is a lot of existing knowledge on purifying biological molecules that can be applied to mRNA, including on the analytics side. We will need improved analytical techniques to better understand what the molecule is that we have in hand."

—Andreas Kuhn

What are the key areas for improvement in the analytical toolkit?

"One of the challenges at this moment is the diversity of methods used to analyze the same parameter. One example is measuring RNA integrity, which indicates the amount of full-length RNA versus the amount of degradation products or truncated transcripts. Analysis of RNA integrity can be performed by using a large variety of techniques and you can question how the results of these different techniques correspond to each other. Harmonization and standardization of analytics are very important moving forward."

—Andreas Kuhn

Watch the webinar here

Read the full transcript here

In partnership with: ThermoFisher SCIENTIFIC

CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Let the cells thrive: the next generation of cell expansion

Hugo Fabre, Maura Barbisin & Maria Knaub

The cell therapy field has experienced tremendous growth in the number and variety of applications as well as disease targets under exploration for commercialization. At present, manufacturers face a wide range of decisions when it comes to implementing automated solutions in their processes. Committing to an unsuitable manufacturing technology early in development can result in expensive and unpredictable delays in late process transfers. Key steps in the cell therapy manufacturing process, such as cell expansion, can benefit from modular automation, allowing for easy integration into an established or growing manufacturing process. This article describes a cell expansion platform using hollow-fiber perfusion technology to provide an optimized cell culture environment for cell therapy manufacturing, from process development to clinical manufacturing.

Cell & Gene Therapy Insights 2023; 9(6), 675–686

DOI: 10.18609/cgti.2023.086

Engineered T cell therapies, such as chimeric antigen receptor (CAR)-T cell therapy, have surpassed expectations, driving an ever-expanding number of clinical trials for the treatment of cancer. The cell and gene therapy field has grown exponentially to include over 1,300 cell, gene, and tissue-engineering therapeutic developers worldwide. This substantial growth has led to an increase in technical innovations enabling robust clinical manufacturing in functionally closed and often automated systems. Cell therapy manufacturing workflows often encompass the integration of modular processes and analytical technologies to design, analyze, and control the manufacturing processes. Modular solutions within a cell therapy protocol can include cell washing, activation, gene modification, expansion, and cryopreservation technologies.

QUANTUM FLEX CELL EXPANSION SYSTEM

Quantum Flex is a compact and functionally closed platform designed for the expansion of adherent or suspension cells used in cell therapy. It allows for scalability from process development to commercial manufacturing and can support autologous as well as allogeneic applications for a variety of cell types. The system makes use of hollow-fiber technology

and comes with two sizes of bioreactors, small and standard, which allows for seamless upscaling. The cell processing application is a robust software application that facilitates good manufacturing practice (GMP) compliance and device management.

The core of the system is the hollow-fiber bioreactor (Figure 1). The hollow fibers have a diameter of approximately 200 µm and are made of semi-permeable membranes. The bioreactor's structure creates two environments: The inside of the fibers is known as the intracapillary (IC) side and the void between the fibers is known as the extracapillary (EC) side (Figure 1A). Both capillary sides make use of inlets and outlets to create independently controllable loops that enable fluid circulation (Figure 1B). The IC loop is part of the cell compartment, shown in orange in Figure 1B, whereas the loop shown in blue is part of the EC compartment and provides medium and gas exchange. Small molecules such as glucose and lactic acid can freely pass through the pores of the membranes. Cells and larger molecules, such as proteins and cytokines, are confined to the IC loop. The unique dual-loop system allows for much higher cell densities than achieved in manual cultures, thereby mimicking physiological conditions found, for example, in the human lymph nodes.

For cells cultured on the Quantum Flex platform, thriving means:

- Ready access to fresh media, waste removal, and gas exchange over µm distances
- A robust environment for both suspension and adherent cell culture
- Precisely controlled, low-shear expansion
- Reduced usage of valuable reagents

ADHERENT CELL EXPANSION

To demonstrate adherent cell expansion on the Quantum Flex system, mesenchymal stem cells (MSCs), derived from three different donors, were loaded onto the small and standard bioreactors, and expanded for 7 days. In total, 30×10^6 and 8×10^6 cells were loaded onto the standard and small bioreactors, respectively. Because lactate is one of the key waste metabolites of cell culture, it can be used as a surrogate measure of cell expansion. The lactate generation rates for

FIGURE 1

Schematic overviews of the interior of the Quantum Flex bioreactor. Cells are expanded inside the hollow fibers (IC) while a continuous nutrient and gas exchange takes place via the EC loop.

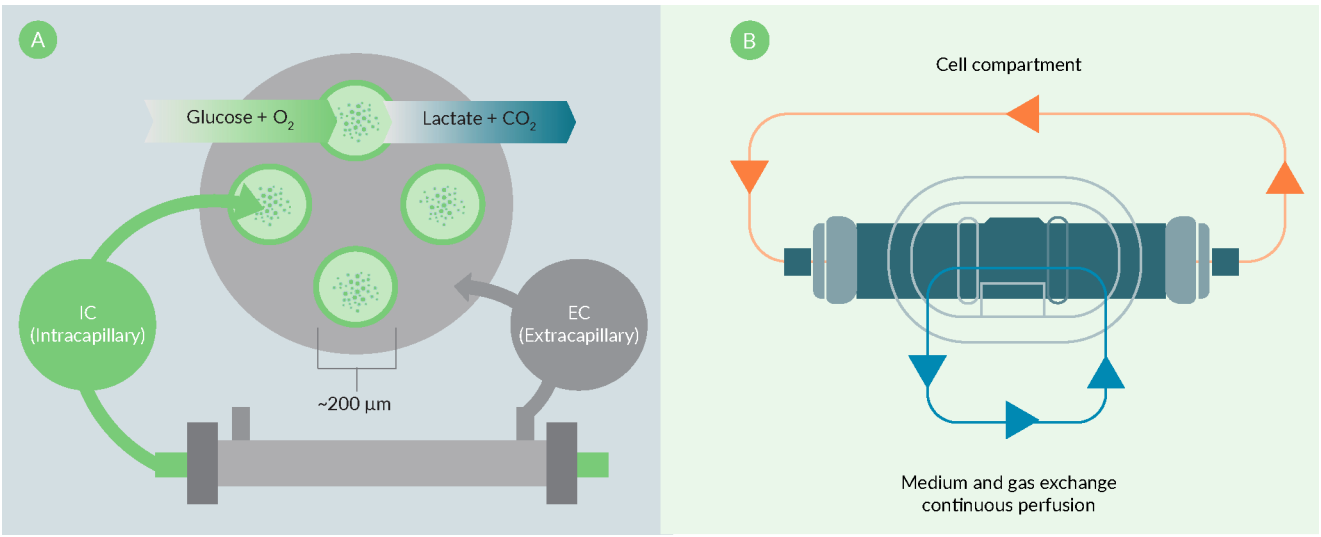

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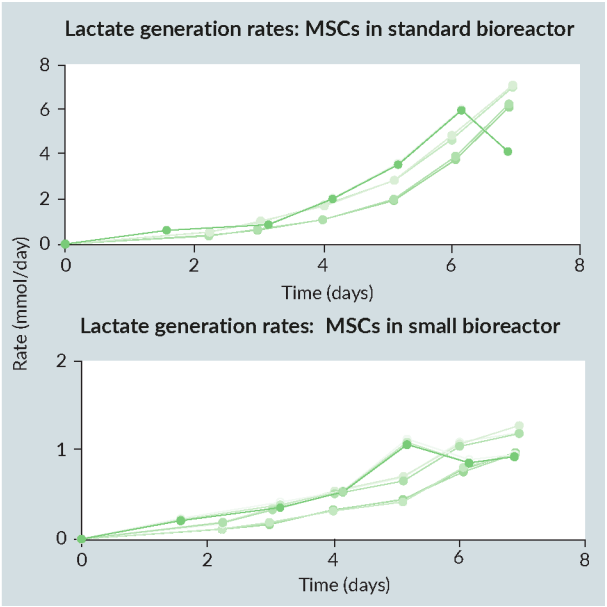
FIGURE 2

Lactate generation rates of a 7-day expansion period of MSC cells, using the standard (top) and small (bottom) bioreactor.



MSC: Mesenchymal stem cells.

FIGURE 3

MSC expansion kinetics expressed in harvest counts, viability at harvest, and population doublings demonstrate seamless scalability between the two bioreactors.

the MSC culture are shown in Figure 2. The lactate generation rate increases over time with a peak of 6 mmol/day between days six and seven for the standard bioreactor and 1.2 mmol/day for the small bioreactor, demonstrating a successful cell expansion.

Viability at harvest, harvest counts, and population doublings were used as the parameters for expansion kinetics. The results are depicted in Figure 3. The viability at harvest for all three donor samples was reported as excellent in both the small and the standard bioreactors. Population doublings, although variable between donors, all range between three and four point five and are comparable between the two bioreactors. The calculated yield from the small bioreactor is approximately 20% of the yield from the standard bioreactor. The difference in yield between the small and the standard bioreactor is similar to the differences measured on lactate production and media consumption (data not shown),

which demonstrate the seamless scalability between the two bioreactors.

SUSPENSION CELL EXPANSION

After confirming a successful growth of adherent cells, the next step is demonstrating suspension cell expansion on the Quantum Flex system. In this experiment, 6×10^6 T cells were loaded onto the small bioreactor and 30×10^6 cells on the standard bioreactor. The cells were loaded with CD3/CD28/CD2 soluble T cell activator and amplified in media containing 2% human AB serum and 200 IU/mL of IL2. T cells were expanded for 8 days.

Meeting the requirements for clinically relevant doses

Figure 4 illustrates the expansion kinetics of suspension cultures in both bioreactors. Similar to the adherent cell cultures, the viability at harvest of the suspension cells is high. A very promising result is the number of cells harvested after 8 days: over 22×10^9 cells from the standard bioreactor and 2.8×10^9 cells from the small bioreactor. Assuming 2×10^9 cells is the target number for an autologous T cell dose, this can be achieved in 5–6 days with the standard bioreactor and approximately 8 days with the smaller bioreactor. This translates to a 500-fold expansion and is especially relevant when starting material cell counts are low.

Quality of the T cells

In addition to achieving the required number of cells for a relevant clinical dose, the cells must be of high quality. To evaluate the quality of the cultured T cells, flow cytometry experiments were conducted to investigate T cell phenotype, which is an important indicator of function.

The first part of the analysis was to examine the distribution of CD4+ and CD8+ subsets (Figure 5). Cells were gated based on the expression of CD3, a T cell coreceptor that is expressed on all T cell types. Comparing pre-expansion to post-expansion demonstrates that cell expansion does not result in a change in T cell subset distribution. The graphs reveal a steady number of CD4+ T cells, 80–90% of the total cell population, while the number of

FIGURE 4

T cell production over 8 days. Indicated are viability at harvest, the number of cells at harvest, and population doublings.

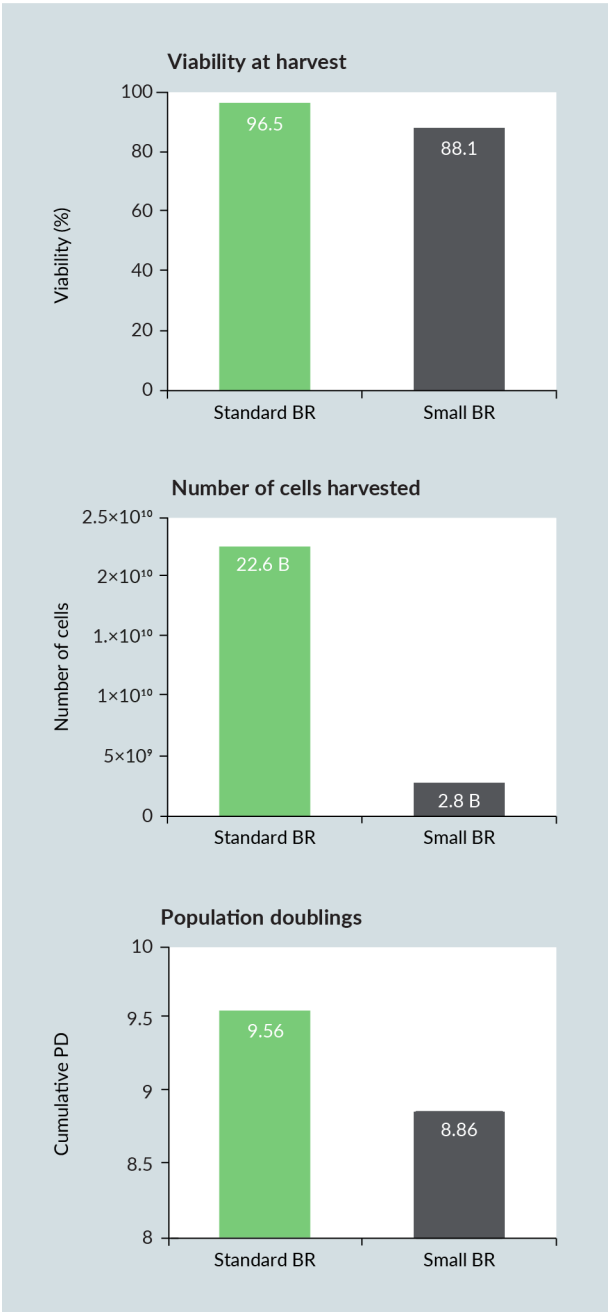


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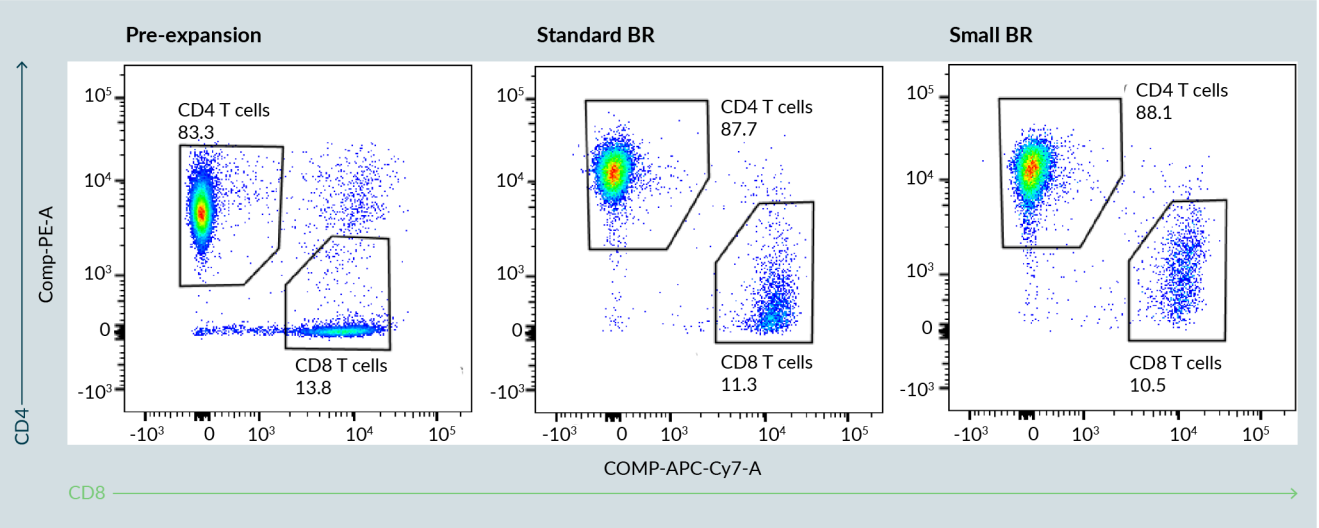
FIGURE 5

Flow cytometry density plots depicting CD4 and CD8 expression. T cell subsets are consistent regardless of seeding and feeding strategy.



CD8+ T cells is around 10% to 13%. These results suggest that the T cell subsets are consistent regardless of the seeding and feeding strategy used during cell culture.

T cells encompass a wide variety of cell types along a spectrum that goes from naïve cells with high proliferation potential to effector cells that are terminally differentiated and showcase a cytotoxic function. To better understand the specific subsets of T cells generated throughout the cell manufacturing process using Quantum Flex, the distribution of naïve, central memory, and effector memory T cells was determined by flow cytometry analysis of CCR7, CD45RO, and CD45RA after expansion. CD45RA is an expression marker for naïve cells, while CD45RO is used as a memory T cell marker, and CCR7 is a secondary lymphoid organ homing marker. Having populations of cells positive for these markers typically suggests the presence of long-term proliferative persistent cells. Figure 6 illustrates the flow cytometry results of these three markers on CD3+ gated T cells with CCR7 presented on the y-axis and CDR45RA or CD45RO on the x-axis.

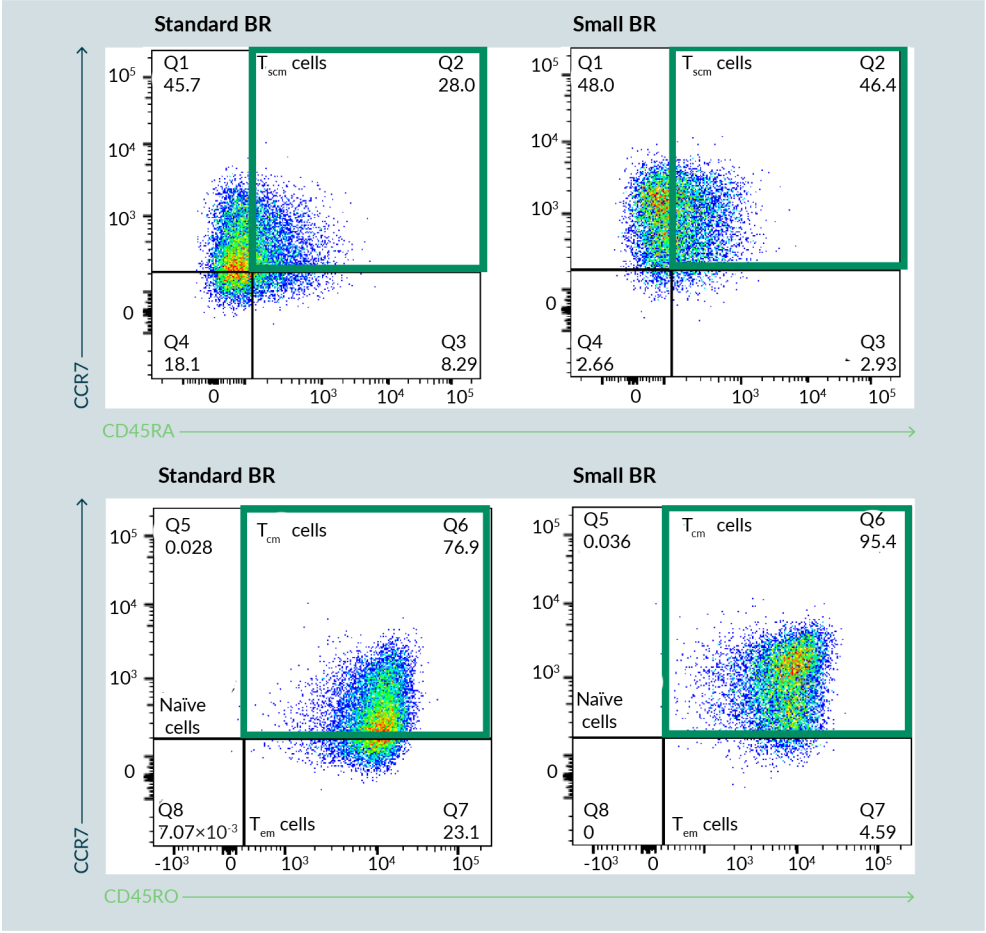
In both bioreactors, the bulk of the expanded cells exhibit a double-positive phenotype for CCR7 and CD45RO (Figure 6, bottom), representing a subset of central memory T cells (T_{CM}) which is a type of cell considered of high importance for use in T cell therapies. Interestingly, these cells were cultured without the addition of any cytokines aside from IL2, indicating that the cells were not directed in any other way by using additional cytokines. Furthermore, the results show the absence of purely naïve cells and only a small population of effector memory T cells (T_{EM}) present at this stage of the cell culture.

The cells positive for CCR7 and CD45RO were further analyzed by looking at the CD45RA expression (Figure 6, top). This analysis reveals the identification of a subset of stem cell-like memory T cells (T_{SCM}). Because these cells have long-lasting effector and differentiation potential, they are considered very desirable for cell therapies. Together, these results show that T_{SCM} cells as well as T_{CM} cells are generated in bioreactor of both sizes during the cell expansion process.

In the final set of experiments, exhaustion of the expanded cells was investigated in terms of over-proliferation and over-stimulation. A co-expression analysis was performed

FIGURE 6

Flow cytometry density plots revealing the phenotype of the T cells based on CCR7, CDR45RA, and CD45RO expression. T¬SCM cells are stem cell-like T cells, TCM cells are central memory T cells, and TEM cells are effector memory T cells.



for two exhaustion markers, PD1 and Tim3, whose co-expression has been shown to be correlated to terminally exhausted cells. Exhaustion was measured in CD4+ and CD8+ subsets. In addition, exhaustion was determined at different steps of the culture process; pre- and post-expansion, post-thaw, and 24 h post-thaw. Results are shown in Figure 7, demonstrating that only a small portion of the cells is truly exhausted. In addition to expression analysis of the two T cell subsets, co-expression analysis during the different steps of the cell culture process was investigated and demonstrated that the percentage of cells being exhausted throughout all the steps remains low.

CONCLUSION

To summarize, Quantum Flex hollow fiber perfusion technology:

- Generates a thriving environment for both adherent and suspension cell expansion.
- Enables smooth scalability from process development to commercial production.
- Allows for clinical-grade production of adherent and suspension cells in both the standard and small bioreactors.
- Delivers healthy subset populations of T_{SCM} and T_{CM} cells that are not terminally exhausted.

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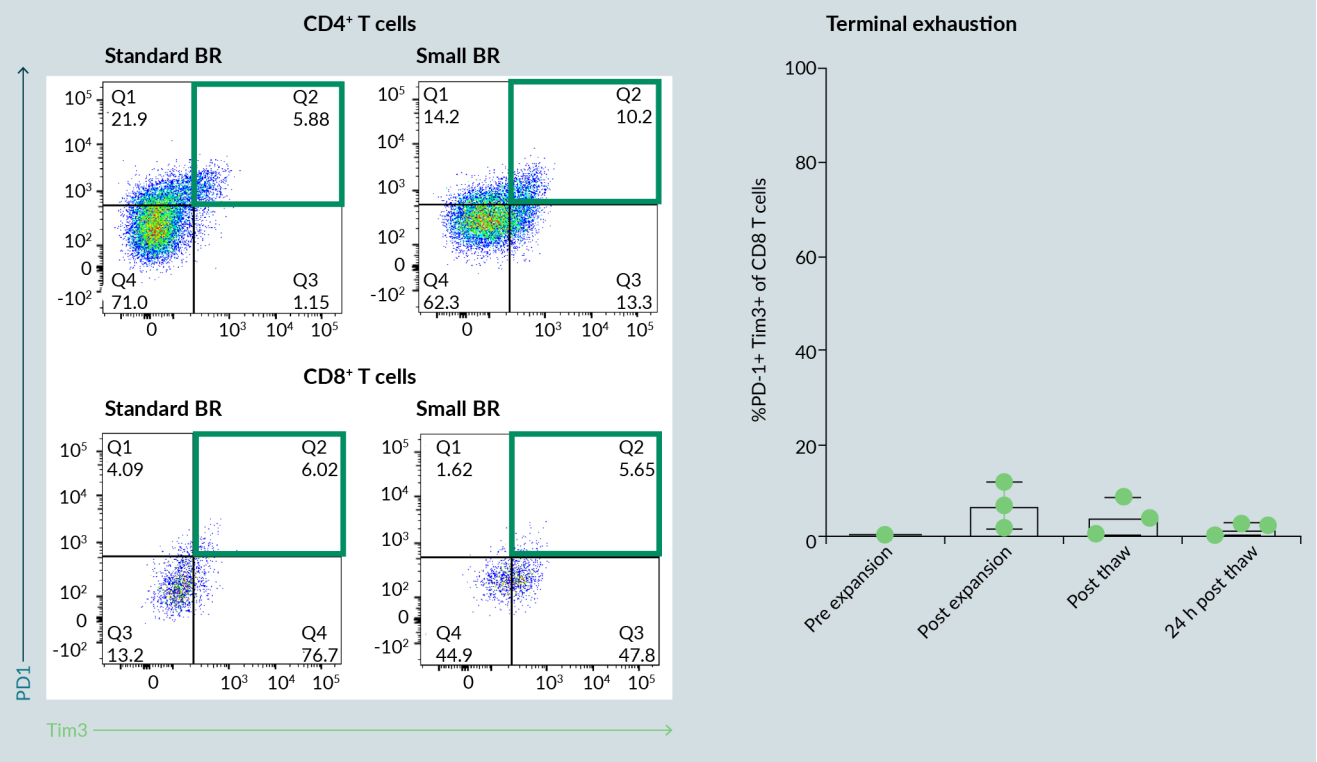
FIGURE 7

Data showing that expansion does not exhaust T cells. CD4+ and CD8+ T cell subsets reveal a low percentage of terminally exhausted cells (left). Similar results are shown throughout the different steps of the culture process (right).



Q&A



Charlotte Barker, Editor at BioInsights, speaks to (from left to right):
Hugo Fabre, Medical Science Liaison, Terumo Blood and Cell Technologies,
Maura Barbisin, Field Application Scientist, Terumo Blood and Cell Technologies and
Maria Knaub, Field Application Scientist, Terumo Blood and Cell Technologies

Q Why is perfusion so important in cell culture?

MB: Perfusion is really the core of the system. It is important because you can continuously pump fresh media and fresh nutrients into the culture and remove waste. For example, you can reduce the lactate concentration in the cell culture. Plus, oxygen is readily available to the cells by using the extracapillary (EC) pump. The oxygen can pass through the membrane separating the EC side from the intracapillary (IC) side and then needs to travel only around 100 µm into the fibers to be readily accessible to the cells. It's an active process, compared with a static culture such as a flask or bag, where the oxygen must pass from the surface of the media through the whole media layer and finally to the cells. The system can generate an environment that is close to a physiological environment, which allows them to not only expand but really thrive.

Q How can you maintain such high cell numbers in such a small volume?

HF: This goes back to the first question. The one thing I would add is the physiological aspect related to this question. Quantum Flex enables the solid growth of cells in good condition and at high density. When you consider what happens physiologically in the human body, for example in the lymph nodes, where the cell density often reaches several hundred $\times 10^6$ cells per mL and up to 1.5×10^9 cells per mL, it is not surprising that a hollow fiber-based perfusion system is able to sustain the growth of these numbers of cells in a similar manner.

Q To what extent is cell stress and its consequences, including cell death, a problem during the cell production process?

MK: As highlighted in previous questions, the unique advantage of this system is that it is close to the physiological situation, especially compared to manual cell culture. When our customers perform quantitative and qualitative analysis in terms of viability, differentiation, and specific markers for their desired cell type, they see that the cells have the same expression and usually either the same or higher viability. We do not usually see problems with cell death or stress.

Q What non-adherent cell types do you have data for and, more specifically, do you have any data for NK cells?

HF: At this point, there is data generated using hollow-fiber technology for T cells, T-regs, CD34 cells, and the K562 cell line, not only in our hands but also in customers'. We have a big pipeline to assess a wide variety of cell types and are always on the

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lookout for opportunities of external collaboration for new data generation. Natural killer cells are in the pipeline and so are CAR-T cells.

Q Is the system completely closed?

MK: Whatever comes into the system is either going through a filter or is sterilely welded on. Thus, the Quantum Flex system is closed. The only open step is when you fill a bag, which takes place outside of the system.

Q Is fluid sampling also closed, and how is the sterility of the fluid in the reactor guaranteed when drawing the sample?

MK: There are two ways of taking a sample. One is from the IC side by welding, meaning removing a piece of the IC-tubing, which contains the sample, while simultaneously closing the IC-loop with a sterile weld. The other is from the EC side, where you attach a syringe to take a sample. Everything that leaves the system goes through a filter.

Q Did you use any activation methods such as Dynabeads™ when expanding T cells?

HF: No beads were used. At the loading phase, human CD3/CD28/CD2 T cell activator was used. This gives robust activation and expansion without the use of feeder cells, antigens, or beads. It provides a good stimulus that maintains high viability. During the expansion phase, only serum-free media, IL2, and 2% human AB serum are used.

Q Can the system be used to expand and activate adherent and suspension cells simultaneously; for example, T cells that need to be stimulated with monocyte-differentiated dendritic cells?

HF: I do not see why not. It would require collaboration between the customer and Terumo Blood and Cell Technologies to make sure that the process development is optimal and that the seeding phase for the adherent and suspension cells is carried out correctly. In this way, each step can be assessed and optimized where needed, plus you can consider what readouts to use.

MK: Our main goal is to help our customers understand how to use the system and let them independently work with it. A combination of different cell types is possible, but similar to a manual culture, it is more challenging than having a single cell culture.

Q For the T cell data, you have shown 30×10^6 cells in the initial seeding. Have you attempted higher numbers such as 100×10^6 cells per mL in the initial seeding?

HF: A lot of process development work has been done to tailor the number of cells used for seeding. Up to 100×10^6 cells have been successfully tried.

MB: Seeding with 85 and 100×10^6 cells in total has been successfully performed, internally and with customers. The results were very similar to the data shown with 30×10^6 cells: very good viability and good expansion rates.

Q How do you maintain the cells in suspension within the hollow fibers?

MK: The basic idea is to create a bidirectional flow. You push the cells into the center of the bioreactor from both sides in order to obtain high concentrations. There is no need to take the whole bioreactor volume or the IC loop into account. By having that bidirectional flow, you maintain a high concentration of cells in a low volume, which closely mimics physiological conditions.

BIOGRAPHIES

DR HUGO FABRE joined Terumo BCT as Medical Science Liaison for Cell Collections and Cell Therapy Technology in February 2021. He is responsible for medical and scientific support, in-house or external associates training, stakeholder engagement and management as well as business intelligence related tasks. Dr Fabre has a PhD in Tissue Engineering and a Master's degree in Cell Biology & Genetics. He gained knowledge as a researcher and entrepreneur from 10 years of translational research experience in mid-sized and start-up CROs as well as in academic and hospital settings with comprehensive intercultural and international experiences in global teams. He is recognized as a stem cells characterization specialist through the use of proprietary protocols for GMP-compliant and state of the art flow and image cytometry. He was also the CEO and co-founder of a Luxembourg-based cryptocurrency management fund that was successfully sold in 2019. Before joining Terumo BCT, Dr Fabre was the Head of Business Development at Firalis, a mid-sized CRO and R&D company in the Basel area bringing lncRNA diagnostics solutions to market for a variety of neurologic and cardiac diseases.

MAURA BARBISIN joined Terumo BCT in 2018 as Field Application Scientist in EMEA to support customers in the Cell and Gene Therapy field and facilitate their process automation. In her role, she provides full technical training on Terumo BCT Cell Therapy device suite and continuous scientific support during device evaluations and post-sale implementations. Previously, she worked as Sr. Staff Scientist R&D at Applied Biosystems-Life Technologies, now part of Thermo Fisher (Foster City, CA, USA) for 10 years with increasing responsibilities from individual contributor to project leader and then also people manager, developing products based on qPCR and sequencing technologies. Among other roles, Maura Barbisin worked as contract researcher at SISSA-International School for Advanced Studies (Trieste, Italy) with a research assignment in the Neuroscience Department-Prion Biology Laboratory to study the whole transcriptome of animal models affected by prion diseases. Furthermore, she was a Scientist at ISS-Istituto Superiore di Sanità (Rome, Italy) in the Virology department-HIV Laboratory to evaluate the efficacy of a protein-based

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HIV vaccine. She studied the role of cell-mediated immune response during vaccination in animal models. Maura Barbisin holds an MSc. in Biological Sciences from the University of Trieste, Italy. She is an experienced molecular and cell biologist with extensive knowledge of both academia and biotech/medical device industry.

DR MARIA KNAUB joined Terumo BCT in 2021 as a Field Application Scientist for Cell Therapy Technologies in EMEA to support customers in their process automation. In her role, she provides full technical training on Terumo BCT Cell Therapy device suite and continuous scientific support of customers during device evaluations and post-sale implementations. For the Quantum Flex launch, she applies her cross-functional knowledge and experience to support globally. Dr Maria Knaub studied biomedical chemistry in Mainz, Germany, and worked in parallel as a paramedic and first aid instructor. In Heidelberg, Germany, she completed her PhD in molecular biology, where she focused signalling pathways in liver cancer development and supervised interns and trainees. Afterwards, she worked as a project manager, where she led different international customer projects in GxP environment and mentored new employees into their roles.

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TERUMO BLOOD AND CELL TECHNOLOGIES

AUTHORSHIP & CONFLICT OF INTEREST

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TERUMO BLOOD AND CELL TECHNOLOGIES

CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Navigating bottlenecks in cell therapy manufacturing: a versatile & scalable platform solution

Peter Yates

As cell therapies quickly advance from the preclinical stage into clinical and commercial applications, the design and implementation of scalable manufacturing platform technologies continues to be a focus of the field. Despite the successful commercialization of several cell therapies, bottlenecks in manufacturing throughput and capacity continue to hinder patient access to these life-saving treatments. This article will explore the current landscape and limitations of cell therapy manufacturing platforms and will share insights on how to choose a technology platform that assures flexibility in process development (PD) and scalability for clinical and commercial success.

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INTRODUCTION

There has been a tremendous amount of interest and investment within the cell and gene therapy (CGT) sector over the past 10 years. This has been primarily driven by the efficacious nature of the therapies that are being developed. Rare genetic disorders, cancers, and other diseases that have either run out of treatment options or never had curative treatments to begin with are now seeing renewed vigor within this field. This has culminated in over 2,200 CGT trials currently underway globally, with over 1,400 different CGT manufacturers, with 14 expected regulatory decisions for licensing and commercial approval in 2023. GCT development encompasses a wide range of manufacturing providers including startups, biotechnology companies, pharmaceutical companies, as well as universities and hospitals. Investment in this sector has

seen tremendous growth, reaching a high watermark of $23 billion in 2021, driven by venture capital, initial public offerings, and seed funding. However, in 2022 a decline in investment occurred, with only $12 billion raised.

CURRENT CHALLENGES IN PATIENT SCALE CELL THERAPY

The 2022 decrease in funding can be seen as a realignment of greater market forces, but it also speaks to the broader nature of CGT manufacturing. There has been tremendous initial success with the lead candidates of a select few groups, but there are several challenges pertinent to manufacturers that need addressing before renewed investment can be expected within the field.

In commercial cell therapy manufacturing, groups are currently only manufacturing for a small minority of the patient populations that they can treat. Certain groups have seen bottlenecks in expanding their manufacturing despite a tremendous demand for their product. These bottlenecks are product quality, quantity, and cost. Bottlenecks are often tied to antiquated technologies or open and manual handling steps within manufacturing processes, as the race to market has led to a lack of forward-looking sustainability.

A lack of automation leads to batch failures, as the majority of batch failures are related to operator error or mishandling. There are several regulatory challenges and logistics, which can also add to the cost of these products. Working in a centralized facility adds additional cold chain logistics and considerations for testing and traceability. Due to the complex nature and manual handling or semi-automated handling of these processes, they have an inherent inability to quickly and efficiently scale, leading to a high cost of goods. Disruptive solutions are needed to truly enable cell therapy commercialization and the scale of manufacturing needed to support these therapies in the marketplace.

THE COCOON® PLATFORM

The Cocoon® Platform is a disruptive solution for cell therapy manufacturing. This automated bioreactor and bioprocessing device can link many of the unit operations traditionally used for cell therapy manufacturing into a single, easy-to-use cassette with minimal operator intervention. After acquiring the technology in 2018, Lonza commercialized the Cocoon® in 2020. Since then, over 100 units have been placed in the field and there are three active clinical trials on the platform. The Cocoon® Platform can substantially reduce the cost of cell therapy manufacturing through its closed functionality, flexibility, and scalability.

There is a broad understanding in the space that process automation and closed-cell processing systems will be crucial for the advancement of scaling cell therapy manufacturing. Automation is now facilitating adoption through flexibility. The Cocoon® Platform is uniquely designed to meet the challenges of cell therapy manufacturing, as it is functionally closed, allowing a sterile environment for processing to occur while also reducing the number of touchpoints needed from operators and manufacturing technicians. This reduces the amount of human error and the number of batch failures that occur. With reduced human interaction, the quality and efficiency of the manufacturing processes whilst also increasing the reliability and repeatability of processes and allowing for ease of technology transfer from site to site. With reduced human interaction, a reduction of labor costs is seen and with the Cocoon®'s small footprint, a lowering of overhead needed for facilities and infrastructure is feasible.

The Cocoon® Platform consists of three primary components: the environmental unit, the single-use consumable cassette, and the Cocoon® Platform software.

The environmental unit controls the atmosphere, the temperature, and several sensors built into the instrument. It has a unique dual-zone temperature control system

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in which the upper portion is 37 °C to enable cell processing and cultivation, and the lower portion of the unit is 4°C for onboard reagent and waste storage within the cassette. A built-in precision peristaltic pump connects to the back of the cassette to drive fluidics within the cassette. Dissolved oxygen, pH, and gas are monitored through optical sensors within the unit. There is also a built-in barcode scanner to allow for the traceability of consumables and reagents into the electronic batch record.

The Cocoon® cassette is a single-use, functionally closed consumable. The lower half, known as the reagents storage zone, is home to onboard media, reagent, and waste storage at 4°C. There is the ability to connect additional bags of media or reagents, as necessary. Cell cultivation and processing take place in the upper half of the unit. A dedicated proliferation chamber is where the cells reside and where cell concentration, buffer exchange, media exchange, and other functionality occur. Built into the upper half is a series of ports that allow sterile connection for the ability to add reagents, remove samples for in-process quality control, and connect to other devices, such as Lonza's 4D LV Nucleofector® electroporation unit, allowing for inline integration of non-viral genetic modification.

The Cocoon® Platform software allows easy and flexible monitoring and control of the temperatures, gases, and fluidic pathways of the environmental unit and the corresponding consumable cassette. It has a built-in protocol designer that allows the creation of individual protocols from scratch, editing of existing protocols, or making one-time process decisions. It monitors pH and dissolved oxygen in real-time and has built-in PID control for the pH setting. Information is logged and kept in electronic batch records, and the software is 21 CFR part 11 and Annex 11 compliant. A built-in barcode reader allows sample and product traceability within the batch record. Graphic information is displayed on the operator console tab in easy-to-read tiles that clearly display the current status of the instrument. A direct control tab allows the end user to have full control over every parameter of the instrument in real-time.

UNIT OPERATIONS & PROCESS OPTIMIZATION

The Cocoon® Platform automates and integrates multiple unit operations, including processing upstream and downstream of cultivation, into a singular consumable cassette. These operations include magnetic cell selection, activation of the isolated population, genetic modification of cells, cell expansion, and final formulation. All these unit operations are performed in an automated manner with minimal hands-on interruptions. Overall, this reduces the number of errors and batch failures from operator personnel and eases tech transfer across multiple sites. It produces a high-quality product that is reproducible and robust, and with the lack of operator intervention, it lowers labor costs, allowing fewer operators to produce more batches.

The Lonza Cocoon® has been designed to allow process development (PD) and process optimization flexibility. The Lonza Cocoon® offers several ways to optimize and parameterize a process while maintaining flexibility throughout PD. The parameters that are flexible within this automated system are presented in Figure 1. Once a process is locked down, access to protocols that would prevent them from being edited can be inhibited to ensure compliance for good manufacturing practice (GMP) manufacturing.

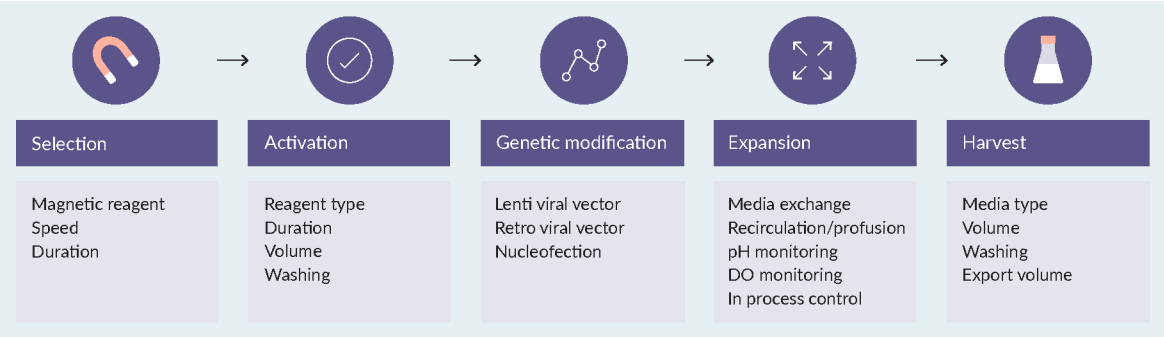
A chimeric antigen receptor (CAR)-T manufacturing workflow based on a full 8 h workday performed on the Lonza Cocoon® is shown in Figure 2.

PROCESS TRANSFER TO THE COCOON®

The Cocoon® Platform's ease of use and flexibility extends to process transfer. As the Cocoon® is a commercially available product,

FIGURE 1

Flexible automation of parameters on the Cocoon® Platform.



customers can build their own processes independently of Lonza, although Lonza's customized PD team is readily available and specifically tailored to the Cocoon® Platform.

A transfer process from a small research use only (RUO)-phase to GMP-ready can take as few as 9 months to complete. This can be done as Pre-Investigational New Drug Application or as an additional arm in a Phase 1 trial. Early incorporation of scalable automation facilitates time to market and availability of treatment to patients. Comparability studies, especially the need for additional dosing in patients, can dramatically increase timelines and costs. With early process translation, there are often lowered costs due to reductions in facility costs, labor costs, and batch failures, along with a simplified tech transfer and scale out. A clear path to commercialization helps to establish goals for quality, scalability, and sustainability for the cost of goods, and understand what those costs will look like at the scale of thousands of patients. This allows for a phase-appropriate level of quality development. Having a process that is trackable and changeable will allow easier interaction with regulatory authorities when changes are needed. Overall, early incorporation of scalable automation helps to facilitate market availability and treatment to patients.

FIGURE 2

A typical 9-day CAR-T manufacturing process on the Cocoon® Platform.

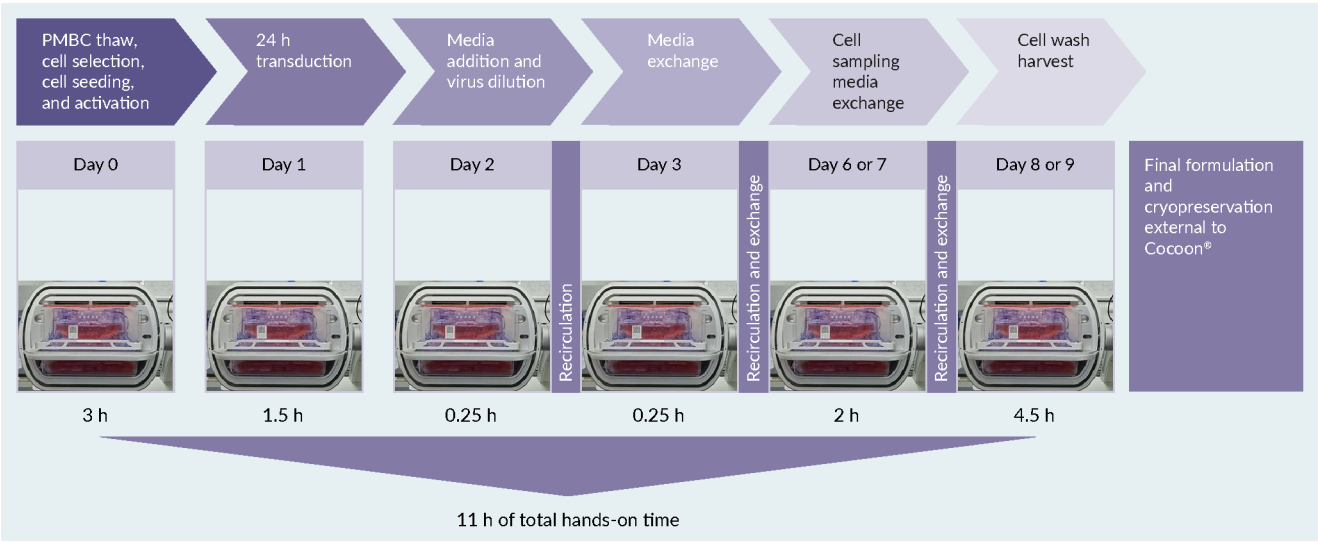


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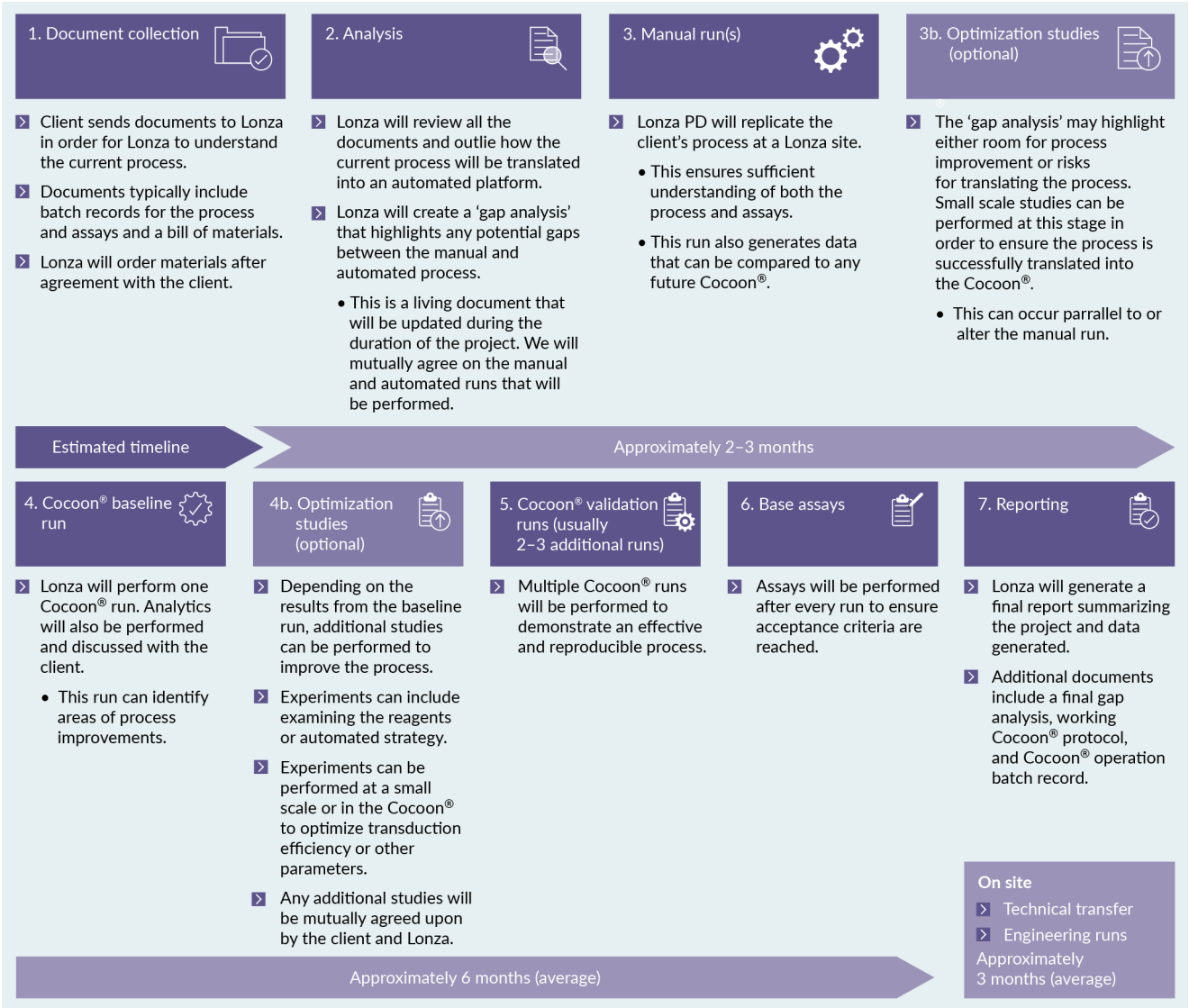
FIGURE 3

Timeline of translating a process to the Cocoon® Platform with Lonza's personalized medicine PD team.



Lonza's personalized medicine PD team can be utilized to accelerate the transition from manual processing to automated Cocoon® manufacturing (Figure 3). The team can provide a full-scope service for tech transfer of manual or semi-automated processes to the Cocoon®, and process and development optimization for a personalized process on the Cocoon®. In addition to processing services, the PD team also provides analytical development, using the in-line process analytics on the Cocoon® for dissolved oxygen levels and culture pH, as well as other services for off-line flow cytometry, metabolite profiling, cytotoxicity, and multiplex and single-cell real-time cytokine profiling.

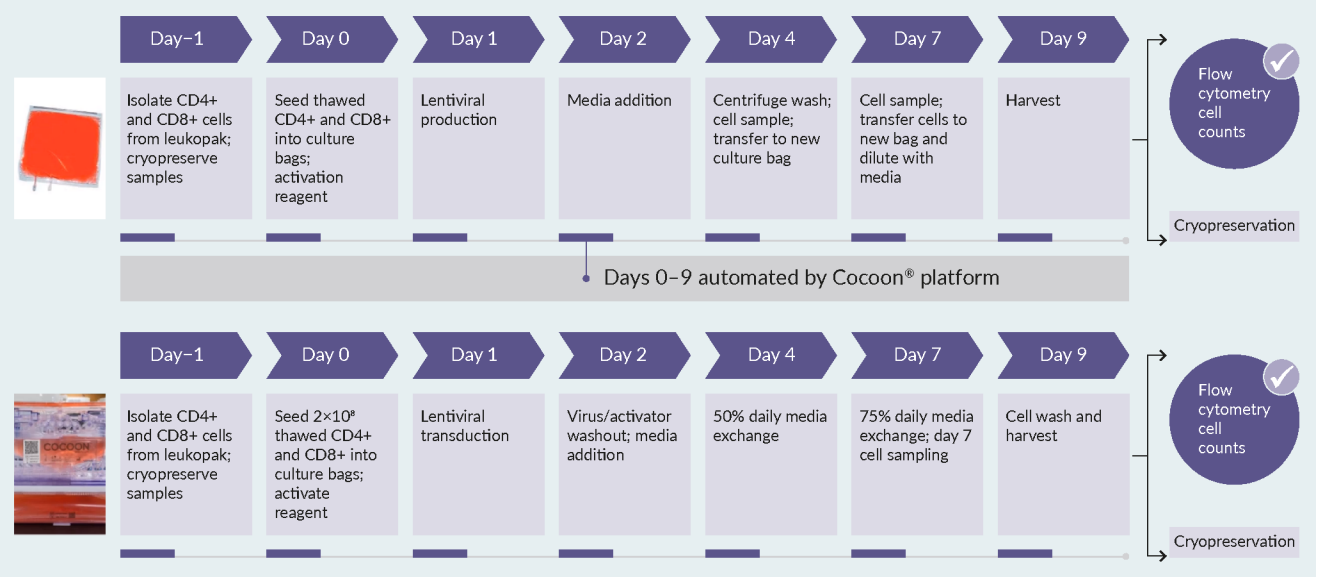
CASE STUDIES OF PROCESS TRANSLATION

Case study 1: novel CAR-T process translation

The first case study of the translation of a novel CAR-T process onto the Cocoon® Platform is outlined in Figure 4. The

FIGURE 4

Translation of a novel CAR-T process onto Cocoon®: an open and manual process (top) was transferred to a functionally closed and automated process (bottom).



results of this process transfer are described in Table 1. The success criteria were determined by the client and set forth by the optimized parameters desired from their bag-based process. After the first baseline run, subsequent runs for both optimization and validation all exceeded the denoted success criteria.

Case study 2: clinical CD19 CAR-T process translation

The second case study was a product already in a Phase 1 clinical trial. This involved a bag-based process that was manual and difficult for operators to complete. The group was looking for increased efficiency and capacity of manufacturing through the inclusion of an automated platform, the Cocoon®. As this was already in a clinical trial, there was limited process development flexibility.

Lonza mapped out several key acceptance criteria, highlighted in Figure 5, to ensure there was clinical comparability with the data that had already been generated. Lonza was able to meet the base criteria needed for acceptance within their study, exceeding their cell numbers for dosing, and having comparable transduction efficiencies for CAR-T cells and killing

TABLE 1

Novel CAR-T process Cocoon® Platform translation data summary.

Test	Success criteria	Cocoon® run 1 MOI=0.5 45 IU/mL IL-2 Donor A	Cocoon® run 2 MOI=1 45 IU/mL IL-2 Donor A	Cocoon® run 3 MOI=1.5 100 IU/mL IL-2 Donor B	Cocoon® run 3 MOI = 1.5 100 IU/mL IL-2 Donor B
Yield	N/A	2.38×10^9	1.97×10^9	2.02×10^9	1.87×10^9
Subject dose	$>3\times10^8$	1.16×10^9	1.00×10^9	1.09×10^9	1.24×10^9
% transduction efficiency	≥50%	48.7 (control= 10.7	51 (control= 26.2	54.2 (control= 29.5)	66.4 (control= 30.9)
% viability	≥70 post-thaw	89.8	77.9	88.7	95.8
% CD3+ cells	≥95%	97.5	97.4	96.5	98.2

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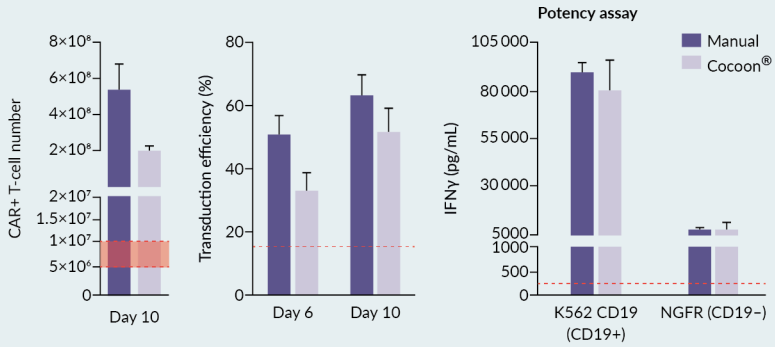
FIGURE 5

Clinical comparability in the Cocoon®: translation of a CD19 CAR-T process in clinic.

Test	Time point	Acceptance criteria
Cell viability	Final product	≥ 70%
CAR+ T cell number	Final product	1×10⁶ CAR⁺ T cells/kg
% CAR+ T cells (identity)	Day 9 or 10	≥ 15% double positive
% B cells (cellular impurity)	Day 9 +/– 1 day	≥ 5% CD19+, CD3–
Potency, in process	Day 5–9	≥ 200 pg/mL, 2-fold higher than control

1. 10 day γ-retroviral CD19 CAR-T process
2. Original process open, manual
3. Final product: 1×10⁶ CAR+ cells per kg



Considerations
- Limited PD flexibility due to existing clinical data
- Time and effort required

Cocoon® final product met all comparability criteria

- Exceeded required cell number for dosing
- Similar transduction efficiency (CAR+ cells)
- Comparable killing and functionality (IFNγ)

and functionality assays for IFN-γ production. This process was translated in 12 months.

ACHIEVING SCALABLE CELL THERAPY MANUFACTURING

A recent workforce report by the Alliance for Regenerative Medicine provides a gap analysis for the CGT sector highlighting the status of the industry and what needs to be overcome from a workforce perspective to successfully commercialize these cell therapies. Workforce gaps were identified in manufacturing, analytical development, testing, and quality control. The gap in manufacturing is expected to widen the most. 40% of respondents claimed that difficulty in finding the right talent has negatively impacted manufacturing or clinical development timelines.

CGT manufacturing processes are taking place in large-scale centralized manufacturing facilities requiring around 10,000 m^2 of processing space to manufacture 4,000 patient doses annually, which is only a fraction of the total available market. Needing to produce more doses means more square footage, larger buildings, and more personnel. Automation is required for scaling out, as there are not enough personnel in place to satisfy the demand for these life-changing therapies.

With Cocoon®, the move from large facilities into spaces of around 200 m^2 is possible. To manage the same number of doses in this smaller amount of space, the Cocoon® Tree has been designed as a scaffolding structure to allow the vertical orientation of multiple Cocoon®s in ~1 m^2 of floor space. These Cocoon®s use the same cassettes and processes as the benchtop version of the device but allow for maximized manufacturing productivity through the utilization of vertical space. The Cocoon® Tree is based on a track system where instruments are lowered or raised to operator height when intervention is needed. The prototype unit is currently being studied for usability and manufacturability in the long term.

COST & PROCESS ANALYSIS

Cost and process analysis was performed to identify the benefits of the Cocoon® Platform. Automating individual process steps into separate systems (i.e., semi-automation) was found to still be very labor-intensive, even at scale. A similar process on the Cocoon® showed a reduction

of the overall cost of around 30%, broken down to a 10% saving in material cost and a 20% reduction in overall labor and suite costs due to a reduction in footprint and operator handling.

SUMMARY

Bottlenecks in CGT manufacturing continue to hinder patient access to these life-saving treatments. To help solve this problem, the Cocoon® is a technology platform that assures flexibility in process development (PD) and scalability for clinical and commercial success. The Cocoon® Platform has a smaller footprint than many other benchtop devices and can unify multiple unit operations into a singular platform and cassette. A singular device simplifies logistics, with fewer devices to maintain, fewer consumables to track, and fewer operators to be trained. The integrated electronic batch record simplifies the unification of several different unit operations, and automation allows more robust production and leads to lower rates of batch failures.

Q&A

Charlotte Barker, Editor at BioInsights, speaks to, **Peter Yates,** Director, Product Management-Personalized Medicine, Lonza.

Q Is the Cocoon® only suited for T cells or can it support manufacturing processes for natural killer (NK) cells or hematopoietic stem cells (HSCs)?

PY: The Cocoon® cassette is designed to support T cells, HSCs, NK cells, and adherent cell culture work. Very simply, the Cocoon® is a culture vessel that we automate fluidic movement through in a closed and sterile manner.

Q Have you explored shorter processes for CAR-T manufacturing?

PY: We have explored these. We have current processes that are under development that would unify the unit operations for day zero to three activities, including selection, activation, and genetic modification. This would allow the formulation of an infusible product that

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allows *in vivo* expansion to take place. As the processes change throughout the marketplace, we are making sure that we are adaptable to current trends.

Q Are you able to connect to other devices or manufacturing platforms?

PY: The Cocoon® Platform can be connected to other devices and platforms including Lonza's 4D LV Nucleofector® Platform. The flexible nature of the cassette works within the standards of the industry, with input and output lines that can be connected to various processing devices to incorporate additional functionalities. The flexibility of the platform lends itself to connectivity to other devices. The pump system that drives fluidics in and out of the Cocoon® can be married to other devices.

Q What volume of media or regions can be stored in the cassette?

PY: We currently have two different cassette options, both of which are standardized for onboard fluidics of about 1.5 L. We can add additional bags of media to increase that to roughly 3 L of media usage overall.

On day zero, we try to ensure the ability to integrate all the required reagents and fluidics so that additional operator intervention is not required for either the removal or addition of media. We are developing new Cocoon® cassettes that would increase the amount of onboard volumetric storage and the capacity of cellular output that would be generated.

Q How do you dispose of the single-use cassette?

PY: The single-use cassette would typically be disposed of like any other biohazardous material. There are filters in the cartridge to draw on sterile air for some mechanisms. We are focused on the application of post-use integrity testing of those filters and access to those filters may be required. However, once your material is exported from the cassette, it can be disposed of as any other biohazardous material as per local regulations.

Q How would you perform media exchange?

PY: The Cocoon® operates as a modified perfusion culture. We can keep the cellular material maintained within the proliferation chamber, which is where we are actively cultivating the cells, but we can remove most media or buffer. That way we can do washes, cell concentrations, and reformulations. We also do profusion to exchange media and reoxygenate media. We have a recirculation line that allows for passive gas diffusion for oxygen back into the media which helps efficient media use.

Q When would you anticipate a manufacturer moving from benchtop Cocoon®s to tree-based Cocoon®s?

PY: We anticipate this as soon as the Tree is ready and commercially available. We are designing the Tree version of the Cocoon® to be as identical to the existing Cocoon® version as possible. The model we expect is using benchtop instruments to get started during process development, process optimization, and preclinical work, and then as scale-up is needed, utilizing the existing floor space within clean rooms to implement a Tree. We are designing the Tree to be usable in most clean rooms without special construction or modification.

We want it to be seamless as the need for increased throughput grows concurrent with demand from clinical trials. We anticipate transfer around the Phase 1/2 area, but that would not preclude someone from using a Tree in a modified version with only a few pods in use. We want it to be as flexible as possible so that we can support different groups and their different needs when it is convenient for them.

Q What does your onsite support look like in practice?

PY: We are commercially available for use. Customers are not obliged to use Lonza Contract Development and Manufacturing Organization (CDMO) services, but those are certainly available, and we welcome you to use them. We have several groups that use the Cocoon® in third-party CDMO facilities or their internal manufacturing facilities. For those groups, we offer support from our external field teams, who are teams of dedicated, cell therapy experienced field application support specialists.

For qualifications and preventive maintenance, the infrastructure has already been built to ensure that we can support our increasing number of units in the field. We are being proactive in our support of those units ahead of time to make sure that everyone can move as quickly as possible.

Q Where does the R&D for the Cocoon® take place?

PY: Lonza acquired this technology from Octane Biotech in Canada. Our primary research and development facility is in Kingston, Ontario. We also have several facilities around North America that contribute to research and development.

We are rapidly expanding to keep up with demand and the overall need for continuous improvement on the platform. As we grow out with Tree and our process development services, we have expanded to additional facilities around North America. We have a number of different sites that are currently supporting those activities.

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Q What specific infrastructure is required for the Cocoon®?

PY: For the base unit itself, the indicated use is in a Grade A or B environment, though there is sometimes a gap between intended use statements and what is practically being used in the field. We have groups that are using it in lower classified rooms at their own discretion. In terms of facility requirements, typical cleanroom infrastructure, power requirements, and networking for connecting these units are needed, including O_2 and CO_2.

Q Can adherent cells be expanded in the Cocoon®?

PY: Yes. In many ways, the proliferation chamber that is utilized is similarly structured to a T-flask. It is a hard rigid structure for performing cell suspension, cell culture, or adherent cell culture. We are working on several additional applications to support this as we have recently seen more regenerative medicine therapies come online.

Our target indications expand beyond oncology. We want to serve as many applications as we can and because of the open programmable nature of the Cocoon®, we are not locked into only CAR-T manufacturing and unit operations. Much of the underlying functionality supports mesenchymal stem cells and other cell types that could be cultivated within the Cocoon® itself.

Q Have you started any work with existing cell therapies that have been granted FDA approval to increase capacities and reduce costs? If not, what has been the resistance to that?

PY: The further you go within clinical trials towards a Biologics License Application (BLA) approval, the harder it is to make changes, especially in terms of the style of cultivation, for example from a bag to a rigid structure or from manual to automated media exchanges. The level of scrutiny increases throughout the phases of the clinical trial you are in, peaking at the BLA point. It is a very costly endeavor to initiate changes to already approved products.

Where we have seen the most interest is from groups that have had some success and are working on additional pipeline products upon realizing the manufacturing challenges that are ahead of them. Instead of trying to reinvent the wheel for a commercially approved product, it is more apt to focus on the pipeline products that are coming up and iterate on those instead. It is a continual work in progress. Most of the groups that we have been working with are in preclinical or Phase 1 activities.

BIOGRAPHY

PETER YATES is the Director of Product Management for Lonza's Personalized Medicine Business Unit. Peter earned a doctorate in Microbiology and Molecular Immunology from the University of Southern California in 2013 where he studied epigenetic regulation of hematopoietic development. Joining Lonza in early 2021, Peter's focus is on the continued development and advancement of the Cocoon® Platform, a closed and automated cell therapy manufacturing solution.

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AUTHORSHIP & CONFLICT OF INTEREST

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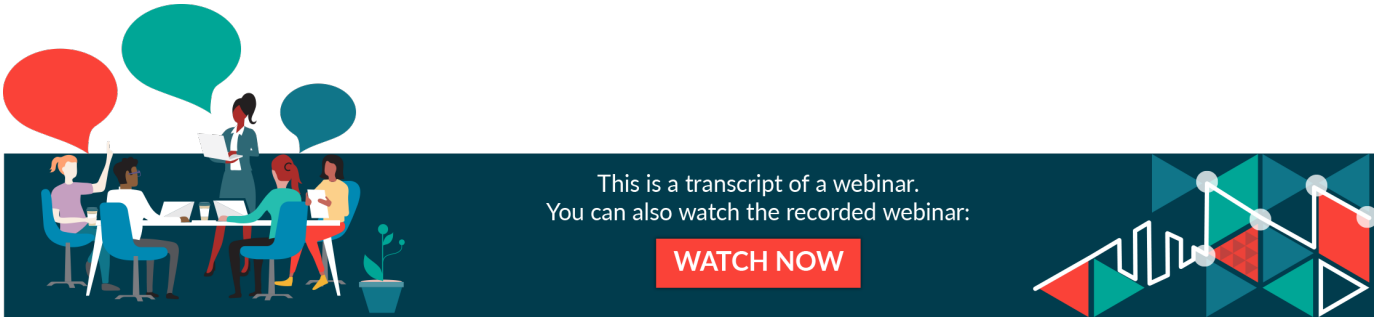

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Cocoon® Platform

The next step in cell therapy manufacturing

- Improved quality and efficiency
- Increased reliability and repeatability
- Reduced human error
- Less labor intensive = lower costs

Learn more

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Enabling instrument connectivity through digital automation

Bruce Greenwald & Elizabeth Wahl

Automation of the manufacturing process for cell therapies could significantly increase manufacturing success rates while reducing manual touchpoints and labor. Innovative instrumentation to address the various unit operations across the manufacturing process have been developed at Thermo Fisher Scientific, which are specifically designed to facilitate digital integration and automation. Gibco™ CTS™ Cellmation™ Software for DeltaV™ System is an off-the-shelf, digital solution that allows users to connect cell therapy instruments within a common DeltaV network to control workflows across multiple instruments in a 21 CFR Part 11 compliant environment. This article will introduce a closed, modular end-to-end manufacturing process and demonstrate how instruments can be integrated, automated, and incorporated into existing workflows.

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From 2016 to 2023, the number of global chimeric antigen receptor (CAR) T cell therapy clinical trials grew by 62% [1]. The market is dominated by the USA and China with the US leading the industrialization and commercialization of CAR T cell therapies worldwide. However, the expansion of clinical trials to other countries has been rapid, and the geographical reach of this technology is growing.

The traditional cell therapy workflow includes cell collection, processing, and distribution. This complex and labor-intensive process requires many open manipulations, thus increasing the risk of error. It can be difficult to synchronize different instruments and products while making the workflow traceable and compliant with regulations. Furthermore, there are high costs associated with lengthy and expensive QC and scale-up,

and the automation of workflows. Regulatory compliance requires supporting documentation, which also takes time and resources.

Traditionally, the focus in this sector has been firmly on reducing time to market, rather than aiming for process improvement. However, a complete suite of dedicated devices and reagents could help improve the process whilst also helping accelerate time to market.

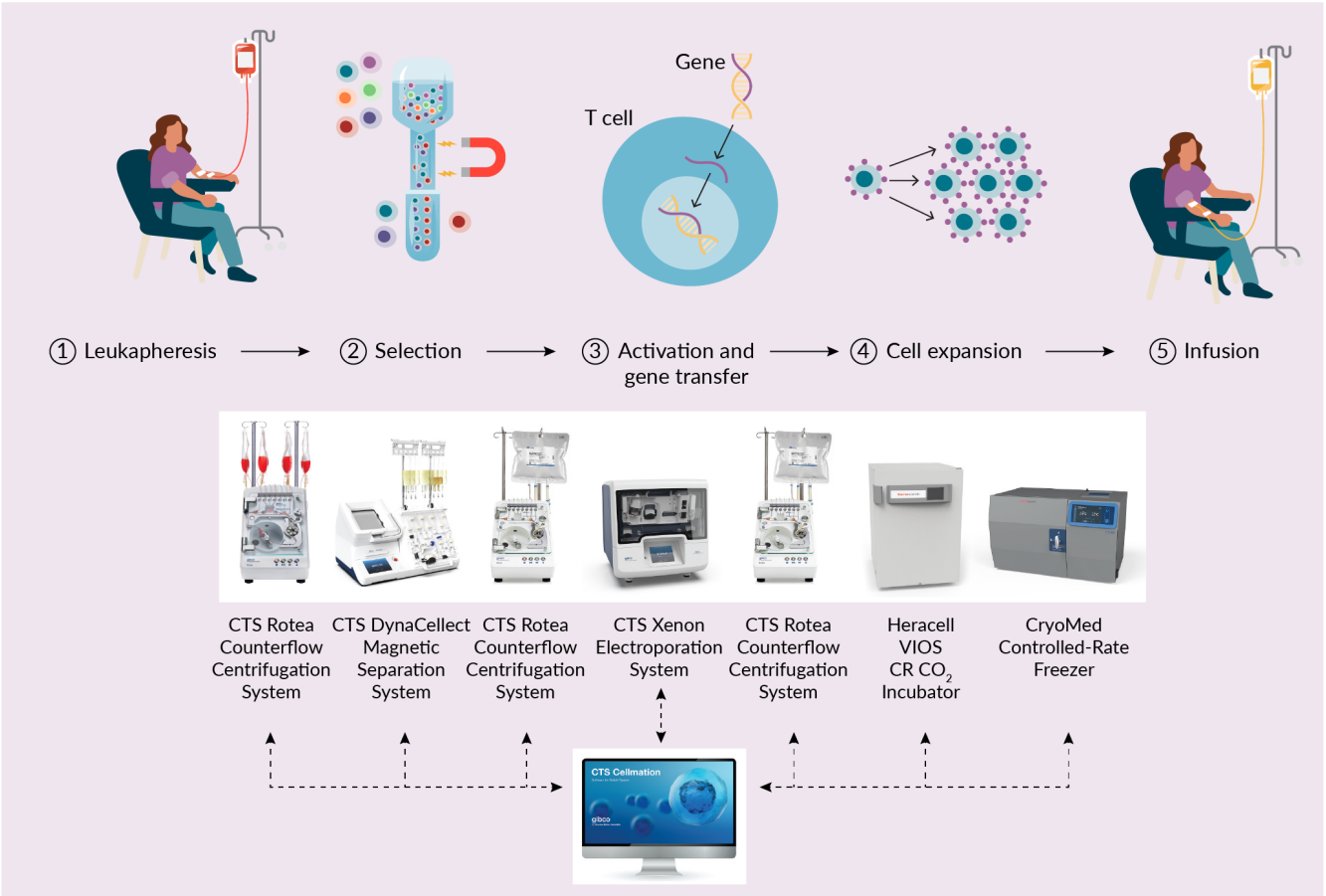
A CLOSED, MODULAR & AUTOMATED CELL THERAPY WORKFLOW

Thermo Fisher Scientific offers instrument solutions for multiple steps of the T cell therapy workflow, as shown in Figure 1.

To automate this workflow, all these instruments can be connected to the Emerson™ DeltaV™ Distributed Control System (DCS) to be controlled and managed in the same network with the same interface. To date, there has not been an off-the-shelf validated software solution to automate the cell therapy workflow. To allow scalable process control data management and to manage the cell therapy workflow through distributed control system (DCS) automation, a comprehensive, off-the-shelf validated software solution—Gibco™ CTS™ Cellmation™ Software—has been developed to connect cell therapy instruments to the DeltaV DCS controller directly. This saves money and time while reducing the number of manual touchpoints and facilitating digital integration.

FIGURE 1

Thermo Fisher solutions for the T cell therapy workflow.



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DeltaV is one of the most reliable and widely used process control systems in the industry. There are currently five instruments in the Thermo Fisher portfolio that are part of the software workflow solution, including Gibco™ CTS™ DynaCellect™ System, the Gibco™ CTS™ Rotea™ System, the Gibco™ CTS™ Xenon™ System, Thermo Scientific™ HeraCell VIOS™ CR CO_2 incubator, and the Thermo Scientific™ CryoMed™ Controlled Rate Freezer. Compatibility and control across these instruments helps ensure traceability, repeatability, and secure data connectivity.

CTS DynaCellect, Rotea, and Xenon Systems can be connected for a smooth transition from one instrument to the next with individual software modules for each instrument. Furthermore, multiple instruments of the same type can be connected and run on CTS Cellmation Software, offering a solution to help accelerate time to market and optimize the process as software validation is already complete.

CTS Cellmation Software includes an OPC Unified Architecture (OPC UA) Interface Module and a Phase/Equipment Module. The Interface Module maps the data between DeltaV and the instrument to allow data to be read and written. The Equipment Module executes commands to the instruments, and higher-level batch recipes can control the Equipment Module using phases. The DeltaV Batch Executive is used to create batch protocols for different workflows and collect data from all instruments. An example of one of the modules showcasing the CTS Rotea System graphical interface is shown in Figure 2.

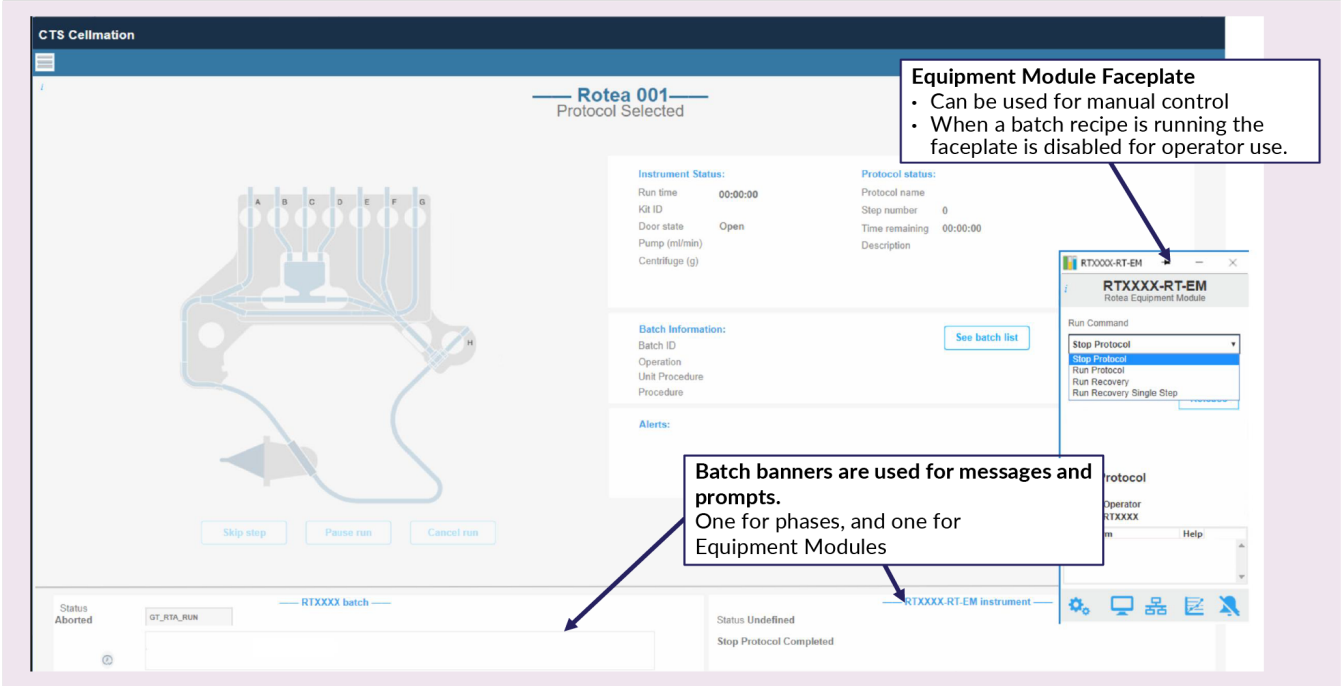
OVERVIEW & BENEFITS OF DeltaV

The digital plant maturity model is designed to help evaluate the maturity of a facility and is split into five levels:

1. Pre-digital plants, primarily with paper-based systems;

FIGURE 2

Example of CTS Rotea System status display with DeltaV Live graphics.



2. Digital silos with some manual processes and islands of automation;
3. Connected plants with digitization and some vertical integration;
4. Predictive plants with internal integration to the value chain;
5. Adaptive plants with full end-to-end integration from suppliers to patients.

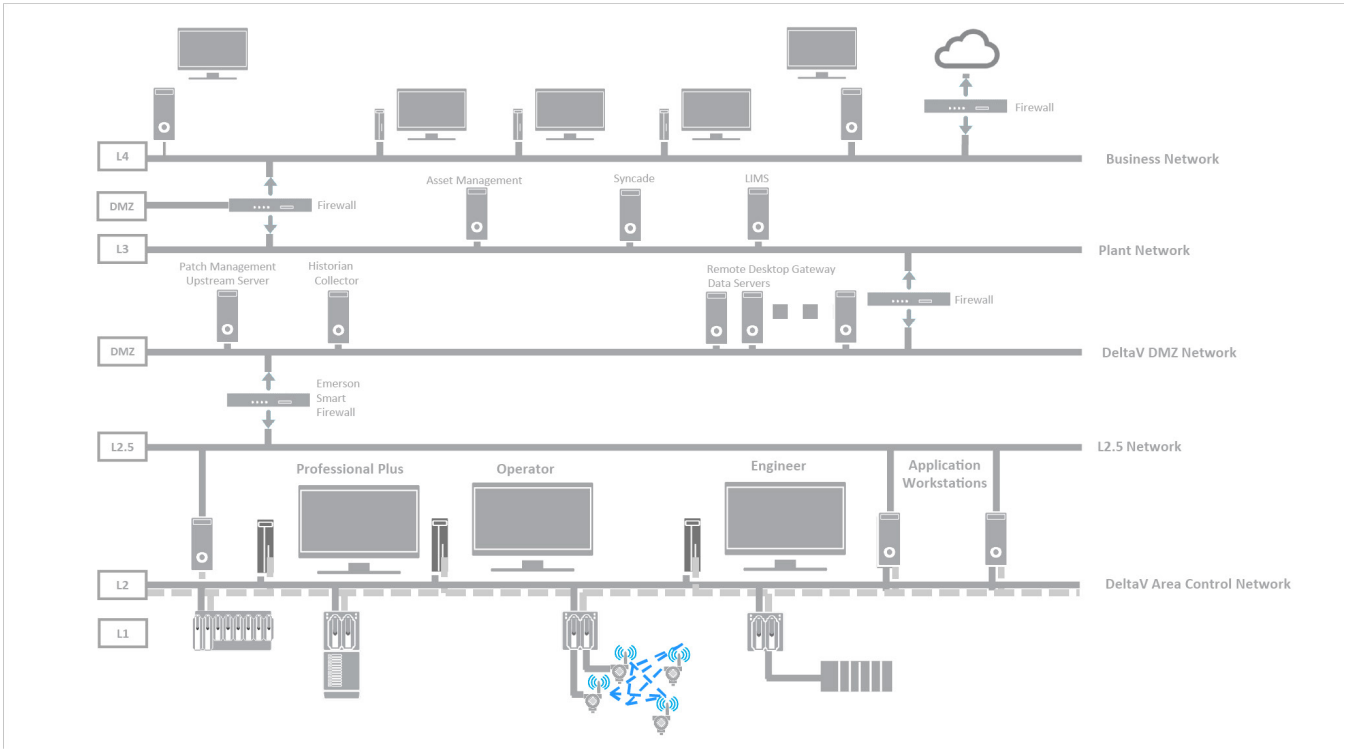
Manual integration of islands of automation are often associated with hidden costs. Each level requires engineering validation on a per-island basis, which can be highly complex. People may even attempt to skip steps in the process, which could result in a greater number of manual steps and more complexity. DeltaV supports a harmonized operations experience bringing together various levels of the digital maturity model without the added costs and complexity.

Released in the late 1990s, DeltaV is designed to be both easy for engineers to deploy and easy for operators and scientists to use. Integrating standard Dell PCs, Microsoft operating systems, and off- the-shelf components is key in the DeltaV architecture. Native batch with supervisory control support, electronic marshalling, easy virtualization, and smart commissioning makes DeltaV easy to deploy and use. The complete architecture of DeltaV spans a variety of process instruments and unit operations (along with auxiliary equipment), integrated DeltaV-ready components, utilities, building management systems, and third-party integration. The goal is to bring all of these processes together whether they are directly controlled or managed by DeltaV or not, to help provide a harmonized operational experience and offering contextualized data across all instruments in a single unified architecture.

The DeltaV single integrated solution combines input/output (I/O) subsystems, controllers, the engineering environment,

FIGURE 3

Process suites to business systems integration.



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user management, data contextualization, advanced process control capabilities, and recipe management. The system is a one-stop shop to configure control strategies, add unit operations or instruments, manage alarm and recipe settings, and manage historization requirements in a single database. DeltaV can automatically aggregate all data from the process, including continuous, alarms, operator interactions, and batch data, and contextualize this information within the various databases. A common, easy-to-use interface facilitates data retrieval and contextualization.

For electronic records, data integrity is very important in the GMP space. DeltaV can help pharma and biotech companies easily become compliant with 21 CFR Part 11, supporting compliance across three main categories: configuration, runtime, and historicization.

One of the key enablers of digital transformation is the use of OPC UA. OPC UA allows CTS Cellmation Software instruments to act as data servers to communicate with DeltaV, which then can also act as an OPC UA server to allow contextualized data to be pushed out for analytics, cloud applications, or reliability requirements. Within the whole S95 model, DeltaV can be used to communicate securely through firewalls up to the demilitarized zone (DMZ) network and the business network (Figure 3).

SUMMARY

CTS Cellmation Software is an off-the-shelf validated solution that allows users to connect Thermo Fisher cell therapy instruments within a common DeltaV network to control workflows across multiple instruments enabling regulatory compliance. DeltaV provides the ability to easily build a connected plant with the knowledge that transition to an adaptive plant in the future can be easily made possible. The software provides data contextualization across all instruments and unit operations and supports full data integrity and compliance. The ability to scale as a business grows is also supported by DeltaV.

REFERENCE

1. Clinicaltrials.gov search words: “chimeric antigen receptor” (Not all trials are listed on the map view).

Q&A

Abigail Pinchbeck, Assistant Editor, BioInsights speaks to (pictured left to right) **Bruce Greenwald,** DeltaV Platform Business Development Manager, Emerson Automation Solutions and **Elizabeth Wahl,** Product Manager, Cell and Gene Therapy, Thermo Fisher Scientific

Q What DeltaV version is compatible with CTS Cellmation Software?

EW: Cellmation is currently compatible with DeltaV version 14.

Q What are the options for starting small and expanding the DeltaV system size as needed?

BG: A single Cellmation piece of equipment or unit operation can be connected to the DeltaV system, and then it can still expand as needed. For example, from an I/O perspective, a single DeltaV system can handle 30,000 I/O. It is easily scalable by adding additional instruments and controllers to the system, and these can communicate in a peer-to-peer manner.

Q Are there plans for integrating additional Thermo Fisher instruments with CTS Cellmation Software in the future?

EW: Yes, we are discussing outfitting more instruments with Cellmation modules in the future to expand across the workflow. The key piece that those instruments need is the OPC UA.

Q How do I go about integrating other process equipment?

BG: Integrating other process equipment is easy. OPC UA is one method for communication. Others include Modbus TCP/IP or conventional I/O systems. DeltaV can handle all those subsystems and industrial ethernet protocols. It is simply a matter of identifying what protocol you need, and we can then easily bring that information into DeltaV.

Q What is the recommended approach for managing updates to software and hardware components including DeltaV, CTS Cellmation Software, and Thermo Fisher instruments?

EW: Guardian support from Emerson will be the best way to receive any support needed regarding DeltaV. We align closely with Emerson to ensure any updates they have are installed and that we are aligned to offer the best support. In the case that there is an instrument update that affects connectivity, a corresponding update will be available for Cellmation. However, we do try to limit the number of updates necessary. For some instruments, software updates can be downloaded directly online. Others, such as the CryoMed Controlled-Rate Freezer, do require service tech, but these instruments require updates much less frequently.

Q Are there tools available to extract runtime data from DeltaV for analysis and reporting?

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BG: Yes, all the data is contextualized at the DeltaV level, whether using our batch historian or continuous historian. Then, we have documented interfaces for pulling out data, such as OPC for continuous historical data or other methods for extracting continuous data. That can then be pulled out and brought into, for example, the Syncade manufacturing execution system (MES) platform, where we can generate full batch reports and exception-type documentation can be put together at the level 3 MES layer.

Q What support and training options are available to companies that adopt CTS Cellmation Software?

EW: In general, DeltaV support is provided by Emerson and its local impact partners. Guardian support is available from Emerson. Support for Cellmation-specific queries will be provided by Thermo Fisher via remote or onsite channels.

We have training for operators through our Thermo Fisher Education Connect portal and if additional training is needed, we can send somebody onsite. Additionally, Emerson has its own training courses around DeltaV that are beneficial. There is support and different training available to those who want to learn more about DeltaV and Cellmation.

Q Does Emerson automation offer Internet of Things (IoT)/Lots of Things (LoT) or digital twin solutions for biotech industries?

BG: We have a full digital twin solution available that is becoming increasingly popular for process design, process implementation, validation, and operator training. This allows a training system with the operator's interface to mimic processes as they run, to offer hands-on experience prior to working on real processes. That same platform can be used for validation activities—if you are making changes to configuration, validation requirements can be met in that operator training system environment before moving to the production environment.

BIOGRAPHIES

BRUCE GREENWALD is the DeltaV Platform Business Development Manager for Emerson Automation Solutions, located in Austin, TX. In his current role, Mr Greenwald assists customers in understanding the features and benefits of DeltaV to improve their automation experience. Mr Greenwald is a 1979 graduate of the University of Kansas with a degree in Chemical Engineering. He started his career with the Dow Chemical in Freeport, TX and joined Fisher Controls in 1983 as a systems engineer. Mr Greenwald joined the RE Mason Company, an Emerson Impact Partner, in 2000, executing PROVOX and DeltaV projects. He held several positions at RE Mason, and in 2011, re-joined Emerson. His 4 decade-long career has been focused on process control.

ELIZABETH WAHL has over 10 years of experience working in cell culture and molecular biology and supporting scientists in their R&D. She is a Product Manager focusing on workflow automation for Cell and Gene Therapies at Thermo Fisher Scientific. Previously she was

the Strategic Product Manager and part of the Operating Leadership Team at GeminiBio, a raw materials manufacturer for the CGT market. Being part of a small company allowed her to wear many hats and drive the company's strategy while fully understanding the importance and difficulties of each functional area while driving products to launch. Elizabeth has an extensive educational background, including an MBA from the University of Illinois at Urbana-Champaign, a doctorate from TU Munich in experimental medicine with a focus on regenerative medicine and tissue engineering, an MSc from TU Dresden in molecular bioengineering, and a BSc from Kent State University in biological anthropology and studio art.

AFFILIATIONS

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Emerson Automation Solutions

Elizabeth Wahl
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Thermo Fisher Scientific

PRODUCT USE STATEMENT

AUTHORSHIP & CONFLICT OF INTEREST

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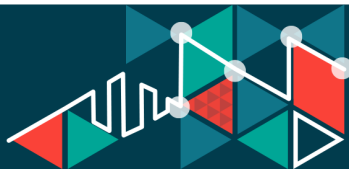

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Cell therapy

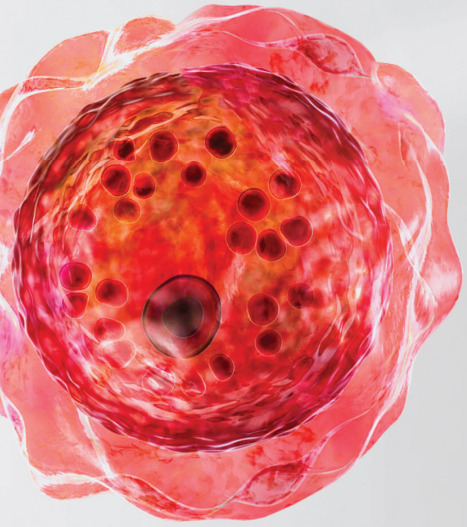
CTS Cellmation Software for the DeltaV system

Connecting the instruments of your cell therapy workflow

Gibco™ CTS™ Cellmation™ Software for the DeltaV™ system is an off-the-shelf solution designed to simplify and optimize cell therapy manufacturing processes. It provides digital connectivity to modular cell therapy instrumentation from Thermo Fisher Scientific while enabling compliance with 21 CFR Part 11/Annex 11.

CTS Cellmation Software offers:

- An off-the-shelf solution—no need for costly custom software projects and extensive validation, which helps save time and resources in the manufacturing process
- Seamless integration—connect and control your cell therapy workflow across multiple instruments through a single user-friendly interface
- Data security and compliance—help ensure traceable, reproducible, and secure data storage with the DeltaV™ Continuous Historian, which enables compliance with the 21 CFR Part 11/Annex 11 requirements for current good manufacturing practices (cGMP)
- Streamlined data management—a direct connection to the DeltaV™ system layer simplifies the transfer of data streams to the manufacturing execution system (MES) and the enterprise resource planning (ERP) system
- Workflow control—create user-specific batch recipes to control the flow of your cell therapy manufacturing process



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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

INTERVIEW

Modernizing cell therapy manufacturing to reduce vein-to-vein times

In this episode, **Charlotte Barker,** Editor, BioInsights, speaks to Curate Biosciences' CEO **David Backer** and CTO **Tony Ward** about the need for CAR-T cell therapy manufacturing to evolve, and how a new cell separation technique could boost efficiency.

Cell & Gene Therapy Insights 2023; 9(6), 843–850

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Q Dave, you have been in this field for some time now. How have you seen CAR-T cell therapies evolve?

DB: Yes, I have been in cell and gene therapy for over 25 years, almost since the inception of the technology itself. First of all, we would not even be having this discussion if not for the scientists and clinicians at UPenn who initiated the first clinical trials using

genetically modified T cells. The initial studies and products were primarily around a single antigen found on a cancer cell—CD19. While there have been many additional trials that are variations on the theme, there has been a search for new antigens, multiple antigens, and utilizing other cell types such as NK cells. Throughout this, there has been a drive towards more precision, focusing on specific subunits. More precision means you need to capture and keep as many target cells as possible in their initial state.

Q What are the key challenges and priorities for CAR-T cell manufacture?

DB: We have already seen that it is a successful therapy that can be commercialized. The products currently on the market are primarily for leukemias but there is increasing discussion about moving into areas such as multiple myeloma.

CAR T cell therapies are moving from fourth-line to third-line and even second-line treatments. That is expanding the patient population and exacerbating the problems with the original manufacturing process, which involves doing leukapheresis on the patient's own cells, shipping them to a centralized manufacturing center to carry out various manipulations, release test, and send the cells back to the patient to be reinfused. This process is highly expensive and time-consuming and is normally carried out in centralized manufacturing facilities due to the GMP requirements.

We and others are looking at the different unit operations and improving them, utilizing newer technologies to move production to regional centers (a distributed model) rather than a centralized manufacturing model.

TW: As Dave noted, the T-cell field is learning more and more about what works and what doesn't. An example is better understanding of the importance of younger and naïve T cells, leading to a greater focus on how best to recover and maintain that smaller subset of T cells. This is especially difficult when considering the patient profile, which includes elderly patients with lower levels of naïve cells, and leucopenic patients in second-, third-, and fourth-line treatment.

Q Why are the initial steps in apheresis or debulking particularly important?

TW: Apheresis samples degrade quickly over time. At a high level, there are two main aspects of this: a) physical cell loss and death over time; and b) the response of the innate immune system to cell death, which includes a lot of cell signaling.

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The industry is split on how to handle this. It is recognized that fresh cells are the better starting material, but for 2–8°C controlled samples, the quality degrades beyond 24–36 hours, when the innate immune system response kicks in. The industry's response to this has been to use frozen samples. However, the freeze-thaw process is far from perfect, even with a lot of improvement and a significant effort by a lot of companies.

> "We are all trying to do the same thing—find a way for the patient to get their cells re-infused in the cleanest and fastest way possible."
>
> **— David Backer**

The process is still highly variable and there is a significant amount of cell loss, especially when targeting a smaller subpopulation of cells. The freeze–thaw process also requires a recovery period after thawing, which adds time and variability to the overall manufacturing process. There is also variability in the ability of patient cells to withstand the process.

At an industry level, there is no consistency in where the freezing process takes place—at the collection site or at a regional processing center. So, the variability is exacerbated not only by the patient's state of health but also by the process used and the timing of processing. The use of 30–50-year-old techniques only adds to the variability.

For T cells, there was always a sense in the market that you could, 'Grow your way out of the problem'. However, recent data from multiple groups show the therapeutic benefits of a shorter time in culture. In fact, most developers of these commercial therapies now state that their best yields and out-of-specification products are directly correlated with the quantity and quality of the starting material.

DB: As Tony mentioned, there are a variety of different protocols that people are using as we move into the next stage of autologous cell therapy manufacturing. We are all trying to do the same thing—find a way for the patient to get their cells re-infused in the cleanest and fastest way possible. We are doing everything we can to make sure that those precious patient cells are protected so that downstream activities to create a gene-modified cell can occur in the best way possible. That is the imperative that we have put into our company.

Q Let's talk about how Curate Biosciences is addressing some of those problems. What does Deterministic Cell Separation™ (DCS) microfluidics involve and how does it differ from commonly used techniques?

TW: DCS involves the microfluidic separation of cells on the basis of size, rather than density. It is a fully closed system, and within our consumable is a 3.5 million

micro-post array where each micro-post is around double the size of a typical monocyte. These posts are precisely arranged so that large cells (in this case, leucocytes) are gently deflected into a parallel clean buffer stream, effectively washing the cells individually, while letting the smaller cells (platelets in apheresis) and plasma pass straight through. The red cells, being discs, flip on their axis around the posts and roll through the device and come out in the small cell and plasma fraction. This eliminates the need for an acidic lysis step, which is metabolically damaging.

A key distinction of DCS separation versus other techniques is that it is amazingly efficient and consistent. This results in a uniquely powerful process that combines four best-in-class metrics into one platform:

- The array is over 95% efficient at recovering large cells and is unbiased when compared to density and lysis combinations;
- The overall process has <7% coefficient of variation;
- It is >99.5% efficient at removing platelets;
- It achieves a >3 log wash efficiency for soluble factors as part of the process.

We believe the technique has the potential to be a revolutionary technological advance. Individually, these metrics would be a significant improvement on the existing manufacturing process. In combination, they are uniquely powerful.

Q How can DCS optimize the expansion of T cells and reduce manufacturing time?

TW: In the simplest terms, the objective in CAR-T cell engineering is to maximally recover the youngest T cells, keep them young and as 'fit' as possible, while most efficiently targeting them to the tumor. So, the practical goal for the developers is achieving the best recovery, with the most predictable cell engineering and the ability to consistently make the highest quality dose.

We have third-party data showing recovery of up to 30% more of the naïve or younger T cells in their first step with DCS compared with a new elutriation approach. In another third-party study, almost twice as many total T cells were recovered with DCS when compared to an automated Ficoll centrifugation approach.

Going into the engineering process with the knowledge that you can reliably get the most out of the sample is the first step—and this is especially important if specific T subsets that require multiple cell-specific selections are involved.

This recovery alone will enable much shorter processes to be more easily considered—one of our collaborators is now targeting a 2-day manufacturing process, for example. For TCR-T processes where more cells are currently needed, in addition to the initial recovery our internal data shows an approximately 30% better fold expansion at day 12.

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"...a Deterministic Cell Separation™ cell preparation that is about 50% less pre-activated, generates about 50% less regulatory T cells (which is critically important to dose quality)..."

— Tony Ward

Q Does cell separation have an impact on any subsequent genome editing steps?

TW: There are four parameters that come together. We see a biological advantage that is derived from the DCS wash/separate process itself. When doing comparison studies, we found that density-based separations still contain platelet aggregates, which persist for several processing steps. Biologically, this is important because the platelet aggregates are still releasing pro-inflammatory cytokines and chemokines, which then act upon the T cells and start signaling events.

This results in a DCS cell preparation that is about 50% less pre-activated, generates about 50% less regulatory T cells (which is critically important to dose quality), and has also been shown to have 30–50% improved lentiviral transduction efficiency—all benefits that are directly related to the efficient and timely removal of cytokines and chemokines.

So, it's not just improved editing steps—it's an overall improvement in the ability to recover, engineer, and reliably and rapidly deliver the cells of the right phenotype. And it's not just in fresh cells—cells that have been DCS prepped and subsequently frozen retain these positive attributes.

The same broad benefit of less activation and fewer T-regulatory cells means DCS-prepared cells experience less lag in the early days of expansion, which we expect to be especially important in shorter processes.

Q Can you elaborate on how manufacturing processes can help ensure the clinical efficacy of the product by removing unwanted factors that can impact transduction efficiency?

DB: We clearly cannot say clinical efficacy at this point, but things are heading in the right direction. We just launched our product commercially earlier this year and we are in the process of getting into clinical trials. However, getting the right cells to the starting line in the right condition is potentially a huge step forward for the industry.

If we follow the clinician's credo of 'First, do no harm', it's clear based on our findings so far that it will be better for the patient if unwanted signaling can be stopped. We think that the

right thing for both patients and the industry is to perform DCS processing as soon as possible post-collection and then engineer from there—whether frozen or not.

BIOGRAPHIES

DAVID BACKER has over 25 years' experience in cell and gene therapy. He was Owner, Founder, and CEO of Molecular Medicine BioServices, focused on GMP manufacturing of viral vectors. Dave sold the company to Sigma Aldrich and stayed on in a variety of commercial, strategic, and operational roles. After Sigma Aldrich became MilliporeSigma, Dave expanded his responsibilities as Head of Commercial Development for its Gene Editing and Novel Modalities business. Most recently, Dave was SVP of Commercial Development at ElevateBio and then Chief Commercial Officer at Oxford Biomedica.

TONY WARD previously worked in R&D at Becton Dickinson prior to moving to commercial roles, leading up to being Global Strategic Marketing Director for Becton Dickinson's research cell analysis business. He was recruited to lead commercial operations for eBioscience from startup through sale to Affymetrix, where he served as SVP/General Manager for the business unit. Tony's background includes invention, development and commercialization multiple cell processing and assay technologies.

AFFILIATIONS

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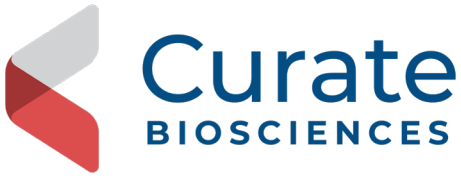
Tony Ward
CTO, Curate Biosciences



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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Scale-up and delivery of allogeneic cell therapies to large patient populations

Marcos Langtry, Krishna Panchalingam & Inbar Friedrich Ben-Nun

Cell therapy has become a therapeutic reality for many patients, with several successful autologous drug approvals over the last few years. Beyond the known off-the-shelf benefits provided, allogeneic cell therapy cost structure makes this technology substantially more economical, increasing the ability of cell therapy to treat large patient populations. As allogeneic cell therapy continues its exponential growth, a scale-appropriate model to start clinical trials early, while aiding a smooth transition into large scale when the need comes, will be required for companies to successfully deliver from first-in-human to commercialization. This article will explore how the use of outstanding technology, alongside a tried-and-tested standardized systematic approach and reliable analytics, can enable the delivery of a robust and commercially viable process; ensuring an adequate supply to achieve therapeutic and commercial goals.

Cell & Gene Therapy Insights 2023; 9(6), 523–535

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SUSTAINABLE SUPPLY FOR ALLOGENEIC CELL THERAPIES TO TREAT LARGE PATIENT POPULATIONS

Current threats to cell therapies

Scale appropriateness and cost of goods are at the forefront of the current threats to cell therapies. Scale appropriateness requires agility to scaling-up to meet market demand, as clinical and commercial needs can be vastly different. Defining critical quality attributes (CQAs) and developing reliable analytical methods early on are key to ensuring a robust output and a successful scale-up.

Market-induced challenges include the limitations posed by the need for speed to

market. The cell therapy field is highly competitive, with certain indications being targeted by many, and limited access to funding and revenue streams. In addition, a lack of talent can pose challenges, as there are not enough personnel available to keep up with market demand. Having the right people with high expertise and the right training can make the difference between failure and success.

Good manufacturing practice (GMP) tissue sourcing capabilities are a critical piece for cell therapies. It is an area prone to misconceptions, where the quality of tissue will determine the product quality. One must establish an appropriate tissue source for the desired cell type with specific acceptance criteria predictive of successful manufacturing. Donor eligibility is paramount, including donor consent for the intended indication and phase. GMP quality tissue sourcing is required to meet unique specifications and regulatory requirements. It is important to note that there are stringent safety and testing requirements for tissue to be used, especially in the creation of cell banking and cell lines. In the end, the sponsor must be able to support the rationale for how regulatory requirements are met, articulating the risks. Lonza is accredited by the American Association of Tissue Banks which establishes standards and accredits tissue banks based on verified compliance.

New product introduction & lifecycle process

To anticipate and address potential FDA observations, Lonza has created tried-and-tested quality systems and standards to bring a product to market using a standardized, systematic approach. This system enables new products to meet strict quality requirements, standard global technology transfers, and compliance throughout the product lifecycle.

The stages of the new product introduction and lifecycle process are outlined in Figure 1.

THE DEVELOPMENT APPROACH TO SUCCESSFUL COMMERCIALIZATION

Process development (PD) is the foundation for enabling success in the commercialization of cell and gene therapies. The initial stage of Lonza's step-by-step approach to commercial readiness is diagnosing to de-risk. This means establishing the baseline process, identifying the major manufacturability gaps of the process, and defining the scope of development activities. Through the identification of the major manufacturability gaps, risks are identified from low to high risk (e.g., where an example of a high-risk gap results in a safety issue or a batch failure). The second step is development and industrialization, where process optimization and development occur to meet the intended manufacturing design specifications and CQAs. Following the establishment of this GMP-ready process, the process is then transferred to manufacturing. This transfer starts with pilot and training runs, engineering runs, and proceeds into clinical production. For a commercial process, manufacturing would execute process performance qualification (PPQ) runs in support of the commercial manufacturing runs.

Analytics are key to establishing a robust and reliable manufacturing process and should be developed and evaluated during the development phase. Implementing analytics for in-process monitoring can result in greater process insight and control, including defining and assessing the impact of the critical process parameters (CPPs) on the critical quality attributes (CQAs) of the process. Additionally, the development of release assays that can interrogate and establish the therapeutic identity is critical in ensuring the production of the intended therapy. This includes establishing the identity, safety, purity, and potency of the product. Stability testing is also a requirement to understand the stability of the product formulation and support the commercial lifespan of the product.

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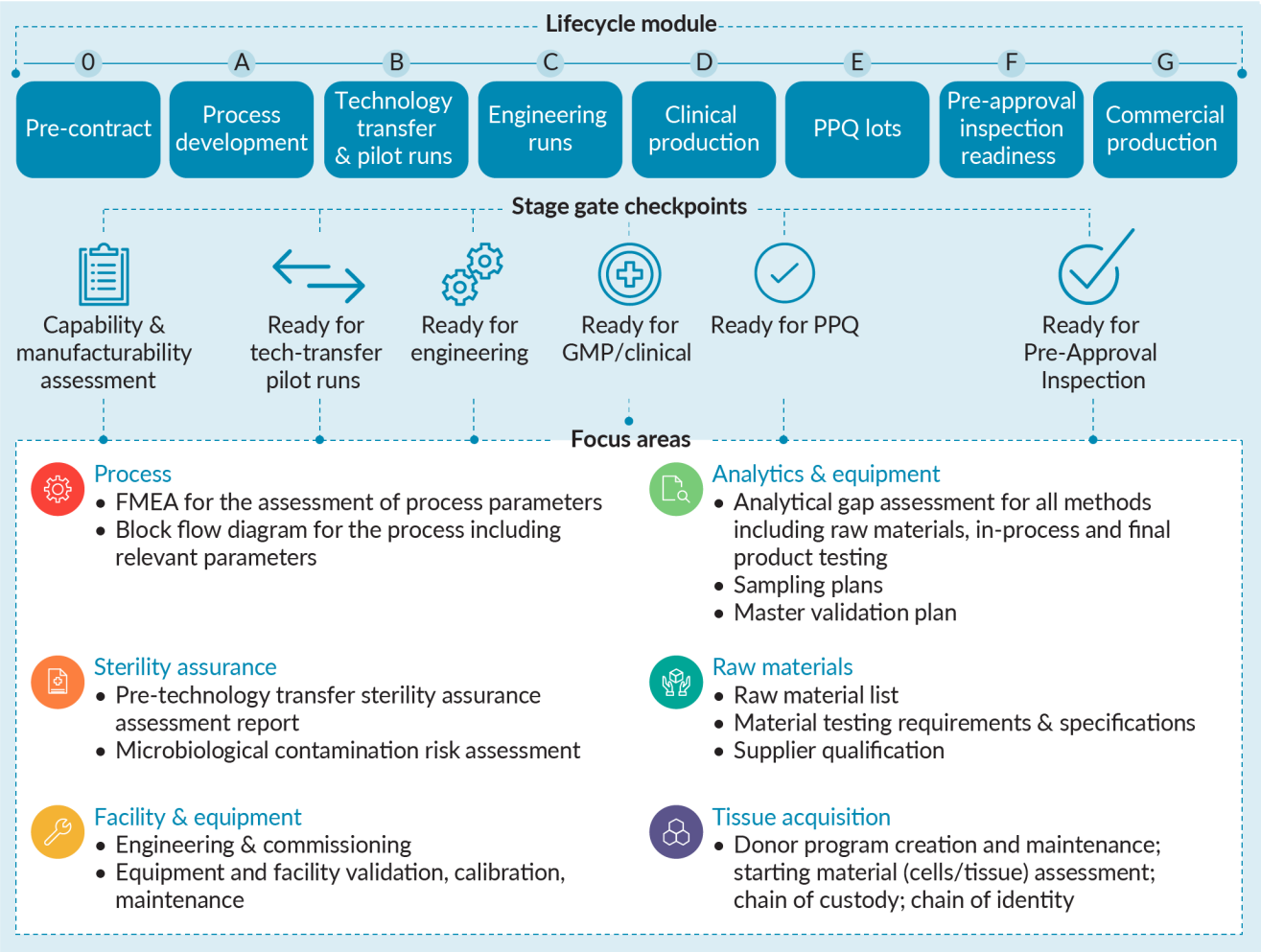
FIGURE 1

Lonza's standard New Product Introduction & Lifecycle Process.



To consistently manufacture a high-quality therapeutic product, it is critical to lock down the starting material and identify the potential CPPs. By Phase 2/3, these requirements are crucial to have defined to have a well-defined manufacturing process to ensure consistent manufacturing of cell and gene therapy products (Figure 2).

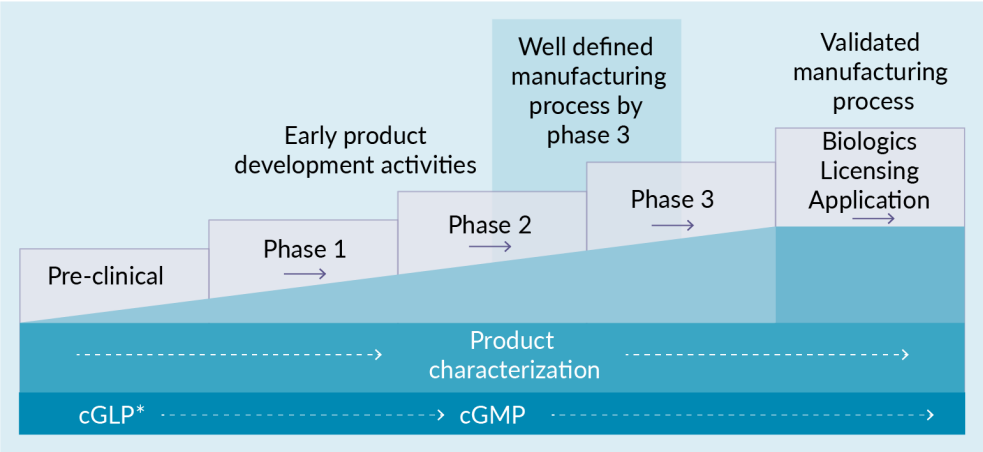
To support this commercialization push, Lonza offers a step-by-step streamlined late-stage commercialization readiness pathway. This includes a collaborative 3–5 day workshop to analyze past studies/runs (preferably at scale) and identify potential critical process parameters through a failure modes and effects analysis (FMEA). FMEA is performed to define the actual focus of process characterization or the need for process improvement studies.

TRANSITION TO A COMMERCIALLY VIABLE LARGE-SCALE PROCESS

To enable the execution of scale-appropriate processes various areas of focus are considered. These include but are not limited to, 2D closed processes or 3D bioreactors, optimizing process unit operations, implementing in-process controls and in-process monitoring strategies, and filling/ inspection automation and downstream technologies. Focus on these

FIGURE 2

The path to commercialization: manufacturing of cell and gene therapy products with a defined process.



*Good Laboratory Practice.

areas can result in targeted improvements in terms of yield, consistency, efficiency, quality, and most importantly cost.

Lonza has the capabilities to support small-scale to large-scale production of cell and gene therapies and implement varying levels of automation. Automated systems have distinct advantages for large-scale expansion, enabling high-fold expansion to meet cell quantity demands, having controlled and monitored systems for increased batch-to-batch consistency, and reduced labor, duration, and footprint for cost-efficient production. One example is the implementation of stirred tank reactors for up-scaling the production of allogeneic immunotherapies. An example allogeneic process may start with mobilized apheresis or an induced pluripotent stem cell (iPSC) bank for directed differentiation, before proceeding to manufacturing large-scale GMP batches in a bioreactor system. Then, large-scale, closed concentration/wash of cell product occurs, before a streamlined fill/finish, visual inspection, and cryopreservation of the formulated cell product.

For large-scale processes, common scale-up methods are typically based on numerical methods to estimate mass transfer, maximum shear stress, power/volume, or other key hydrodynamic parameters. However, these empirical methods are based on specific bioreactor configurations and can be challenging for characterizing cell therapy-based bioprocesses. In this regard, the implementation of computational fluid dynamics (CFD) modeling approaches can accurately consider the geometry and bioreactor configuration and their influence on bioreactor hydrodynamics. Using this approach, key hydrodynamic values can be determined at any location within the reactor, alongside their volume average values and/or the distribution within the reactor, all independent of the geometry or setup of the system chosen. In this regard, CFD modeling can also reduce the number of biological runs required to develop appropriate scale-up parameters. This results in reduced cost and time going from clinical to commercial scale.

Utilizing CFD modeling and introducing automation, the Ambr250 can also be implemented as a scale-down model or as a development reactor, due to its versatility and throughput. It has up to 12 mini bioreactors in parallel and has been used extensively with exosomes, viral vectors, and cell-based therapies at Lonza.

In addition, Lonza offers multiple liquid handling systems for automated sample preparation, including the BD FACS Duet,

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an automated sample preparation device for flow cytometry, and the Tecan for plate-based assays. These are designed to improve the consistency of the assay performance and reduce deviations and analyst variation.

COMMERCIAL CASE STUDIES

Case study 1: mesenchymal stem cell (MSC) manufacture

MSC industrialization is a notable example to demonstrate how different manufacturing platforms impact cell quantity and ultimately process cost. A single closed automated 200 L bioreactor can generate more doses than large-scale traditional 2D expansion solutions such as Cell Factory or HyperStack. A 200 L bioreactor produces the same number of cells as 315 CF10s while requiring only 1/4 of the footprint. The benefits of moving away from 2D conventional culture conditions into 3D cell expansion systems are summarized in Figure 3.

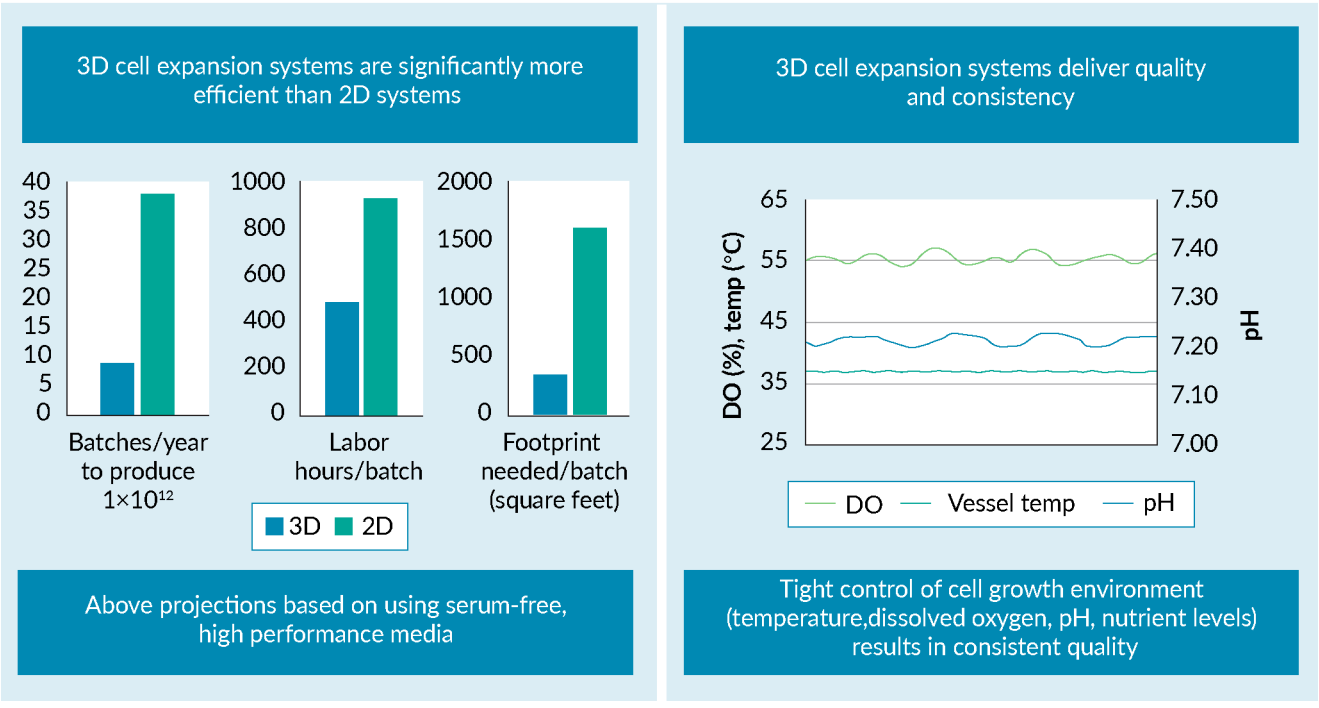
The benefits of 3D expansion systems drove Lonza to develop innovative solutions for 3D expansion for other adherent cell types.

Case study 2: iPSC manufacture

iPSCs can provide a platform for allogeneic therapy standardization and scalability. iPSCs can be banked for indefinite use, and therefore they provide a common and stable source to allow tissue material standardization, avoiding reliance on donor material. These cells could provide the solution for allogeneic cell scale-up, providing a platform in which upfront gene modifications can be achieved with the cells expanding before differentiation. This increases scale and yield while addressing cell exhaustion/senescence of the final cell therapy product. In addition, proprietary iPSC lines enable companies to enhance product exclusivity. An area of concern with iPSCs is having non-differentiated cells in the final cell therapy product. Several

FIGURE 3

Impact of MSC industrialization: 3D cell expansion systems.



approaches are being used to minimize the risks, and Lonza is establishing an assay to detect non-differentiated cells as a part of its quality control and release.

Lonza is providing an end-to-end scalable offering for iPSCs, starting with tissue acquisition and ending with fill and finish. This includes a full range of iPSC manufacturing services tailored to customer needs including microcarrier and aggregation-based processing. Lonza offers the expertise to reprogram, edit, bank, and expand iPSCs and leverages non-viral 4D-Nucleofactor™ transfection technology. Differentiated iPSCs across the three germ layers are offered, including beta cells, immuno-oncology cells, cardiomyocytes, and neural progenitor cells. The iPSCs are of high quality, which is retained even after long-term cryopreservation. The cells maintain their stability, proliferation, and differentiation capacities, providing a homogeneous starting population for cell therapy manufacturing processes and the ability to manufacture large, cryopreserved master cell banks and work in cell banks to sustain commercial demands. For additional information, refer to [1].

In Lonza's platform, human iPSC expansion is performed in a closed, automated, monitored, and controlled stirred tank bioreactor where cells, in this example, are cultured on plastic microcarriers in Lonza proprietary media. The starting material cryopreserved cells are expanded in 2D prior to inoculation in the bioreactor. However, the 2D step is optional and can be eliminated. Expansion is achieved in a short time as there is no need for 2D to 3D adaptation. Labor is reduced as cell passaging is not needed and media is automatically perfused in and out of the bioreactor using Lonza's proprietary deep tube.

Similarly to the expansion step, the downstream processes of cell release and separation from the microcarriers, followed by cell concentration, are performed in a closed manner reducing contamination risk and enhancing compliance with GMP requirements.

FIGURE 4

Expansion data from iPSC bioreactor runs on Lonza's end-to-end scalable platform.

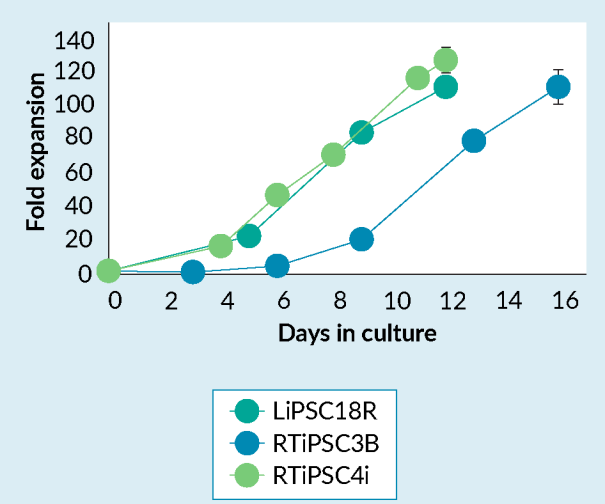


Figure 4 shows expansion data from three bioreactor runs inoculated with different iPSC lines. In all runs, over 100-fold expansion was achieved in 12–14 days post-inoculation. The iPSCs expanded in the bioreactor were also shown to have typical pluripotent stem cell morphology when plated back onto 2D. They retain the expression of self-renewal markers such as Oct4 and SSEA4. Directed differentiation assays were performed on these cells showing their ability to directly differentiate into neural stem cells, cardiomyocytes, and definitive endoderm.

It was also demonstrated that iPSCs expanded in the bioreactor could be cryopreserved successfully at high cell densities. The ability to cryopreserve iPSCs at high cell densities enables the use of the cryopreserved cells for expansion and differentiation in suspension without the need for a 2D seed train. Reducing, therefore, time, contamination risk, and labor.

In Figure 5, iPSCs, cryopreserved at 120 million cells/mL were thawed into a 3 L bioreactor, and 50-fold cell expansion was observed on day nine after inoculation. As a control, cells were thawed onto 2D, followed by alkaline phosphatase (AP)

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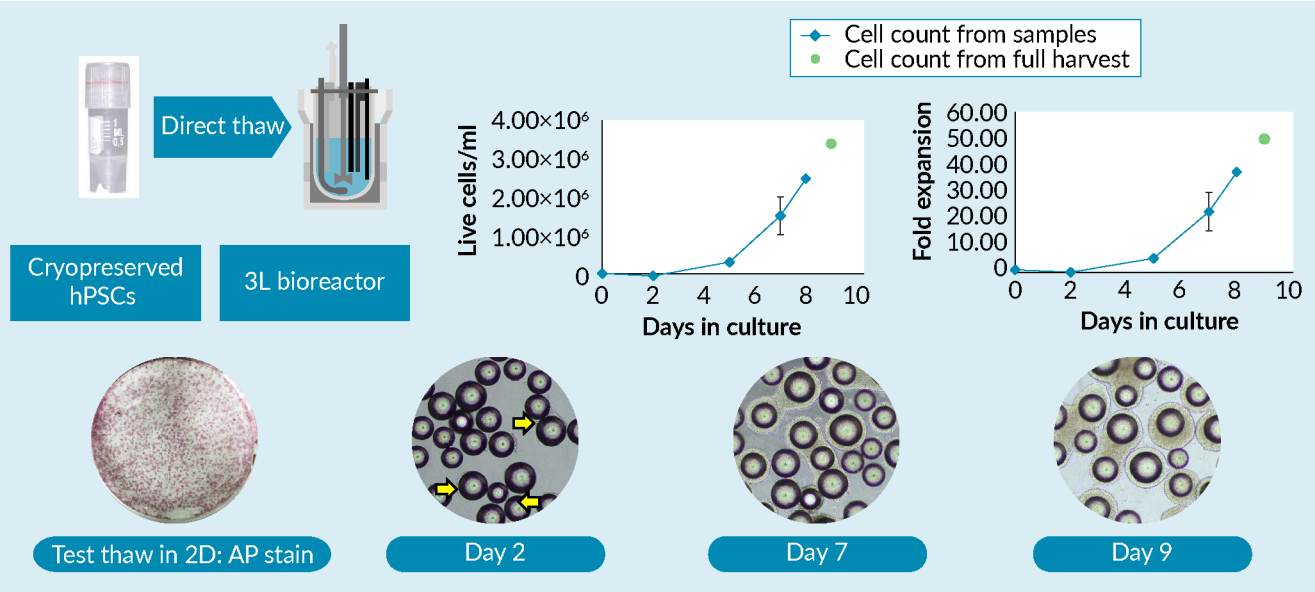
FIGURE 5

Human iPSC large-scale expansion study [4].



stain 2 days post-thaw. Likewise, images of samples from the 3D bioreactor were taken 2, 7, and 9 days post-inoculation. For additional details on Lonza's platform for iPSC expansion, refer to [2].

Case study 3: allogeneic T cell manufacture

T cells are required in large cell quantities for immuno-oncology applications. Lonza's end-to-end platform for T cell manufacturing uses a scalable, closed, and automated stirred tank bioreactor. The key steps of T cell manufacturing – activation, expansion, and selection – are performed inside the bioreactor relieving the need for additional unit operations and ensuring cells are kept under optimal culture conditions. The process is automated and media is changed through perfusion.

T cell viability remains high throughout the run with cell density reaching 35×10^6 cells/mL on day 14. Expanded T cells were characterized by stemness markers as well as for senescence and exhaustion markers. Low levels of senescence and exhaustion markers were found, indicating that T cells are actively expanding and have good potency potential. High levels of central memory and memory stem cells were also found, indicating that T cells have the potential for long-term persistence in patients.

In-vessel magnetic selection enables the T cells to be kept in their optimal culture

TABLE 1

In-vessel magnetic selection performed for CD4+ cells.

Parameter	Run 1 Low % of CD4⁺ cell		Run 2 High % of CD4+ cells	
	Before CD4 depletion	After CD4 depletion	Before CD4 depletion	After CD4 depletion
CD4+ %	17	0	52.4	1.64
Depletion (%)		> 99		96.87
CD8+ %	80	97	39.8	85.4

conditions throughout selection to preserve cell quality. Table 1 outlines the efficiency of in-vessel magnetic cell selection in two runs before and after $CD4^+$ cell depletion. For additional details on Lonza's platform for allogeneic T cells, refer to [3].

SUMMARY

In summary, a scale-appropriate model for the rapid initiation of allogeneic cell therapy clinical trials and transition into large-scale when needed is necessary to enable the field to fulfill its potential in treating a large number of patients. Lonza's allogeneic cell and gene therapy solution is designed to enable scalable and commercially viable manufacturing processes. As an established partner supporting three commercial cell and gene therapies, Lonza has the cell therapy industry expertise to offer:

- A scale-appropriate model to start clinical trials early, aiding a smooth transition into a larger process, when the need comes;
- A robust and commercially viable process with reliable analytics defined early on to enable adequate supply to treat large patient populations;
- The right partnership to guide and de-risk your path through the journal from early stage to commercial manufacture.

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Q&A



Here, **Marcos Langtry,** Director, Commercial Development, Allogeneic Cell and Gene Therapy, Lonza, **Krishna Panchalingam,** Associate Director, Development Services, Cell and Gene Therapy, Lonza & **Inbar Friedrich Ben-Nun,** Director, Research and Development, Cell and Gene Therapy, Lonza (pictured from left to right) answer your questions about the scale-up and delivery of allogeneic cell therapies to large patient populations.

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Q Can a therapeutic company approach Lonza for only PD work with no manufacturing commitment, and how is this handled?

ML: We can do PD work alone. We have recently expanded our labs both in Geleen and in Houston to be able to cater to this demand. We want to enable people to move forward to the next milestone, and to enable this we have launched an on-demand PD service. The intention is to offer first-class expertise to companies so that they can benefit from it. We have developed a series of simple, standard documentation to help with this. We have a seasoned PD team and would encourage companies to utilize this expertise.

KP: We offer PD services separate from our manufacturing services. As an example, this could be the non-GMP generation of material for R&D studies or pre-clinical work. We can also go through media development, process optimization, and scale-up modeling. On the assay side, we could also support routine testing using either our predefined assays or performing development/optimization of your own assays prior to testing. We are flexible and open to collaboration.

Q Directed differentiation of iPSCs might require single cells or cell aggregates as starting material. How would cells expanded in the micro carrier-based expansion platform be used for those processes?

IFB: When iPSCs are cultured on plastic microcarriers, we show that the cells are expanding well and that there is an option to release them from the microcarriers to achieve single-cell suspension. Those cells could be taken to the next step for differentiation. You have the choice of differentiating the cells on the microcarriers or for processes that necessitate single-cell suspension as a starting point, this can be enabled by releasing the cells from the microcarriers.

Q Can you expand on the complexities of handling tissue acquisition for a cell therapy product that will be sold globally?

ML: We now see more companies going global and needing to meet global requirements. This brings the challenge of complying with several regulatory agency guidance. Global intended donors must comply with all, sometimes conflicting, regulatory standards. Examples include the EMA requiring CE-marked tests, the FDA requiring Clinical Laboratory Improvement Amendments (CLIA) certified tests, the FDA excluding donors who spent more than 5 years in Europe from 1980, or the Pharmaceuticals and Medical Devices Agency specific donor re-testing requirements. Therefore, you need to find a way to comply with all. This is an intriguing field, and you need a seasoned team with the right level of expertise to make the right decisions to enable a successful BLA.

Q How would the in-vessel magnetic selection be used in manufacturing?

IFB: We showed the use of the in-vessel magnetic selection for T cell manufacturing. It can also be applied to other cell types. It can be used to negatively select T cells that still retain their original T cell receptor post-gene editing, eliminating the cells in which the knockout did not work. Another example would be generating T cells from iPSCs and removing undesired or unpreferred T cell populations. This was shown through the example of selecting out $CD4^+$ cells, thus enriching the $CD8^+$ cell population.

Q What is Lonza's viewpoint on the biggest analytical roadblock to successfully transferring cell and gene therapy processes from the lab to the market?

KP: There are several roadblocks to discuss. First, having well-defined assays to characterize your product and understand the CQAs can be an issue. This includes having appropriate potency assays to assess the potency of a product *in vitro* that would have the same mechanism of action *in vivo*. We work with all our clients to support their development of proper potency assays that also consider the maturing regulatory viewpoint. In addition, automation can be a roadblock. As we move towards large-scale processes and commercial applications with multiple batches being generated in a short amount of time, we need to ensure we have consistent testing of the product and in-process characteristics. This is where automation could be valuable.

Q How does Lonza approach process characterization limit evaluation studies for large-scale allogeneic processes?

KP: The first step is to work through an FMEA. In this, we look at interrogating the production process and analytical methods around a certain product and understanding the process parameters that impact the CQAs and the therapeutic product profile. From this, we then go through a risk assessment to identify the potential CPPs. We look to then develop a scale-down model for performing process characterization studies on a smaller scale in which we perform DoE experiments. This enables us to have a better understanding of the actual design space in which our manufacturing process needs to stay within to ensure the generation of a consistent product that meets the CQAs.

BIOGRAPHIES

MARCOS LANGTRY is Director Commercial Development, Allogeneic Cell Therapy at Lonza. He is responsible for the allogeneic commercial strategy, current and future offerings, and

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portfolio optimization. Before joining Lonza, Marcos Langtry held several senior level positions with TiGenix, Astra Zeneca and Sanofi in the areas of external manufacturing, process development, technical operations and industrial strategy; where he has acquired extensive experience with aseptically filled, biologic and cell therapy products.

KRISHNA PANCHALINGAM is an Associate Director of Process Development, Cell and Gene Technologies at Lonza, focusing on the development of cell and gene therapies for clinical and commercial applications. He has over 16 years of experience in stem cell bioengineering, bioprocessing and in the cell and gene therapy (C>) field. Dr Panchalingam holds a PhD degree in Chemical Engineering from the University of Calgary (Calgary, Canada) where he focused on the development of viable, scalable, and defined bioprocesses in the field of regenerative medicine.

INBAR FRIEDRICH BEN-NUN is Director of Research and Development, Cell Therapy at Lonza. She has joined Lonza 10 years ago as a Scientist, after completing post-doctoral research at the Scripps Research Institute, La Jolla, where she obtained an extensive experience with iPSCs. In her current role as a R&D Director at Lonza, Inbar is responsible for driving and executing innovating solutions for allogeneic cell therapy processes and platforms.

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AUTHORSHIP & CONFLICT OF INTEREST

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Development of anti-AAV assay for detection of pre-existing AAV immunity

John Chappell & Zhe Liu

The high natural prevalence of AAV wild-type viruses has resulted in a high frequency of capsid-directed humoral immunity in human populations and many animal models, which may interfere with the effectiveness of AAV-based gene therapies if not identified and managed properly. This case study describes the development of an anti-AAV antibody assay for use in preclinical studies that involve cynomolgus monkeys. This will allow the ability to screen for pre-existing AAV immunity and identify potential low-responding individuals to increase the likelihood of successful transduction and to maximize the utility of the preclinical investigations.

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AAV & RECOMBINANT VECTORS

AAV belongs to the parvovirus family and is a small non-enveloped virus with an icosahedral capsid and a single-stranded DNA genome. To date, 13 serotypes have been identified with different tissue tropisms, which can transduce a wide variety of dividing and nondividing cells in mammalian hosts. As the name indicates, AAV is replication-deficient and dependent on helper viruses such as adenovirus or herpes simplex virus to replicate and complete its life-cycle. The virus infects humans but does not cause any known disease.

Easily modified into vector delivery systems, recombinant adeno associated virus (AAV) has become a popular and promising tool for gene therapy. Advantages include minimal vector-related toxicities and long-term gene expression—up to several years. Approved products are already on the market using different AAV vectors to treat genetic diseases of the skeletal muscle, retina, and central nervous system, with many more in preclinical and clinical development.

Many humans and animal models have already been exposed to AAV and developed capsid-directed humoral immunity.

This creates a challenge for AAV-based gene therapies since pre-existing neutralizing antibodies (NAbs) to the wild-type virus can block vector transduction and gene delivery and activated immune memory may destroy the transduced cells. Treatment-emergent or boosted anti-drug antibodies (ADAs) can result in failure of AAV re-administration and loss of efficacy over time.

CASE STUDY: DETECTING PRE-EXISTING AAV IMMUNITY IN CYNOMOLGUS MONKEYS

The goal of the current study was to develop an assay to detect antibodies to AAV in serum to support a preclinical trial of an AAV-based gene therapy in cynomolgus monkeys (cyno). Using this assay, monkey colonies can be screened for pre-existing AAV immunity, and negative or low-responding animals selected for the trial, to maximize the likelihood of successful transduction. For this purpose, the assay must be quick, simple, sensitive, and robust.

A ligand-binding assay for total antibodies (TAbs) was selected over cell-based assays for NAbs. The presence of ADA, regardless of neutralizing activity, is a biomarker of previous exposure to the virus, and even non-neutralizing ADA could alter biodistribution and accelerate clearance and therefore reduce the biological activity of the vector.

We selected the Gyrolab® immunoassay platform primarily due to its automation, high throughput, and flexible assay design. The small sample volume is particularly well suited to preclinical animal work.

ABOUT GYROLAB TECHNOLOGY

The core of the Gyrolab technology is the compact disc (CD), which is the equivalent of a microtiter plate for an ELISA. The Gyrolab Bioaffy™ CD is made up of highly reproducible microfluidic structures and operates at a nanoliter scale. The microstructure has a defined volume and is fully automated, to avoid human error.

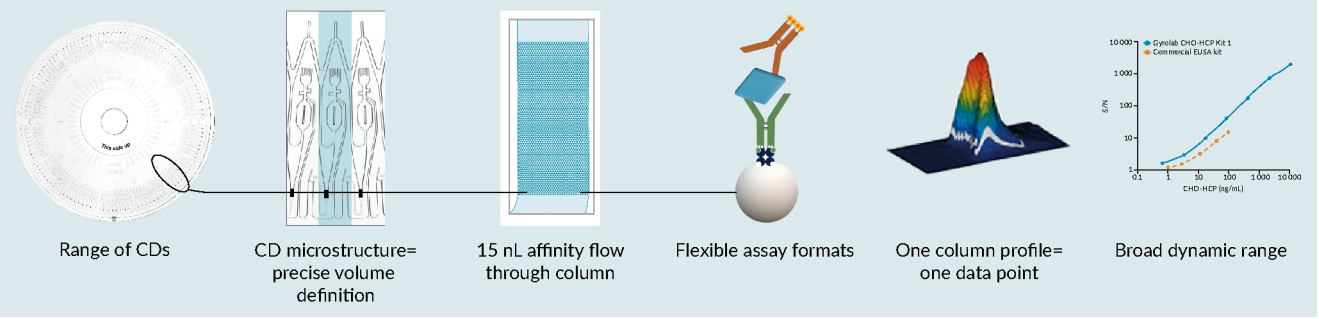
A combination of centrifugal force and capillary action drives movement through the microstructure and into an affinity column below. The 15 nL affinity flow-through column contains streptavidin beads and is the basis for the immunoassay (Figure 1). Various assay formats can be used, including sandwich assays.

Using a fluorophore-detection antibody results in a laser-induced fluorescent peak. There is effectively one column profile per microstructure, which avoids assay drift or other plate or CD effects because everything is processed at the same time. The resulting viewer profile allows interpretation of the binding happening within the column.

Characteristics of the automated Gyrolab immunoassay platform include:

FIGURE 1

Key features of the Gyrolab platform.



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- Fully automated, with minimal hands-on time and fewer errors resulting from manual operations;
- Nanoliter scale, saving both sample volume and reagent volume;
- Uses laser-induced fluorescence allowing a broad dynamic range;
- An open platform with excellent flexibility. Ready-to-use kits or custom-developed assays can be provided, to support applications from discovery to clinical.

Gyrolab assay protocols—pre-qualified methods using commercial reagents—speed up assay development and provide high-quality data.

BRIDGING ASSAY DEVELOPMENT

The project was initiated with a bridging assay, in which virus capsid is biotinylated as capture and Alexa Fluor labeled as detection. Materials and methods are summarized in Table 1.

Briefly, we labeled AAV2 empty capsid in-house with Biotin and Alexa Fluor™ 647 separately. Monoclonal anti-AAV2 antibody was used as a positive control. Conjugated checkerboards performed and both conjugates at one in six dilution were selected for good signal-to-noise ratio and minimizing virus used.

Bioaffy™ 1000 CD was used initially, before changing to the 4000 CD for increased sensitivity. Together with the highest PMT setting of 25%, this gave the optimal response.

Using pooled serum from low-responding cyno, we titrated the positive control antibody at different serum levels. Matrix interference was minor and MRD one in four (25%) was selected.

Serum from 25 cyno individuals was screened in three runs. Results were very consistent, and several high-responding animals were identified, proving the utility of the assay. The same animals were identified as outliers in each run, and all gave very similar cut points (Figure 2 & Table 2).

However, the bridging assay often gave a high coefficient of variability (CV) from a single sample well. CVs higher than 20% were highlighted in red in Table 2. They mostly happened in samples with low signal responses; therefore, for the cut point calculation, high CV samples were not excluded, as to do so would skew the result.

BRIDGING ASSAY CHARACTERIZATION & OPTIMIZATION

Assay specificity was confirmed from capsid depletion of the positive control signal in a dose-dependent manner. A Gyrolab slow spin method further improved assay signal and sensitivity.

With a high prevalence of pre-existing antibodies, it was decided to use capsid inhibition to generate pseudo-negative samples for the cut point. A 1 in 40 dilutions was used for sample inhibition. Signals were well depleted apart from one animal, which likely had too high ADA. Sensitivity was approximately 150 ng/mL using cut point from the pseudo-negative samples.

Again, there were some high CVs, mostly in samples with a low signal response. The slow spin and extra mixing did not fully resolve the issue of high CVs, resulting in the need to test an alternative assay.

TABLE 1

Materials & methods.

AAV2 empty capsids 1013 PP/mL (SIRION BIOTECH GmbH)
In-house Biotin and Alexa Flour™ 647 capsid labeling
Positive control: anti-AAV2 (intact particle) monoclonal antibody, A20 (PROGEN)
Rexxip™ F for all samples and reagent dilution
Checkerboards with both conjugates at one in six dilution (c. 1012 PP/ML nominal) (Figure 2)
Bioaffy™ 1000 CD, PMT=25%

FIGURE 2

Bridging assay profiles for individual animals in runs 5–7.

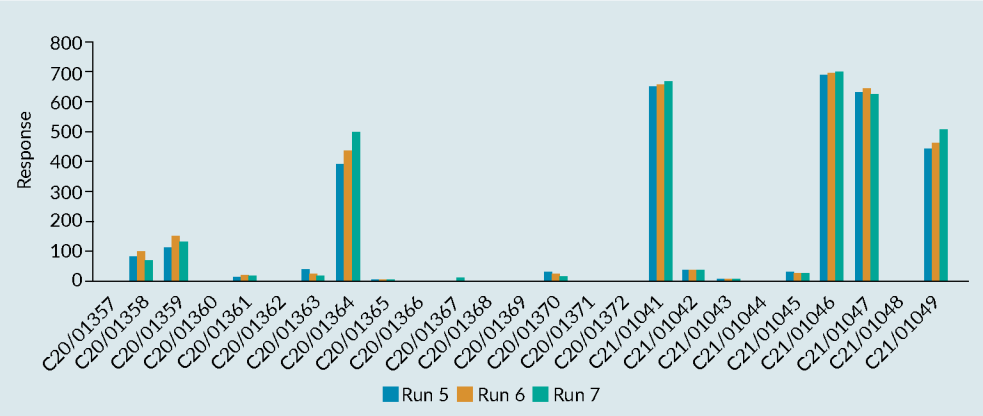

TABLE 2

Bridging assay results for runs 5–7.

Sample ID	Run 5		Run 6		Run 7	
	Mean	%CV	Mean	%CV	Mean	%CV
C20/01357	2.200	42.6	2.302	6.0	5.070	29.5
C20/01358	86.486*	14.1	102.447*	6.1	76.592*	1.8
C20/01359	115.964*	7.0	152.503*	12.3	136.123*	5.9
C20/01360	3.169	37.4	2.595	14.5	5.014	13.9
C20/01361	18.161	14.2	19.719	5.1	20.213	3.3
C20/01362	2.176	224.0	2.227	51.2	3.860	26.2
C20/01363	42.258	28.9	27.917	1.3	26.806	20.3
C20/01364	400.437*	9.4	446.359*	0.4	507.687*	1.4
C20/01365	10.021	14.1	9.673	6.4	9.480	6.4
C20/01366	0.757	28.9	1.485	64.7	2.129	55.1
C20/01367	2.193	11.4	1.648	4.4	13.745	91.0
C20/01368	5.243	13.5	4.739	1.3	5.409	11.7
C20/01369	1.885	7.5	2.670	18.5	2.204	16.7
C20/01370	33.604	7.6	28.0048	6.2	20.951	0.8
C20/01371	2.969	17.7	2.745	25.0	6.131	11.8
C20/01372	2.861	1.4	2.962	3.4	4.422	6.8
C21/01041	665.382*	1.0	671.207*	1.1	685.726*	1.8
C21/01042	42.937	11.7	41.725	5.8	44.956	7.5
C21/01043	13.465	22.2	12.873	3.2	13.264	1.7
C21/01044	2.392	14.5	1.190	12.4	3.576	68.8
C21/01045	35.752	8.4	32.695	5.0	32.229	11.5
C21/01046	706.104*	1.0	715.975*	1.1	719.144*	0.7
C21/01047	647.474*	1.1	656.058*	2.4	641.373*	2.9
C21/01048	2.154	9.2	1.667	1,6	1.821	96.7
C21/01049	454.426*	4.3	472.272*	3.5	521.615*	1.6
Mean	12.455		11.049		12.294	
SD	15.222		13.058		12.218	
Cut point	59.5		51.4		50.0	

*Outlier

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GENERIC ASSAY DEVELOPMENT

Next, a generic assay was developed, with the ability to easily screen ADA to different AAV serotypes without capsid labeling. The Biotin pan anti-AAV capture antibody was used, which binds all AAV serotypes except AAV9.

The anti-AAV2 positive control antibody from the bridging assay was tested using anti-mouse IgG detection and Bioaffy™ 1000 CD. The results showed that the generic format worked well and the overlap of the matrix curve with the buffer curve indicated that cyno serum does not interfere with mouse detection (Figure 3).

With no cyno anti-AAV positive control antibody available, the cyno serum from the bridging assay was used to develop the method. For the detection, three commercial anti-monkey antibodies were tested. However, the negative control serum showed a higher instrument response than the positive sample. Another experiment with different serum samples confirmed the issue. This was caused by nonspecific binding of the capture antibody to the cyno serum components, which was subsequently picked up by the anti-monkey IgG detection.

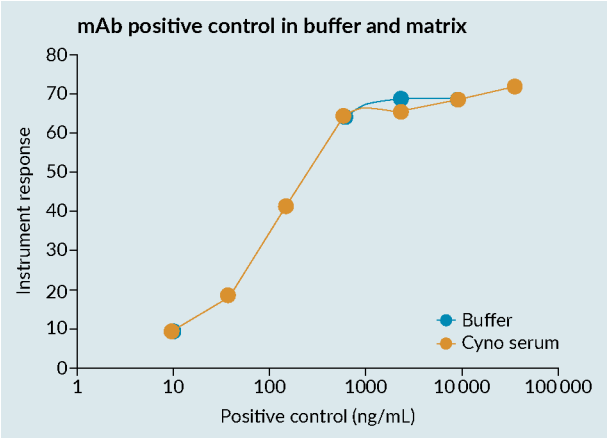
GENERIC ASSAY CHARACTERIZATION & OPTIMIZATION

To overcome the problem of high signals for negative samples, blocking using anti-camelid antibodies or unlabeled camelid capture, or even sample extraction was considered. However, this would add additional steps to the process and be unlikely to work with such a large background.

Instead, two measurements were run in parallel for each sample. In addition to the anti-capsid measurement, a second measurement was made in which the capsid was removed, so that only the non-specific signal was measured. By subtracting the nonspecific signal from the total measurement, a specific anti-capsid signal was obtained.

FIGURE 3

mAb positive control in buffer and matrix.



After the background signal was subtracted, the negative control serum responses were very low, while positive control responses remained high.

The following parameters were selected for further experiments:

- Capsid 1 in 5 dilution
- MRD 1 in 4
- Detection antibody 25 nM
- PMT 5%.

Serum from the same 25 cyno individuals used earlier was analyzed with the generic assay, with a similar profile to the bridging assay (Figure 4). There was good inter-assay consistency, and the generic assay did not suffer from the high CVs seen with the bridging assay.

With the pre-existing antibodies, the cut point resulting from individuals was too high to safely determine the negative animals. Using the variation of negative control response, the cut point was more conservative.

For future experiments, a cut point control (CPC) will be used for this assay at a positive control dilution of 1 in 2000. The CPC will give a more consistent result than the negative control and will be run alongside the samples.

Samples with results at or above CPC will be deemed positive, and below, negative.

To verify the first generic assay, a second serotype (AAV8) was analyzed (Figure 5). Cyno are natural hosts of AAV8 and, similar to AAV2, there was a spread of responses across individuals. Some respond to a single serotype, whereas others respond to both or neither.

Matrix positive control titration showed good intra- and inter-assay consistency. Similar to the anti-AAV2 assay, a CPC at the positive control dilution of 1 in 2000 could be used to define positive and negative samples.

Testing for specificity revealed that AAV8 inhibits the AAV8 ADA response and to a lesser extent AAV2 inhibits this response as well, illustrating anti-AAV antibodies' cross-reactivity to different serotypes. Further research will be needed to assess the cross-reactivity of the anti-AAV8 antibodies.

CONCLUSION

The bridging anti-AAV2 assay had a sensitivity of approximately 150 ng/mL and was deemed specific and day-to-day reproducible. There were issues with poor replicate % CV, albeit mainly in low responders.

The generic anti-AAV assay removed the issue of high CVs seen with the bridging assay. Non-specific reactivity between serum and capture reagent was resolved by subtracting the signal of a capsid unloaded column. The assay was deemed specific, and day-to-day reproducible. Without a characterized cyno-positive control, it was not possible to report sensitivity. However, the matrix-screening profile and capsid inhibition results correlated well with the bridging assay, suggesting a sensitive generic assay that is fit for purpose. The generic assay format has been tested and confirmed on both AAV2 and AAV8 stereotypes.

FIGURE 4

Comparison of generic and bridging assay matrix profiles.

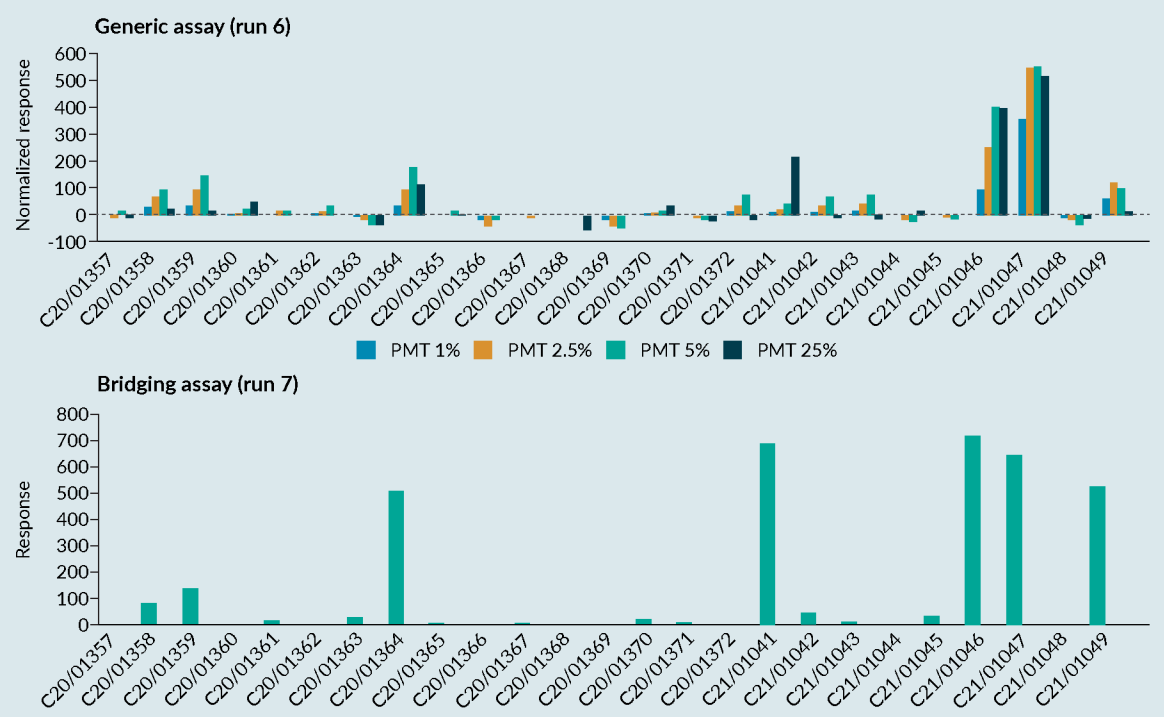


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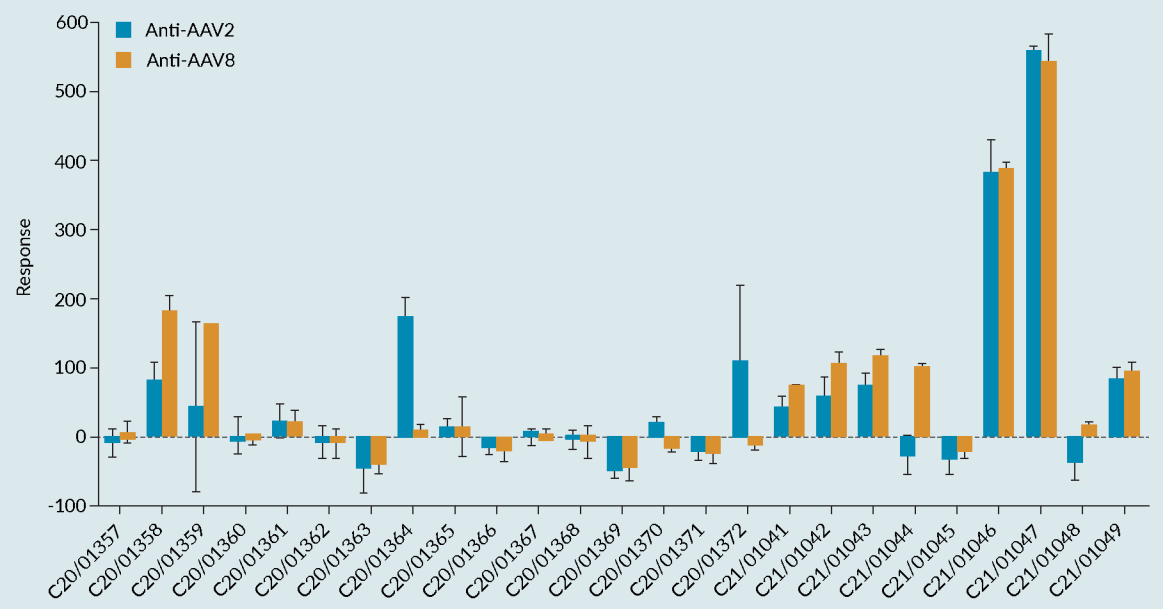
FIGURE 5

Responses for anti-AAV2 versus anti-AAV8.



ASK THE AUTHORS

Here, **Zhe Liu,** Staff Scientist, Labcorp Early Development, and **John Chappell,** Director of Scientific Support, EMEA and Asia Pacific, Gyros Protein Technologies, answer your questions about anti-AAV assays.

Q For the bridging assay format, there's a requirement to specifically label the capsid. Can you comment on the process for labeling, and did you encounter any difficulties?

ZL: We used a commercial kit for labeling. Gyros Protein Technologies were able to share some relevant data with us, so we had very few difficulties.

JC: We had a labeling procedure based on internal work, so we were able to recommend a labeling protocol based on the capsid concentration.

Q Why did Labcorp select Gyrolab for the work?

ZL: For this particular project, we selected Gyrolab primarily due to the automation. The Gyrolab also provided a quick turnaround time, and small sample and capsid volumes so you can better optimize the assay with the reagent available. We also found good matrix tolerance because of the flow-through system, allowing shorter assay incubation.

Q How do you add a small volume of sample?

JC: The Gyrolab Bioaffy CDs use nanoliter volumes of sample, but you need to load excess sample into a microtiter plate that you place in the instrument. For example, for the 200 nL compact disc (CD), you need to add approximately 4 µL of sample into the microtiter plate. Then the instrument itself transfers the sample from the microtiter plate well to the Gyrolab Bioaffy CD. As it adds sample to the Gyrolab Bioaffy CD, the volume is defined within the Gyrolab Bioaffy CD, and excess sample moves into the overflow part of the microstructure. The volume is completely controlled within the Gyrolab Bioaffy CD itself. The Gyrolab Software will tell you when you set your run-up how much volume you need to add to your plate.

Q Can we analyze crude lysate from upstream with your assay for full capsid detection in AAV production?

JC: Yes, this assay can be used with lysate. The presentation today was based on anti-adeno associated virus (AAV) in serum samples, but we also have an assay kit for measuring AAVX or AAV9 titer. The AAVX titer kit can measure all serotypes apart from AAV9 and is specifically designed to support the measurement of AAV titer in upstream and downstream processing. Depending on the amount of cell debris, you may need to carry out another treatment of the sample, but ultimately, you can measure samples from upstream processing.

Q Can you comment on the capability of the generic assay format for clinical assays?

ZL: The generic assay can be used for clinical analysis, and we are currently planning such an application.

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Q Is there a correlation between TAb and NAb titer?

ZL: For this project, we haven't analyzed our final matrix sample in a neutralizing antibodies (NAb) assay because we appropriate it for screening the total. However, there is plenty of evidence in the literature to show the high concordance between the ligand-binding assay total antibodies (TAb) and NAb for multiple serotypes in both non-human primate and human samples.

Q What did you use for the negative control serum?

ZL: We screened cyno individuals from in-house and commercial sources and selected those that were negative for signal response, Ig depletion, and capsid depletion. We made a natural pool from those individuals.

Q When you see positive samples from one serotype, do you observe that they will cross-react with other serotypes?

ZL: Yes, we did see anti-drug antibodies (ADA) cross-reactivity between the AAV2 and AAV8, the two serotypes we tested. Some animals are positive in both serotypes, especially very high responders. This is understandable because of the substantial capsid structure homology.

Q In the AAV titer kit, is there a calibrator included?

JC: In general, we do not supply a standard with the kit. The kit is designed for customers to use with their specific standard. However, if the customer needs to source a standard, we can make a recommendation.

Q Can you discern whether antibodies are neutralizing via the TAb assay only, or does one also need to perform a transduction inhibition assay?

ZL: It depends on what you need. The NAb is a proportion of the TAb. If the animal has a very high response in the total assay, it is more likely to be positive in the NAb assay.

Q When using the generic assay format, is there a requirement to use the specific customer vector or would general serotype control be sufficient?

ZL: The ease of using specific customer vectors is one advantage of this generic assay format. A new capsid can be easily plugged in as you don't need a label or any other preparation.

The use of a general serotype control may not be ideal and could be a compromised approach because the gene therapy vector and the commercial vector are not exactly the same. They may have different antigen epitopes and different manufacturing conditions, so if possible, we should use the same vector in the assay as in the real clinical and preclinical studies, so the ADA detected will be more relevant and complete, and no bridging activity is needed.

Q The background phenomenon with samples is interesting. What do you think is the cause of that and are there any potential solutions?

ZL: We consider that the background comes from the nonspecific binding of this pan anti-AAV capture antibody to the cyno serum component—most likely the serum Ig. We considered different blocking approaches, but these did not look very promising, so we decided to use a background subtraction, which worked pretty well.

BIOGRAPHIES

ZHE LIU is a Staff Scientist at Labcorp Early Development. Zhe received her PhD in neurobiology from King's College London in 2002. She joined Labcorp Early Development at the Huntingdon site as a Staff Scientist in April 2020. Zhe is a subject matter expert for immunoassay and performs development and validation work. She has extensive laboratory experience in cell-based assay, immunoassay, and flow cytometry in both academia and industry. In particular, she has been working in the CRO sector for 15 years with substantial experience in assay development, validation, and sample analysis of ADA, PK, biomarkers, and potency.

JOHN CHAPPELL is Director of Scientific Support, EMEA and Asia Pacific at Gyros Protein Technologies AB. John has approximately 25 years of experience in the Contract Research industry supporting both preclinical and clinical drug development. He has specialized in supporting biological compounds from an analytical perspective e.g., PK, immunogenicity, and biomarker analysis. He is particularly interested in validation requirements and ensuring that the data generated will be acceptable to the regulatory authorities. He now leads the Application Support and Service teams for Gyros Protein Technologies.

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GYROS PROTEIN Technologies

AUTHORSHIP & CONFLICT OF INTEREST

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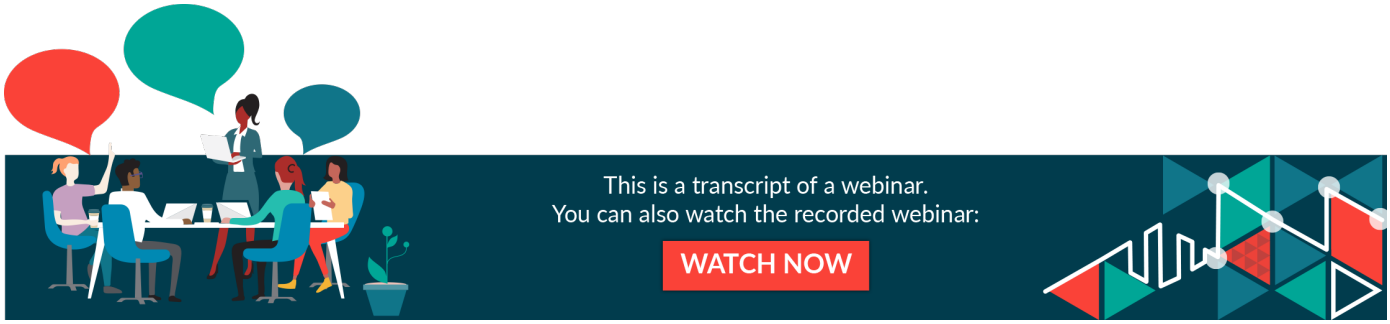

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