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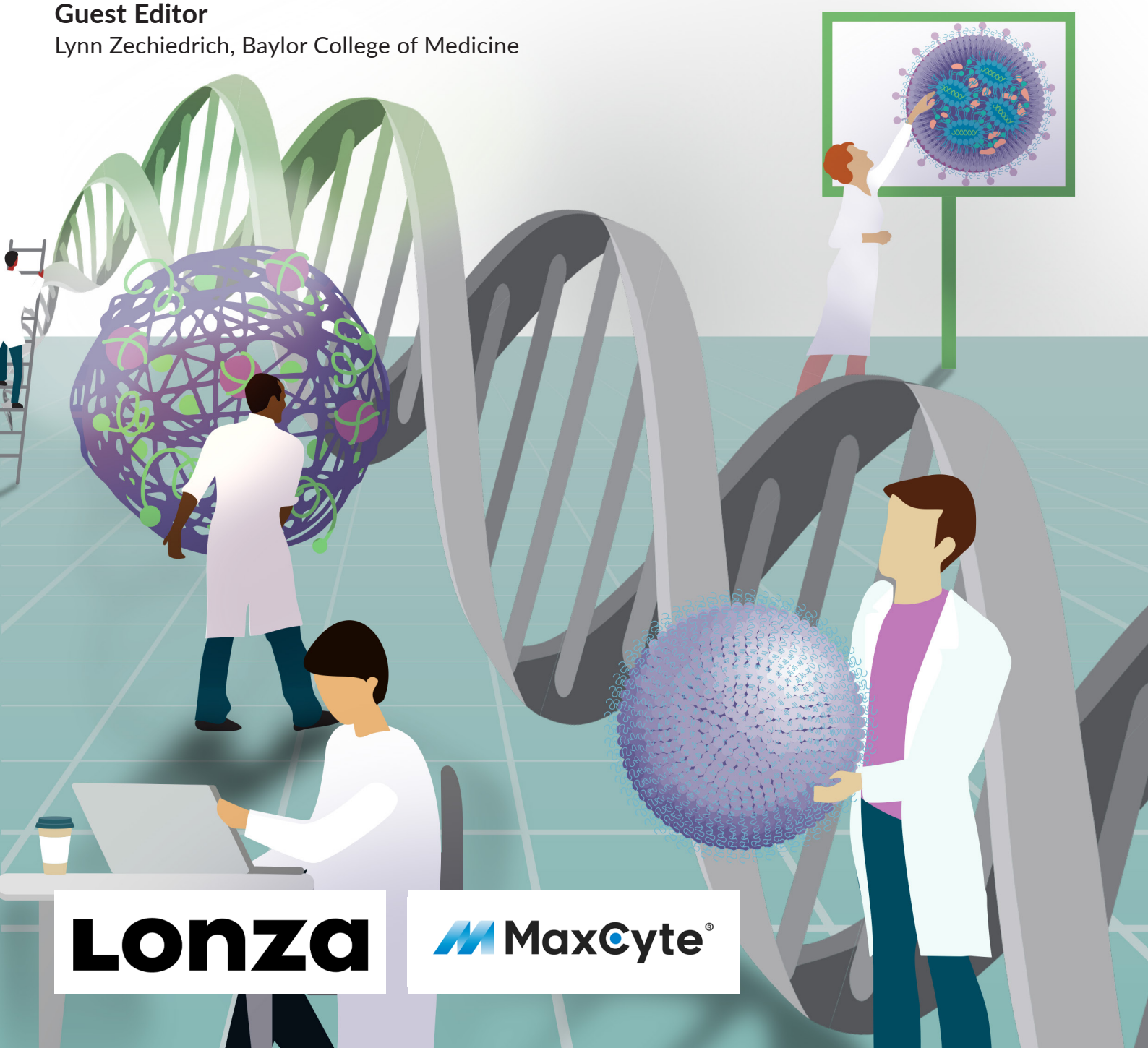
CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON
Gene delivery platform evolution part 2: non-viral

Guest Editor
Lynn Zechiedrich, Baylor College of Medicine



CELL & GENE THERAPY INSIGHTS

Volume 9, Issue 5

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION PART 2: NON-VIRAL

SPOTLIGHT

FOREWORD

Lynn Zechiedrich

"The huge success in saving lives, reducing disease burden, and lessening disease symptoms has bolstered interest and confidence in, and loosened purse strings for, funding of nucleic acid-delivered therapy."

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Many of the 5.55 billion people who received more than 10 billion doses of COVID-19 vaccine [1] were injected with mRNA, hailing in a new-generation vaccine with adaptability to other pathogens and remarkable speed to therapy. The huge success in saving lives, reducing disease burden, and lessening disease symptoms has bolstered interest and confidence in, and loosened purse strings for, funding of nucleic acid-delivered therapy.

To catch up with the latest advances in the rapidly changing research environment of non-viral gene therapy, we solicited reviews from academic and industry leaders in the field. Most were willing to share their views and ideas in either a review or interview format. The results that follow illustrate the wide range of non-viral delivery platforms and the myriad possible applications across cell and gene therapy. Each vector and method in development has promising utility.

In the opening review, Professor Duarte Miguel F Prazeres reminds us that despite so much advancement and promise in the development of alternatives, there remains a major need for DNA plasmids across the advanced therapies space. Plasmids likely will have a place in the field for a long time to come. From

the cellular immunotherapy arena, several interesting interviews follow. Dr Adrian Bot of Capstan Therapeutics discusses developments in lipid nanoparticles for CAR-T cell therapies, Seattle Children's Research Institute's Professor Carol H Miao provides updates on advancing 'doggybone DNA' for treating hemophilia, Professor Uta Griesenbach, from Imperial College London, shares progress in tackling cystic fibrosis with gene therapy approaches, and, together, Drs Julie Shi and Nirveek Bhattacharjee from Bristol Myers Squibb, review their non-viral *ex vivo* approaches to T cell therapy.

The passion and excitement these scientists have for their work improving cell and gene therapy shines through the series. The reader likely cannot help but feel optimistic. Another fact is inescapable—there remains much work to be done. And we are going to need multiple different nucleic acid vectors and thoughtfully designed delivery systems to reach more than a few disease conditions with gene therapy. Learning from one another—both our successes and our failures—is critical for future success. In that spirit, I hope you enjoy this collection of perspectives and opinions.

BIOGRAPHY

LYNN ZECHIEDRICH is the Kyle and Josephine Morrow Chair and Professor in Microbiology at Baylor College of Medicine. She developed minivectors to study DNA function, the enzymes that act on DNA, and the antibiotic and anticancer drugs that inhibit those enzymes. Minivectors also proved to be excellent gene therapy delivery vectors. She holds multiple patents that are licensed to Twister Biotech, Inc., a company she founded in 2011, and has multiple patents pending. Among other honors, she is a Fellow of the National Academy of Inventors and a Fellow of the American Association for the Advancement of Science. At her college, she received the BRASS mentor of the year award, a Woman of Excellence Award, and the Barbara and Corbin J Robertson Jr Presidential Award for Excellence in Education.

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION PART 2: NON-VIRAL

SPOTLIGHT

COMMENTARY

The supporting role of plasmids in gene & cell therapy

Duarte Miguel Prazeres

The biopharmaceutical relevance of producing plasmid DNA at large scale has increased steadily over the years due to the development of a growing number of direct and indirect applications. Be it as biological drugs or as starting materials, plasmids are pervasive across the gene and cell therapy industry of today. With hundreds of biopharmaceutical companies using plasmids in the clinical development of their products, plasmid manufacturing is starting to emerge as a key bottleneck. This commentary provides an overview of the uses of plasmids, discusses manufacturing challenges, and hints at what the future may bring.

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PLASMID AS BIOLOGICAL DRUGS

The rapid growth of the gene and cell therapy industry of the last years dramatically increased the demand for plasmid DNA [1–3]. Plasmids are used to directly deliver genetic information or genes that code for therapeutic proteins, RNA, or antigens to the target cells of patients (Figure 1A and Table 1). Moreover, plasmids are also used as a vehicle to deliver the molecular components of gene editor systems (e.g., editing enzymes, RNA guides) including clustered regularly interspaced short palindromic repeat DNA sequences (CRISPR), zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) [4]. In such *in vivo* uses, the appropriate plasmids are combined with other components (e.g., adjuvants, lipids, etc.) to generate a medicinal product that is transferred to patients [2]. In these applications, plasmids are biological therapeutics, which must be manufactured under current good manufacturing practices (cGMP), regulated, tested, and controlled appropriately [2].

PLASMIDS AS STARTING MATERIALS

Apart from their role as biologicals, plasmids play a supporting role as sophisticated starting materials in the context of the

manufacturing of engineered cell products (Figure 1Bi & Table 1) or of other biologicals (Figure 1Bii & Table 1). For example, plasmids are used as an alternative to viral vectors to genetically modify cells extracted from the patient or donor *ex vivo*, in the context of chimeric antigen receptor T cell (CAR-T) therapies [5], genome editing approaches [4], or mesenchymal stem cells therapies [6]. The first case entails the transfection of a patient's T cells with a plasmid system (e.g., coding for CAR genes, transposases, etc.) with the goal of attaining stable gene transfer, integration, and expression of CARs [5]. In the second case, plasmids are used to deliver the molecular components of gene editors like CRISPR, TALEN, or ZFN [4]. In either case, the plasmid-modified cells are infused back into patients. Finally, plasmids are also used to modify mesenchymal stem cells, for example with the goal of enhancing their therapeutic function *in vivo* [6].

Plasmids are also required in the manufacturing of viral vectors and mRNA, which can then be used either as biologicals on their own (Figure 1Bii), or as reagents for the *ex vivo* modification of a patient´s cells (Figure 1C & Table 1). For example, many

FIGURE 1

Direct and indirect applications of plasmid DNA in gene and cell therapies. Plasmids can be used as (A) biological drugs, e.g., as DNA vaccines or as components of *in vivo* non-viral gene therapy/editing platforms, or as (B) starting materials for (Bi) the *ex vivo* genetic engineering of cells (e.g., CAR-T cells, CRISPR-edited cells, etc.) or for (Bii) the manufacturing of viral vectors, mRNA and eventually RNA biologicals. The later can then be used either as biologicals on their own (Bii), or (C) as starting materials for the *ex vivo* modification of a patient's cells.

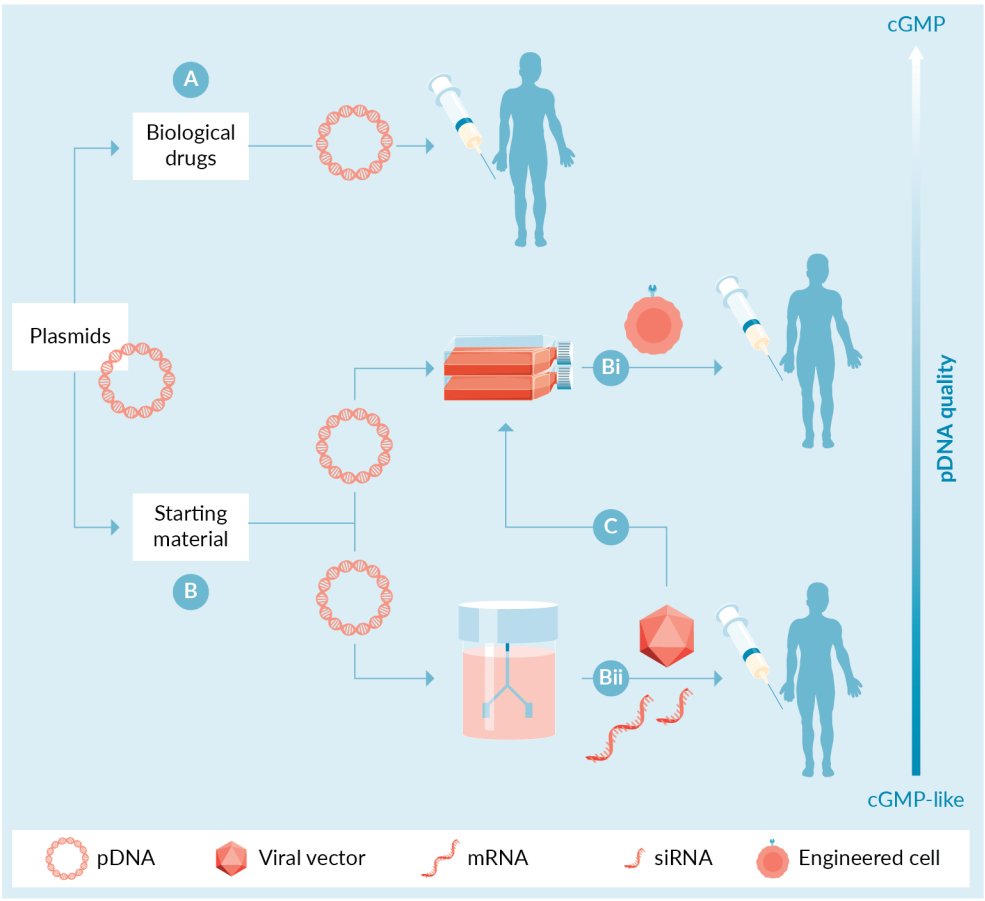

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TABLE 1

Overview of the uses of plasmids as biological drugs and starting materials* in gene and cell therapy.

Plasmid biologicals	Role of plasmids
Gene therapy, gene editing, and DNA vaccines	Plasmids carry genes that code for therapeutic proteins, mRNA, components of gene editing platforms (e.g., editing enzymes, RNA guides) or antigenic proteins. They are combined with other components (e.g., adjuvants, lipids, etc.) to generate a medicinal product that is directly administered to patients. cGMP grade is mandatory.
Plasmid starting materials	**Role of plasmids**
Ex vivo engineering of cells	Plasmids carrying genes that code for elements like proteins, components of gene editing platforms (e.g., editing enzymes, RNA guides) or mRNA are used to genetically engineer cells *ex vivo* (e.g., mesenchymal stem cells, CAR-T cells, CRISPR-edited cells, etc.), which are subsequently infused into patients. cGMP-like grade is required.
Manufacturing of biologicals	Plasmids carry genes that code for elements required to manufacture GMP-grade medicinal products like viral vectors (e.g., AAVs, LVs) or mRNA, which are subsequently administered to patients. cGMP-like grade is required.
Manufacturing of starting materials for *ex vivo* cell modification	Plasmids carry genes that code for elements which are required to manufacture starting materials like viral vectors, mRNA, minicircles, minivectors, and nanoplasmids. These starting materials are subsequently used to genetically engineer cells *ex vivo* (e.g., mesenchymal stem cells, CAR-T cells, CRISPR-edited cells, etc.), which are subsequently infused into patients. cGMP-like grade is required.

*Starting materials encompass all the materials from which active substances are manufactured.
AAVs: Adeno-associated viruses; CAR-T: Chimeric antigen receptor T cells; cGMP: Current good manufacturing practices; CRISP: Clustered regularly interspaced short palindromic repeats; GMP: Good manufacturing practice; LVs: Lentiviruses.

adeno-associated viral (AAV) and lentiviral (LV) vectors are produced by using multiple plasmids to transiently transfect producer cells such as human embryonic kidney (HEK) 293T cells [7]. As a case in point, the manufacturing of AAV particles rely on the use of three different plasmids—an AAV transfer plasmid with the gene of interest flanked by two inverted terminal repeats (ITRs), one plasmid containing AAV genes, and a helper plasmid encoding adenovirus helper genes [7,8]. Likewise, LV manufacturing by transient transfection of cells also requires the use of three or four distinct plasmids [9,10]. The resulting viral vectors can then be administered to patients or used to transduce cells *ex vivo*.

The emergence of mRNA vaccines, which was spurred by the Covid19 crisis, also created a new utility and surge for plasmids [3]. In the context of mRNA technologies, plasmids are extensively used to generate the templates required for the *in vitro* transcription (IVT) reactions that generate mRNA [11,12]. Such templates are most often produced by enzymatic linearization of a purified plasmid or by amplification of the region of interest in that plasmid using PCR [12,13]. The mRNA products resulting from the IVT are then processed and purified further, up to a stage where they can be transferred to patients, e.g., in the context of mRNA vaccination or genome editing (Figure 1Bii and Table 1). Furthermore, mRNA products can be used to modify or edit cells *ex vivo* (Figure 1C and Table 1).

One can also foresee that plasmids and IVT strategies may come to play a more significant role in the manufacturing of small RNA molecules such as antisense oligonucleotides, RNA guides, or double-stranded RNA used in the context of siRNA products [14,15]. At present, solid-phase chemical synthesis, which can generate RNAs up to 50–100 nt in length, is the preferred method for the synthesis of most oligonucleotide-based drugs because of its cost-effectiveness, automated protocols, and remarkably short synthesis cycle times [16]. Nevertheless, IVT, which is widely used to synthesize RNA molecules for structural studies and basic RNA biology (e.g., splicing,

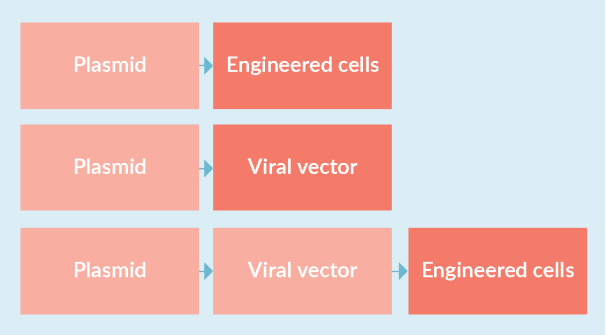
riboswitches, CRISPR, lncRNA), may become an attractive alternative in this context. If this ever comes to fruition, plasmids may well assume a critical role in small RNA manufacturing as they have in the case of mRNA vaccines, therapeutics, and reagents.

PLASMID GRADES

The indirect use of plasmid DNA as a starting material for viral vector or mRNA vaccine manufacturing requires the production of substantial amounts of material. For example, more than one kg of plasmid DNA is required to deliver one billion doses of mRNA vaccine [3]. Because plasmids are not intended to be present in the final medicinal products that are directly administered to patients but are rather used as starting materials for the cGMP manufacturing of other starting materials, biological drugs, or cell products, a cGMP grade is not strictly required. Nevertheless, although not all GMP aspects or a GMP certificate are required, the principles of GMP should be complied with during manufacturing since the starting material can end up in the finished medicinal product at residual levels and potentially impact its quality, safety, and efficacy. Ultimately, it will be up to the sponsor to perform an appropriate risk analysis to define the quality standards applicable to manufacture plasmid DNA suitable for further manufacturing of medicinal products under cGMP [17]. Relevant aspects to be duly considered will include, for example, the quality management system, documentation, raw materials, cell banks, production, specification, testing, and control and storage [17]. Thus, one can opt to produce a cGMP-like/high-quality grade plasmid DNA, which although falling short of all cGMP requirements, is still compliant with many regulatory recommendations [2,18]. Figure 2 highlights stages in the manufacturing of engineered cell products and viral vectors that are reliant on plasmid starting materials, where cGMP and cGMP principles should be applied (adapted from [17]).

FIGURE 2

Manufacturing of plasmids as starting materials for the manufacturing of engineered cell products and viral vectors. Manufacturing activities highlighted in light red should follow the principles of cGMP, whereas those highlighted in dark red should comply with full cGMP.



PLASMID MANUFACTURING AT SCALE

Although reasonably well established, manufacturing plasmid DNA at a large scale is not trivial, and manufacturers are constantly pressed to find ways to increase productivity without affecting quality [3,18]. This pressure to enhance manufacturing performance originates in part from the fact that the available capacity is not sufficient to respond to the increase in demand associated with the development of a growing number of applications of plasmids in a timely manner [3,18].

Currently, large-scale manufacturing of plasmid DNA depends exclusively on one platform host—*Escherichia coli* [1,2,19]. This preference is justified by the ability of *E. coli* to grow and divide rapidly under a range of conditions and to deliver high plasmid DNA yields. Further, many tools exist to support the molecular and microbial engineering of *E. coli*, including the creation of plasmid vectors and improved strains. Modified strains of *E. coli* are available that can be grown to densities of hundreds of grams per liter and produce up to 1–2 g plasmid DNA per liter of culture [20]. Efforts are also being directed towards the development of *E. coli* strains that may circumvent instability problems

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like the ones faced when dealing with ITR-containing plasmids such as those used in the context of the manufacturing of AAV transfer plasmids [8].

One way to improve plasmid amounts generated during manufacturing, as well as to ease regulatory approval and improve plasmid biological functions, is to focus on the engineering of DNA backbones. Efforts have been directed towards the generation of plasmids and plasmid systems (e.g., minicircles, nanoplasmids, minivectors) that are smaller, free from antibiotic resistant genes, increase manufacturing yields and provide high transgene expression [21–25].

The isolation and purification of plasmids from *E. coli* biomass recovered at the end of fermentation is an engineering challenge that has been solved for the most part, especially at smaller scales. The train of unit operations used in the downstream processing of plasmids almost inevitably includes alkaline lysis, tangential flow filtration and chromatography steps [1]. Different combinations of operations are used that deliver plasmid DNA with residual amounts of host impurities (genomic DNA, RNA, proteins, lipopolysaccharides, etc.) that are compatible with regulatory requirements.

Critical issues that have not been solved to satisfaction include poor reproducibility of alkaline lysis, lack of capacity and isoform selectivity in chromatography and loss of supercoiled isoforms during processing due to shear [1,26]. The final sterile filtration with 0.22 µm filters may also be cumbersome when dealing with very large plasmids [27].

Once manufactured, the bulk purified plasmids obtained from each batch should be rigorously characterized. Release specifications of plasmids used as a starting material will essentially focus on the same attributes as those covered when manufacturing plasmids as biological drugs [28]. This means that assays for identity (e.g., sequence, homogeneity), potency (e.g., concentration, homogeneity) and purity (e.g., host impurities, bioburden, residual kanamycin) and the corresponding acceptance criteria must be in place [28].

TRANSLATION INSIGHT

The relevance of producing plasmids at large scale has surged over the last years, not only because of the development of plasmid biological drugs like the ones used in DNA vaccination, *in vivo* gene therapy and gene editing, but mostly due to the supporting role they are currently playing in the manufacturing of many gene and cell therapy products, including viral vectors, viral-vectored vaccines, mRNA vaccines, minicircles/minivectors/nanoplasmids, and engineered cells.

Apart from the current uses, one can anticipate that plasmids may come to play a significant role in the manufacturing of small RNA molecules (e.g., antisense oligonucleotides, RNA guides, siRNA products) by IVT. While at first one may question whether IVT will ever be able to compete with the very well-established chemical synthesis of oligonucleotides, one driver for a move in this direction may come from an unexpected field: agriculture. Specifically, the development of new pesticide tools based on the induction of gene silencing through RNAi in plant pathogens and other pests is pushing for the development of cost-efficient methods for large production of bulk amounts of dsRNA [29]. IVT is emerging as an alternative in this context given that chemical synthesis is most likely not amenable to the large-scale and low-cost manufacturing that is required to implement siRNA.

Looking forward in terms of plasmid manufacturing, a few developments can be envisioned that would facilitate or altogether change how plasmids are made today. For example, while the current performance of *E. coli* as a plasmid producer looks unbeatable, one may wonder if the high demand for plasmids could not justify a search for a bacterial host with characteristics more favorable for manufacturing. Gram positive bacteria would be advantageous as plasmid producers because

they lack lipopolysaccharides—one of the most troublesome impurities associated with plasmids isolated from *E. coli*. As was the case in the production of recombinant proteins, which saw an emergence of producer hosts other than *E. coli*, there might be other hosts waiting to be discovered and developed into plasmid producers.

Engineering of plasmids backbones is also likely to have an impact in the field. Further, one should look out for radical innovations like the use of minimal synthetic constructs such as 'doggybone' and dumbbell-shaped DNA vectors, which are manufactured enzymatically [30,31]. Nevertheless, although these represent important advancements in the field, the likelihood of plasmids becoming obsolete in the future is small. Further, minicircles, mini vectors, and other minimized vectors still depend upon plasmids for their manufacturing [24,25].

Single-use technologies [32], process analytical technologies [33], automation [34], digitalization [35], and continuous manufacturing [36] are industry trends that may change the way plasmids are manufactured in the future [18]. In the latter case, for example, the design of continuous cell lysis processes that are robust and able to consistently deliver intact plasmids is another advancement to look for. The additional coupling of a post-lysis, pre-purification step by tangential flow filtration would significantly improve the productivity of the first part of the downstream processing of plasmids.

In conclusion, the central role currently played by plasmids in the development and manufacturing of many gene and cell therapy products fully justifies that a significant increase in R&D efforts and investments is made towards improving their effectiveness and manufacturing.

BIOGRAPHY

DUARTE MIGUEL PRAZERES is currently a full professor at the Department of Bioengineering and senior researcher at the Institute for Bioengineering and Biosciences of Instituto Superior Técnico (IST) in Lisbon, Portugal. He teaches transport phenomena, downstream processing, bioprocess engineering and gene/cell therapy and pursues research in two critical areas: biomanufacturing of nucleic acids for gene therapy and DNA vaccination, and biosensing and diagnostics. He is also currently Vice-President of the Scientific Council and member of the Ethics Committee of IST.

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FASTFACTS

Reducing risk for cell therapy manufacturing with a battle-tested electroporation platform

Andrew Mancini PhD, Senior Field Application Scientist, MaxCyte®

As the complexity of a final cellular product increases due to the development of strategies like CRISPR/Cas9 editing or transposon integration, so does the complexity of the associated manufacturing process. This, in turn, increases the risk of manufacturing failure. This FastFacts poster will demonstrate how electroporation technology can address key risk factors during the cell therapy manufacturing process.

- Process complexity: manual handling, open processing steps
- Low yield of final product: high cell loss, low viability, low efficiency, poor scalability
- Process variability: donor-to-donor, site-to-site
- Unexpected technical hiccups: slow support, unproven technology
- Regulatory bottlenecks

By modifying the electroporation step of the manufacturing process, where cells are engineered into the final cell therapy product, these risks can be mitigated. This modification can be achieved with a clinically validated electroporation instrument optimized for cell therapy manufacturing, such as the MaxCyte GTx™.

ADDRESSING PROCESS COMPLEXITY RISK

The MaxCyte Flow Electroporation® process, which utilizes specialized closed Processing Assemblies, was designed for simple execution in both the process development and manufacturing space. It allows the user to process up to billions of cells in an automated fashion.

ADDRESSING LOW YIELD OF FINAL PRODUCT

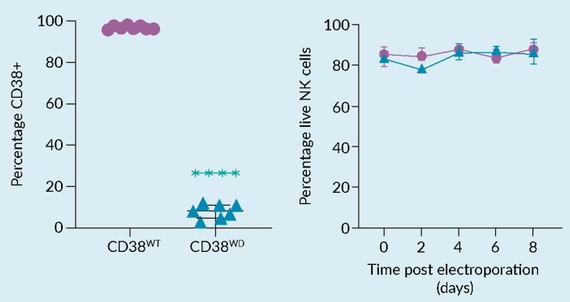
MaxCyte's process maximizes efficiency and viability throughout scale-up without sacrificing cell yield. As shown in **Figure 1**, The R-20K™ Processing Assembly supports excellent cell recovery.

Figure 1. Using the closed process Flow Electroporation® with the R-20K™ Processing Assembly, there was an average volume loss of under 10% across five separate runs.

ADDRESSING PROCESS VARIABILITY

Implementing a high-performing technology can help mitigate process variability. **Figure 2** demonstrates the isolation of natural killer cells from eight healthy donors and transfection with the MaxCyte ATx® instrument in order to knock-out CD38. There was an average of over 90% knockout with no significant differences in cell viability and transfection efficiency observed across different donors.

Figure 2. Demonstrating consistency between donors.



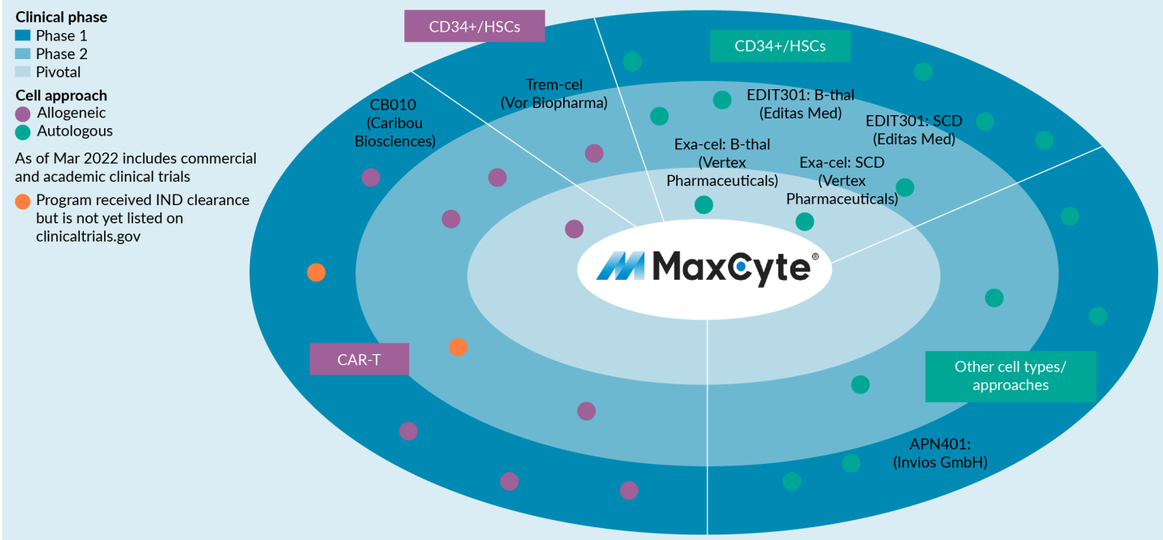
ADDRESSING UNEXPECTED TECHNICAL HICCUPS

MaxCyte provides on-demand global field support from early R&D through clinical and commercial manufacturing. This support is reinforced with in-house R&D and process development teams that can solve complex challenges.

ADDRESSING REGULATORY BOTTLENECKS

MaxCyte's regulatory team has over 20 years of experience and their support has aided in clinical trials around the world, including in leading cell therapy trials **(Figure 3)**.

Figure 3. MaxCyte-enabled active clinical trials.



CELL & GENE THERAPY INSIGHTS

Cell and Gene Therapy Insights 2023; 9(5), 809; DOI: 10.18609/cgti.2023.100

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION
PART 2: NON-VIRAL

SPOTLIGHT

INTERVIEW

Non-viral methods for *ex vivo* cell & gene therapy: is the future non-viral?

In this episode, **Abi Pinchbeck,** Assistant Editor, BioInsights, speaks two industry experts from Lonza, **Valeria Annibaldi,** Group Leader of R&D Transfection, and **Andrea Toell,** Director, Senior Product Manager. They discuss the types and benefits of non-viral methods for *ex vivo* cell and gene therapy in addition to the readiness of electroporation-based technologies for use in GMP manufacturing.

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Q What non-viral methods are currently used in the cell and gene therapy environment, and do you see their use evolving?

VA: Non-viral methods can be grouped into two main categories: carrier-mediated and methods without a carrier. An example of the first group is lipid nanoparticles

(LNPs), which form a complex with the cargo molecule and then are taken up by the cells. Other types of vehicles explored in the field include polymeric nanoparticles and exosomes.

Alternatively, cell permeabilization can be achieved by physical or chemical perturbation of the cell membrane. For example, electroporation is based on the electrical stimulation of the cells and sonoporation utilizes acoustic waves to deliver material into the cells. Other methods rely on mechanical and hydrodynamic forces, for example, in microfluidic squeezing, cells are forced to pass through a constriction and the squeezing makes the cell permeable enabling the cargo to enter. Another microfluidic method is based on vortex shedding, whereby miniaturized posts cause a vortex in the cell solution in a microfluidic channel. This vortex shedding alters the membrane permeability, enabling the cargo to enter cells. Chemical approaches can also be effective, such as Solupore® technology, whereby a solution containing a low concentration of ethanol is delivered to the cells in a specific embodiment enabling the cargo to enter the cells. Electroporation and LNPs are already well established, but some of these other technologies have made big steps forward in the last couple of years, and they are ready or almost ready for clinical use.

Q What are the advantages and disadvantages of utilizing non-viral technology for *ex vivo* cell therapy?

VA: Viral transduction represents the current standard for cell engineering, and it has been investigated and used for both *ex vivo* and *in vivo* gene therapy approaches for decades. There is extensive literature and products on the market in which cell modification was achieved with viral transduction. However, the use of viruses also poses safety concerns related to the random nature of viral integration.

Although significant work has been done to improve the safety and efficacy of viral vectors, in recent years non-viral alternatives are being increasingly adopted because they may provide some advantages over viral transduction due to, for example, lower safety concerns. With non-viral technologies, the risk of insertional mutagenesis is lower or non-existent. Non-viral methods can also offer the option of transient expression, which is considered safer. They can also be used to achieve precise genome editing, which is also a safer option, and they cause less immunogenicity and less toxicity to the cells.

Another crucial aspect is the lower cost of good manufacturing practice (GMP) manufacturing of the therapeutic product. The production of clinical-grade viral vectors can be expensive, time-consuming, and challenging to scale, and there might be long lead times for viral manufacturing. In addition, non-viral methods also offer flexibility with regard to the type of cargo used. In comparison to viral transduction, non-viral delivery has fewer limitations regarding the type and size of the payloads that can be delivered. Moreover, with some technologies, co-delivery of multiple payloads is possible for complex cell modifications.

Regarding potential drawbacks of non-viral methods, in the past, lower efficiency and thus lower expression of the transgene has been seen, and the nature of the expression was normally only transient. However, non-viral technologies have moved forwards tremendously in

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recent years and low efficiency is no longer an issue. Transient expression is now considered an advantage in some applications, and if it is not desired, can be overcome by exploring genome editing tools like transposon/transposase systems or engineered nucleases like ZFN-, TALEN- or CRISPR.

"...by combining non-viral methods with genome editing tools, stable integration can be achieved, with efficiency which is similar or in some cases higher than viruses."

– **Valeria Annibaldi**

Q Cell types used in cell therapy applications can be difficult to transfect using non-viral means. How can these challenges be overcome?

VA: Non-viral methods are often used for blood-related disorders such as leukemia and lymphoma. Primary cells, which are the focus of those therapies, like primary T cells, hematopoietic stem cells (HSCs), or natural killer (NK) cells are historically known to be hard to transfect by non-viral methods. However, this mainly refers to traditional chemical methods. Electroporation can significantly improve efficiency but also requires higher doses of payload, which may be toxic to cells, especially in the case of DNA. Improved electroporation-based techniques like our Nucleofector® Technology can overcome this drawback to some extent by requiring less payload. In addition, by combining non-viral methods with genome editing tools, stable integration can be achieved, with efficiency which is similar or in some cases higher than viruses. Alternatively, in case only transient expression is preferred, mRNA can also be transfected into cells.

Q Can you tell me about the non-viral gene transfer technology that Lonza offers?

AT: The key to the successful implementation of a non-viral technology is to combine the high transfection efficiencies that can typically be achieved by viruses, with the flexibility of non-viral technology. Our solution is an improved electroporation technology, the Nucleofector Technology, which was originally introduced into the market by Amaxa™ in 2001. With this technology, optimized electrical parameters combined with cell type-specific solutions enable the transfer of a molecule directly into the cell's nucleus. Since it does not rely on proliferation due to this nuclear transfer, it can even transfect non-dividing cells like resting T cells. This nuclear transfer makes it particularly beneficial for hard-to-transfect cells and allows for highly efficient transfection of primary cells, including those relevant for *ex vivo* cell therapy.

This technology is based on three key components: firstly, a Nucleofector Instrument that generates unique electrical pulses. Secondly, specified Nucleofection® Vessels are used in

> "...the use of minimalistic DNA vectors encoding transposon and transposase (so-called minicircles) or transfecting the transposase as an mRNA might be a promising alternative that provides significantly higher transfection efficiency and less toxicity compared to plasmid-based approaches..."
>
> – **Andrea Toell**

combination with cell type specific Nucleofector Solutions acting as a supportive environment for high transfection efficiency and cell viability.

The predominant cell therapy applications generated using the Nucleofector Technology are T cells expressing a chimeric antigen receptor (CAR-T cells) or expressing an engineered T cell receptor. It can also be used for genetically modified HSCs or genetically modified induced pluripotent stem cells. The latter can also be generated with the help of Nucleofection by doing the reprogramming step in a non-viral fashion. Natural killer cells might be the next big thing.

Q What unique advantages does an electroporation-based method offer?

AT: Electroporation is relatively easy to establish and can be very efficient, but it needs to be balanced out with toxicity. Here, Nucleofection can be beneficial because less payload is required, for example in case of DNA which can be quite toxic to cells. Differently from other non-viral methods, especially lipid or chemical-based methods, when using electroporation, the naked cargo is directly delivered into the cell through transient pores in the cell membrane. With chemical methods, you may rely on the endosomal pathway for cargo release, which can trigger toll-like receptor pathways and affect the cells in a negative way. Furthermore, electroporation is flexible, as it can deliver nucleic acids like DNA or mRNA, in addition to proteins such as Cas9 ribonucleoproteins for CRISPR-based gene editing, or even combinations of these as required. For example, when performing CRISPR-based knock-ins, you have to co-transfect Cas9 ribonucleoprotein together with a DNA or PCR donor template. As a non-viral method, Nucleofection is suited for both. You can either do transient expression of a therapeutic gene by delivering plasmid DNA or mRNA or aim for stable genetic engineering of cells by combining it with transposon-based systems like Sleeping Beauty™ or piggyBac®, as well as engineered nucleases for more targeted integrations like zinc finger nucleases, TALEN, or CRISPR Cas9.

When using such engineering tools, a few things may require consideration. Similar to viruses, transposon-based modifications are generally more efficient but are less controllable as integration occurs randomly in the genome. In addition, the large amounts of DNA that are

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typically part of these transposon-based modifications can be toxic for cells, especially T cells. Researchers have demonstrated that the use of minimalistic DNA vectors encoding transposon and transposase (so-called minicircles) or transfecting the transposase as an mRNA might be a promising alternative that provides significantly higher transfection efficiency and less toxicity compared to plasmid-based approaches while keeping functional effects comparable to viral vectors. With engineered nucleases, like Cas9, a safer and more controlled modification can be achieved because it can be targeted to a specific locus. Delivering the engineered nuclease as an mRNA or protein would allow for better dosage control of the modification. Another alternative to reduce DNA toxicity can be to transfect CAR mRNA. Such transiently expressed CAR can temporarily limit the CAR-T activity and thus reduce off-tissue toxicity affecting normal tissue.

Q The ability to scale up is an important consideration for GMP manufacturing and clinical translation of cell therapies. Is this technology scalable to meet the needs of the industry?

AT: Our large-scale platform, the 4D-Nucleofector LV Unit, is designed with this need in mind. The LV Unit can handle up to 1–2 billion cells depending on the size of the cell type and thus supports most autologous cell therapy applications. Transfection protocols can be established on the smaller scale Nucleofector units, and then transferred to the large-scale LV Unit without the need for extensive re-optimization. In some cases, re-optimization might be required, and in those cases, a highly skilled scientific support team is available at Lonza, that can help with any optimization or fine-tuning. Furthermore, the use of the LV Unit as manufacturing equipment in a GMP process is supported by various means. For example, the unit itself can be equipped with 21 CFR part 11 compliant software to fulfill documentation needs in a GMP environment. In addition, Lonza offers IQ/OQ services for equipment qualification and also Nucleofector Solutions and Vessels manufactured according to GMP or ISO 13485 rules are available. The system can be closed via weldable connections to upstream and downstream equipment, for example our Cocoon Platform. Early clinical trials are already ongoing involving the use of this technology.

Q What non-viral technologies are currently being used in clinical or commercial applications based on *ex vivo* modifications?

VA: Except for electroporation and LNPs, most of the non-viral methods that I mentioned initially are rapidly evolving but have not yet reached the clinical stage. However, the number of immunotherapy products based on non-viral methods in the pipelines of cell and gene therapy companies has more than doubled over the last 7–8 years. In particular, there are several clinical trials ongoing combining electroporation with Sleeping Beauty or piggyBac transposon/transpose systems, or even CRISPR-Cas9.

Q What are your predictions for the future of this space as non-viral technologies continue to develop?

VA: Up to now, the vast majority of genetic modification has been done with viruses, especially for *in vivo* therapies, but also for *ex vivo* therapies. Nonetheless, there is an increasing interest in non-viral technologies due to the advantages that I mentioned earlier. I expect that in the future, both approaches will coexist, with multiple offerings in the non-viral space, because there may not be a one-size-fits-all method. The type of application will probably dictate the technology of choice, for example, the size of the therapeutic dose, the target indication, or whether the therapeutic approach is autologous or allogeneic. Whether the modification needs to be permanent or if a transient product is desirable could affect the choice. The specifications of the drug product could also play a significant role. For complex gene editing requiring multiple modifications, there are already companies exploring combinations of different technologies, for example, viral delivery and electroporation. Market research shows that growth is expected for both viral and non-viral technologies in the future as the industry evolves and cell and gene therapy approaches become increasingly established.

BIOGRAPHIES

DR VALERIA ANNIBALDI joined Lonza Cologne GmbH, Germany, in 2021 as a Group Leader in the R&D Transfection Team. The team develops innovative solutions for transfection of cells, with emphasis on primary cells. In her current role, she works on the development of next generation Nucleofector Platforms and Consumables, with the focus on cellular therapy applications and large-scale electroporation. Prior to joining Lonza, she worked in the CGT field developing and evaluating transfection technologies suitable for immunotherapy. In her previous positions she gained experience in product and process development formulation and primary T cells. Valeria is a qualified pharmacist, she holds a master's degree in Pharmaceutical Chemistry and Technology from Sapienza University of Rome, Italy and completed a PhD in Electrochemistry, at Maynooth University, Ireland. Results of her studies were published in peer-reviewed journals and presented at scientific conferences.

ANDREA TOELL is a Senior Product Manager at Lonza Cologne GmbH and is responsible for Lonza's Nucleofector Transfection Portfolio. In her current role she is focusing on large volume transfection and cell and gene therapy applications (CAR-T cells, genome editing, GMP manufacturing). In her 20 years' experience with the technology she gained a broad knowledge about transfection technologies and factors that are important for successful transfection experiments. Andrea joined the Lonza team (legacy amaxa) as Scientific Support Specialist in 2001 before moving into Product Management in 2004. She holds a Diploma in Biology from the University of Münster, Germany, and a PhD in Biology from the University of Düsseldorf, Germany.

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AUTHORSHIP & CONFLICT OF INTEREST

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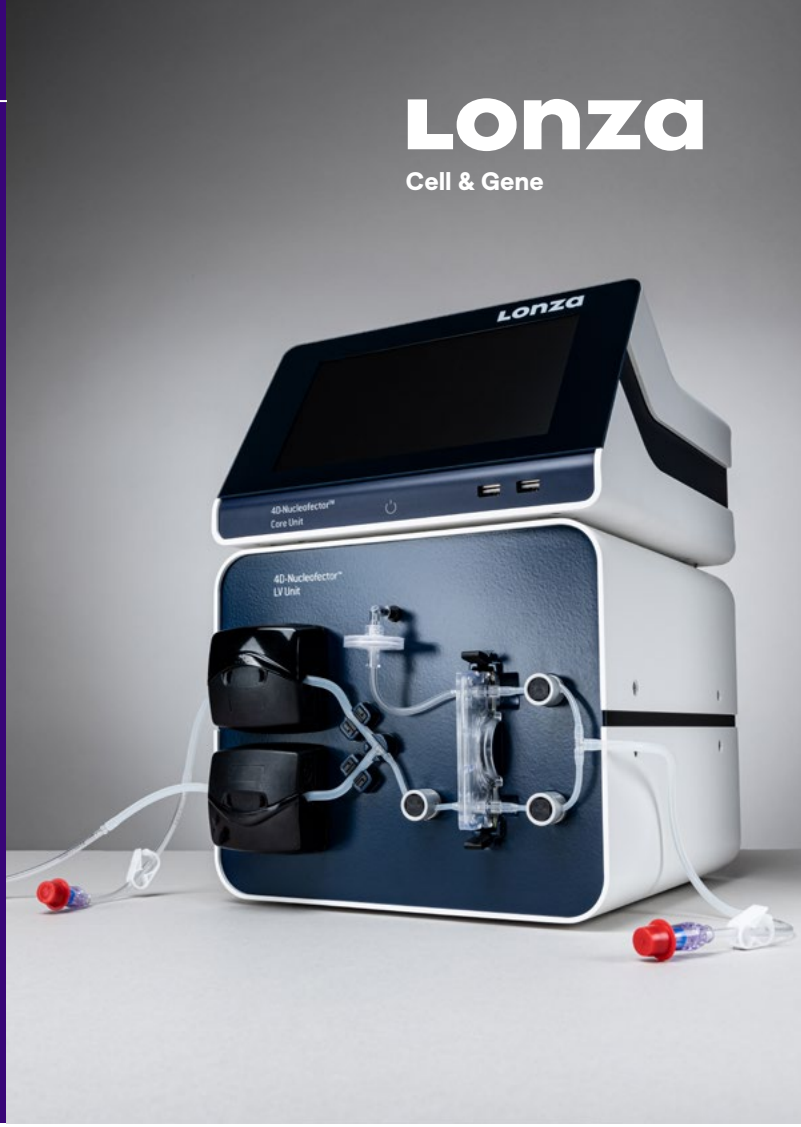

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CT-AD023 03/23

FASTFACTS

Addressing purification challenges: biological pipeline diversity and smaller manufacturing scales

Sushma Nayak Teichert, Global Product Manager, Cytiva

Before the COVID pandemic, scientists working in the field of RNA therapeutics were able to envisage its potential and impact on the field of medicine. The success of mRNA-based COVID vaccines accelerated the market for RNA therapeutics and enabled manufacturers to gain traction. The rise of RNA therapeutics has been exponential and the number of therapies in clinical trials continues to rise. mRNA-based therapies have the potential to treat cancer, genetic disorders, and infectious diseases.

DIVERSIFYING PIPELINE WITH NEW MODALITIES

A rapid growth in novel therapeutics has been seen in the biological pipeline in terms of new modalities entering the market in line with the increasing revenue of biologics, as demonstrated in **Figure 1**. Traditional monoclonal antibodies are dominating the biologics revenue with 45% of the share in 2021 and 42% projected in 2027. Novel therapies have the highest compounded annual growth rate. These novel therapies are drivers of new challenges in manufacturing.

THE THERAPEUTIC PROMISE OF mRNA

With nearly 500 therapeutics in the pipeline, the mRNA landscape is evolving rapidly and rising exponentially **(Figure 2)**. mRNA is showing potential in a wide array of therapeutic modalities, including oncology and infectious disease vaccines, gene editing, cell therapy, and encoding antibodies. The majority of mRNA therapeutics are in the preclinical stage (69%). Increases in indication and therapy diversity are seen, including vaccine, gene editing, protein and antibody replacement, and *ex vivo*/*in vivo* cell applications. The majority of mRNA drugs are vaccines for non-oncological indications (42%), followed by vaccines for oncological indications (16%) and protein replacement therapies (9%).

Figure 1. The growth of new modalities in a diversifying pipeline

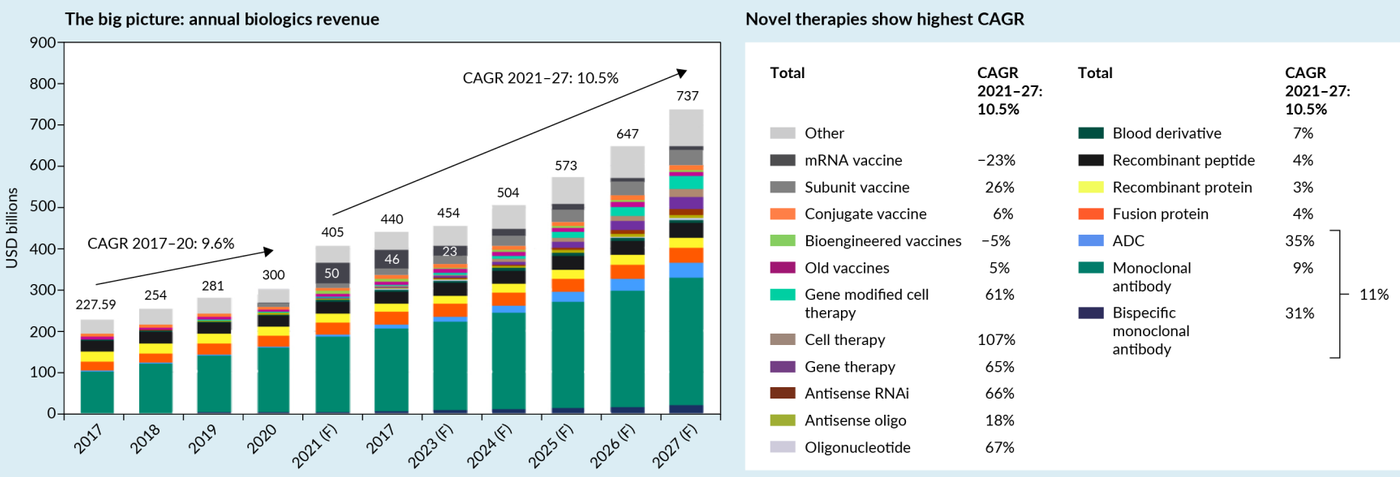
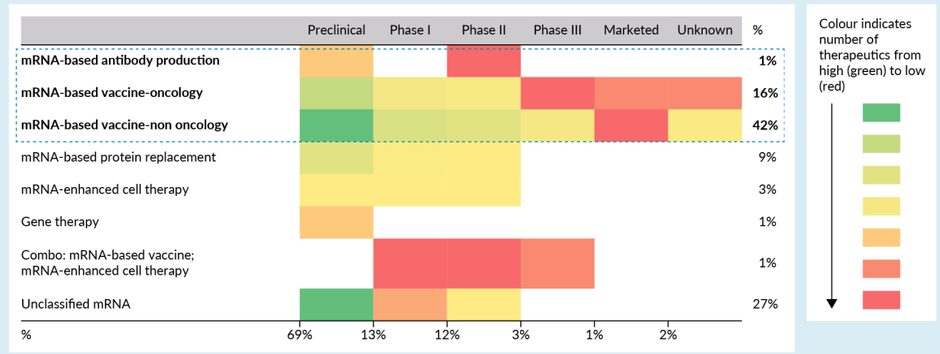


Figure 2. mRNA therapeutic pipeline.



MANUFACTURING EFFICIENCIES FOR NOVEL THERAPEUTICS

The broad spectrum of applications for RNA necessitates manufacturers to adapt to the unknown. There is a choice of types of RNA to use for therapies, each with its own advantages and disadvantages for therapeutic manufacturers. For instance, mRNA is particularly suited to cancer immunotherapy to overcome the hurdle of efficacy. With so many unknowns in manufacturing, it can be challenging for developers to future-proof manufacturing and build in flexibility whilst establishing reproducibility and maintaining control.

Scales can vary from personalized to pandemic response. Cytiva offers scalable equipment from process development to manufacturing in the mRNA workflow, and flexible single-use components to support optimization, when GMP and process development are at the same scale. Equipment designed for small volumes enables the future-proofing of manufacturing, by addressing the smaller scales which novel therapeutics will need. When the size of the process goes down, the quicker the run times and the greater the number of batches possible. Cytiva's range of equipment enables the purification of small drug volumes in a GMP environment.

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In partnership with: cytiva

CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

INTERVIEW

Expanding the potential of *in vivo* cell therapy with tLNP-RNAs (targeted lipid nanoparticle-RNA)

The application of lipid nanoparticles (LNPs) as a non-viral delivery system for advanced therapies, in conjunction with RNA-based medicines, is set to revolutionize the field. In this episode, **David McCall,** Senior Editor, BioInsights, speaks to **Adrian Bot,** Chief Scientific Officer and Executive Vice President of R&D, Capstan Therapeutics, about the potential of these tools to drive the burgeoning *in vivo* cell therapy space in particular, and the broader gene medicine field in general.

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Q What are you working on right now?

AB: Together with my colleagues at Capstan Therapeutics, we are excited about the prospect of advancing this field to the *in vivo* reprogramming of human cells and tissues, which carries a lot of promise in terms of curing a broader range of diseases than *ex vivo* engineered cell therapies or other modalities.

"One feature is that it is targeted specifically for cells of interest. We achieved that by decorating the particles with protein-based ligand antibodies or fragments, which can be thought of as a kind of 'molecular GPS'..."

Q What is the latest R&D progress in the *in vivo* CAR-T cell therapy field, and what evidence do you see that the advantages of *in vivo* over *ex vivo* approaches are beginning to be realized?

AB: The *in vivo* CAR-T cell therapy field is at its inception right now. It is an exciting and rapidly developing ecosystem with a dozen or so companies involved, all in the preclinical stage. There is tremendous excitement and resources are being put behind this concept.

One of the major advantages of *in vivo* reprogramming of the immune system over *ex vivo* engineered cell therapy is that we deal with off-the-shelf synthetic therapeutics as opposed to cell- and viral vector-based products. Another advantage is that we do not use lymphodepletion conditioning, which is a must for *ex vivo* engineered cell therapies and represents a key access hurdle for that type of product. Further, utilizing *in vivo* approaches combined with transient chimeric antigen receptor (CAR) expression concept will allow us to scale-up or scale-down the exposure to regimens in order to fit a broader range of disorders with different therapeutic efficacy and safety bars. These include, excitingly, non-oncologic indications.

Q Why is Capstan's tLNP-RNA technology ideal for the purposes of *in vivo* CAR-T cell engineering?

AB: There are several important features of our targeted lipid nanoparticle-RNA (tLNP) technology. One feature is that it is targeted specifically for cells of interest. We achieved that by decorating the particles with protein-based ligand antibodies or fragments, which can be thought of as a kind of 'molecular GPS', guiding whether or not a certain type of cell will uptake these tLNPs *in vivo*. This is important as you must ensure that your CAR (or any other payload, for that matter) is expressed in the cells of interest at functional levels, instead of other cells or tissues.

Beyond the fact that our platform comprises an off-the-shelf and scalable particle technology with cell targeting capabilities, it also comprises proprietary ionizable lipids with increased performance making them amenable to RNA therapeutic payloads and repeat

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delivery. This is a departure from, and improvement upon, vaccine approaches such as the mRNA-based SARS-CoV-2 vaccines—those products utilize ionizable lipids that do not have to have increased biodegradability and ameliorated immunological activity.

In addition to mRNA fitting the concept of transient CAR T cell engineering *in vivo*, there is the possibility to accommodate a broader range of payloads and include multiplexing - for example, gene editing machinery such as the CRISPR-Cas9 system, which requires delivery of both the enzyme and an RNA guide. You can also multiplex biological response modifiers with your CAR mRNA or another format to achieve higher therapeutic efficacy thresholds in hardier disease indications. Finally, you can think of this platform as a cassette approach or modular design, and select the payloads, targeting binders and dose-regimen depending on the target that you are pursuing, the disease indication, and other aspects.

Q Can you expand on some of the specific advantages of your approach compared to other viral and non-viral gene delivery options? What does the evidence to date tell us about the relative pros and cons of LNPs in terms of safety, specificity, efficiency, durability, and cost?

AB: Over a decade has been spent trying to develop *ex vivo* engineered CAR-T cell products utilizing viral vectors. They proved to be potent and allow delivery of the payload to the cell of interest (T cells), *ex vivo*. Nevertheless, while these products have been transformative and potentially curative in patients with B cell malignancies if combined with lymphodepletion conditioning, most patients eventually relapse, and a subset of patients experience life threatening adverse events (AEs) of an inflammatory nature. One of the major limitations of the integrative vectors is the lack of control on CAR expressing T cells *in vivo*—that could ignite or amplify the AEs of inflammatory nature including cerebral edema, profound cytokine release syndrome, or macrophage activating syndrome. Efforts are underway to pursue the delivery of viral vectors including lentivirus and AAV *in vivo* to reprogram immune cells. However, one of the major advantages of using transient mRNA expression or gene editing systems is the tunability of the dose-regimen fitting broader ranges of safety bars, or precise integration in the genome that can be achieved without utilizing a virus. This gives a greater level of control over safety, toxicity, and efficacy—thereby facilitating the translation of this CAR concept to broader categories of indications including non-oncologic disorders or earlier stage or minimal residual disease cancer indications.

To exemplify this, in the fields of autoimmunity and regenerative medicine, it would be difficult to develop an *in vivo* viral-based approach for broader indications that have high therapeutic safety bars. Instead, for such indications, one would need vectors devoid of the liabilities that integrating viral vectors have displayed in the past, including permanent and/or stochastic insertion in the genome. Moving away from that type of payload makes sense, at least for the first wave CAR products in this space.

The advantage of a targeted lipid nanoparticles (LNP)-mRNA CAR technology is that it allows us to develop a portfolio of *in vivo* CAR products fit for purpose. Depending on disease indication and the cell category that you want to reprogram, you use a different targeting binder and/or payload. For CAR-T cell generation *in vivo*, one would use a binder that targets all T cells, or T cell subsets directly in the body. The payload would be a CAR in mRNA format, optimized for this platform technology, that would correspond to cognate targets relevant to the disease pathogenesis. The format would allow in principle multiplexing to achieve multi-antigen targeting or dialing in biological response modifiers to adjust the potency as needed. For distinct disease categories such as monogenic blood disorders, for example, one would use a binder specifically for long-term reconstituting of hematopoietic stem cells, such as CD34 or CD117. It is difficult to utilize this sort of 'plug-and-play' approach without a targeted LNP based on a protein binder leveraging the exquisite specificity, versatility, and rapid optimization potential of antibody-target interaction. Nevertheless, some of the companies in our ecosystem do not utilize a protein-ligand approach and leverage instead the organ tropism imparted by physicochemical characteristics of lipid particles. This approach shifts the burden to high throughput generation and semi-empirical screening efforts with unclear impact on ability to precisely engineer desired cell subsets.

"The advantage of a targeted lipid nanoparticles (LNP)-mRNA CAR technology is that it allows us to develop a portfolio of *in vivo* CAR products fit for purpose."

I have already mentioned the importance of the lipids used in the particle alongside the formulation process. While physicochemical characteristics would impart organ tropism and even help de-target the liver, targeting binders would greatly facilitate the particle uptake by specific cell types. In addition, developing a new range of ionizable lipids that stand out would allow repeat dosing by avoiding organ-specific toxicities and untoward immune reactions. Then, in a modular fashion, one can build therapeutic treatment cycles that comprise multiple-dose regimens and are designed to fit clinical indications with different therapeutic efficacy and safety bars.

Q Can you go deeper on the specific cell targeting and systemic delivery potential and the broad applicability of LNPs—looking to the future, how do you see these aspects playing out in *in vivo* gene therapy clinical application?

AB: The future of the *in vivo* programming technologies is linked to the possibility to systemically deliver particles carrying safe and effective payloads, dependent on disease indication. Most disease indications will require a systemic type of access,

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including metastatic tumors, regenerative medicine, autoimmunity, and chronic infections. It is important to design a formulation that can be safely dosed to a sufficient level for clinical effect and that is amenable to repeat dosing to build the therapeutic regimens for a particular medical need. That must involve intravenous infusions in most clinical indications to facilitate both the mechanism of action and patient access—standing in contrast to many topical delivery methods.

I am optimistic about the prospect of rationally optimizing and selecting the lipids in addition to the binder that will allow the particle to access the appropriate organs and number of target cells. A major prerequisite is of course to prolong the life span of the particles just sufficiently, by largely de-targeting the liver and reticuloendothelial system, thereby allowing enhanced deposition in other organs. For example, when dealing with *in vivo* CAR generation, you must keep in mind that most of the T cells that are the desired recipients of the targeted LNPs are located in secondary lymphoid organs, including the spleen and lymph nodes. This means one needs to generate and optimize a particle for targeting secondary lymphoid organs, and within the lymphoid organs, the T cells themselves—so, both organ and cell tropism must be considered. The physical-chemical characteristics of the particle will determine the organ tropism, whereas the targeting binder will determine the cellular tropism within an organ. That is the current rule of thumb and we, at Capstan, are posed to leverage this particular path. The next step of course would be selecting and optimizing a payload that would impart a transient (through mRNA) or permanent (through gene editing) change to the target tissue or cells.

Q Capstan's founders include experts from the mRNA space as well as the CAR-T cell therapy field. Can you expand on how the former have informed the application and ongoing development of LNP-RNA technology in this particular context? What learnings have they brought from the mRNA field that can be leveraged for CAR-T cell engineering?

AB: We cannot overestimate the impact of our foundational team at the University of Pennsylvania, comprising luminaries in CAR, mRNA, translational, and regenerative medicine fields. We are privileged to have pioneers such as Drs Carl June, Bruce Levine, and Drew Weissman in addition to the larger team who advanced breakthrough products or solutions that helped millions of patients in need. Our founders' expertise brough together the fields of CAR, mRNA and nano-delivery technologies, providing us with a springboard for our product development.

The CAR technology taught us that one could in principle reprogram a T cell and effectively direct them against pathogenic cells (such as tumor cells) in a biological and clinically meaningful fashion. Owing to the pioneering work of Drs. June and Levine, together with their collaborators and other groups in the field, the goal of 'cure' is now tangible in a range

"One feature is that it is targeted specifically for cells of interest. We achieved that by decorating the particles with protein-based ligand antibodies or fragments, which can be thought of as a kind of 'molecular GPS'..."

of cancer indications. Other Capstan founders, including Drs Jonathan Epstein, Haig Aghajanian, Ellen Puré, and Steven Albelda, showed that one can translate this concept to ablating non-tumoral cells, thereby opening up the possibility to broadly use CAR technologies in non-oncologic indications.

The payload format is very important and amongst our founders we have Dr Weissman, who helped pioneer the mRNA formats that have been widely utilized in the context of LNPs for SARS-CoV-2 vaccines.

More specifically, Dr Weissman and collaborators were able to uncouple the positive effects of mRNA in translation from the negative effects—namely, the stimulation of innate immunity which suppressed protein translation. This was a major discovery in molecular medicine, which led to our learning of how to modify mRNA to be a good therapeutic payload for our programs.

Finally, these were brought together by the concept of delivering the payloads *in vivo*, using targeted lipid nanoparticles—essentially, building on the system used for COVID-19 mRNA vaccination—by Drs Weissman and Hamideh Parhiz. These LNPs were co-developed for the purpose of looking at cancer immunotherapies initially, before being explored in the SARS-CoV-2 vaccine context, which was an opportunistic offload of the original programs. A key piece was added—the LNP targeting aspect—that is vigorously exploited by our team as it imparts cell engineering selectivity. These learnings helped us set up our programs, which will be directed at the concept of transient *in vivo* re-programming of T cells through mRNA or direct delivery of gene editing systems.

Q What's the most surprising thing you have learned in your preclinical work to date?

AB: The most surprising thing that I have learned is that through a transient mRNA system, one can deliver a sufficient payload to generate a biologically meaningful population of engineered T cells *in vivo*—that was a wonderful surprise! For now, these results have been obtained in preclinical models, but we are poised of course to take this concept to the next level: clinical development.

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Q Can you summarize what you are specifically most excited about in terms of Capstan's work over the next 12 months, and over the next 5 years?

AB: In terms of CARs, I am excited about rapidly advancing this technology in non-human primates and the investigational new drug (IND) enabling stage, looking first at validated targets such as a B cell-related antigens to pressure test and optimize our technology, but also developing products for novel and exciting disease indications. In terms of a five-year timeline, I am excited about obtaining proof of concept in clinical studies in a meaningful indication—most likely a B cell-related autoimmune disorder indication, before expanding into oncology—and set the stage for or even initiate registrational studies, depending on the indication. In parallel, our objective is to build a portfolio of product candidates that can be developed internally or through partnering. In terms of delivery of gene editing systems, in the medium- to long-term, it would be wonderful to bring this technology to the clinic for the purpose of correcting gene defects *in vivo*.

Q What do you see as Capstan's biggest challenge right now and what are you doing to address it?

AB: The biggest challenge, as expected, is integrating and scaling up all the components in a novel, transformative line of products. There are three areas that we need to integrate, which is both a challenge and an opportunity to lead. Number one is the nano-formulation and delivery part. Number two is the biology part and corresponding biomolecules, which particularly deals with targets, payloads, and binders. Number three is bringing this together in a manufacturing process that is scalable and can be sustained from Phase 1 to registration and commercial stages. It will be an iterative process that no one has done before, and we will learn a lot along the way.

Q What would you predict might be Capstan's biggest challenge in 5 years' time, and how can you prepare for it?

AB: One of the biggest challenges will be the transition from indications that have a lower therapeutic efficacy bar, such as non-oncology, to more challenging indications like solid tumors with much higher therapeutic efficacy bars. *In vivo* reprogramming has an advantage as this approach allows a holistic reprogramming of multiple arms of the immune system simultaneously. This cannot be done with *ex vivo* CAR-T cell

therapy, targeted small molecules, or antibodies. These must be done one target at a time, or with cocktails of two or three different targets. Co-reprogramming *in vivo* various arms of the immune system may bring together multiple categories of immune effector cells and mechanisms, thereby attacking the cancerous process from multiple angles. You can do much more by directly reprogramming tissues *in vivo*. You can reprogram both T cells and innate immune cells, either separately or simultaneously. You can increase the immune system's fitness through co-delivery of biological response modifiers and checkpoint inhibitors in an mRNA format. This is moving the whole field of protein-based therapeutics to an exciting new era of genetic-based or mRNA medicine. Novel solutions including multiplexing LNP-mRNA formulations or precision delivery of gene editing systems may be enabling in that regard. I think that these types of platform technologies will catalyze that progress.

Q What would you do if you were handed a 2× increase in your R&D budget? How about a 10× increase?

AB: Two times the budget would increase optionality and contingency planning, as this is a cutting-edge area that is largely unexplored. An R&D plan often encounters surprises along the way. By having a parallel track and putting the resources aside to develop a toolbox of binders, biomolecules, and lipids, the likelihood of technical success is increased early on in Phase 1 (first-in-human), and later in Phase 2 and registration of a first-in-class product.

With 10 times the budget, we would be looking at developing a broader product pipeline as soon as we have solid preclinical proof of concept, including next-generation gene editing approaches *in vivo*. This would require a lot of effort and resources past mRNA, which is a simpler payload. When dealing with prime editors, base editors, RNA or DNA writing, the complexity is huge—both from technical and manufacturing perspectives. The amount of work that we would need to do to optimize the delivery system for selectivity, specificity, safety profile, and genome toxicity would be staggering. Lots of resources would be needed to enable the transition to an *in vivo* reprogramming of the genome in specific cell populations—a goal that is still elusive, of course, but one that we strongly believe is attainable.

BIOGRAPHY

ADRIAN BOT is the founding Chief Scientific Officer and Executive Vice President of Research and Development at Capstan Therapeutics, a company developing next generation precision medicines. Previously, he held leadership roles at Kite Pharma and Kite, a Gilead Company, including Chief Scientific Officer and Head of Translational Medicine and Discovery Research, respectively. At Kite, he contributed to the development of first-in-class cell gene therapy products for cancer. Dr Bot has more than 25 years of experience in biopharmaceutical industry with focus on discovery and development of targeted therapies in general, and immunotherapies

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in particular. He obtained his MD in Romania at the University of Medicine and Pharmacy in Timisoara in 1993 and his PhD in Biomedical Sciences at Mount Sinai School of Medicine in New York in 1998. Subsequently, he was a Guest Scientist at the Scripps Research Institute in La Jolla, California. Dr Bot also served in various senior R&D leadership positions at MannKind Corp and Alliance Pharmaceutical Corp, La Jolla, California. His prior activities and appointments include editorial boards (Journal of Immunology, International Reviews of Immunology, Journal of Translational Medicine) and leadership appointments in global professional societies (International Society for Immunotherapy of Cancer).

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

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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

INTERVIEW

Exploring the application of doggybone DNA to facilitate non-viral gene therapy delivery to hemophilia patients

Novel non-viral gene delivery methods have the potential to greatly improve treatment protocols for patients with genetic diseases such as hemophilia. **David McCall,** Senior Editor at BioInsights, speaks with Principal Investigator at Seattle Children's Research Institute, **Carol Miao,** about her work with doggybone DNA (also known as dbDNA™), an optimized DNA platform for non-viral gene transfer. Doggybone DNA is an enzymatic DNA is manufactured in a cell free process using enzymes, rather than bacterial cells. Miao discusses the key benefits to using dbDNA versus plasmid DNA as cargo-carrying transgene expression cassettes.

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Q What are you working on right now?

CM: Using hemophilia as our model disease system, we are developing various gene therapy and modulation strategies to improve treatment protocols for patients. These include novel non-viral gene delivery methods such as ultrasound-mediated gene delivery (UMGD) and lipid nanoparticle-mediated delivery of DNA and RNA. We are also working on viral gene therapy, including the intraosseous delivery of lentiviral vectors targeting hematopoietic stem cells. Recently, we have been particularly interested in

delivering gene editing tools *in vivo* using these novel gene therapy strategies. An optimized DNA platform such as doggybone DNA (dbDNA) is particularly important to facilitate their success.

Another goal of my lab is to understand the basic mechanistic pathways of how factor VIII inhibitory antibodies are generated in hemophilia patients following gene or protein replacement therapy. We are striving to develop immunomodulation strategies to eliminate these inhibitory antibodies.

Q dbDNA is a highly versatile technology—what specific application(s) have you used it for?

CM: We collaborated with Touchlight Genetics Ltd. to optimize and select the best transgene expression platform for the liver to achieve the goal of therapeutic applications of this new technology. We are mainly using non-viral gene delivery strategies to screen for the optimal transgene expression platform to be carried by the dbDNA.

Q Can you expand on your experience in utilizing dbDNA for this particular purpose? What are some of the advantages relative to other viral and non-viral platforms?

CM: For non-viral gene transfer, we used to employ plasmid DNA as a cargo-carrying transgene expression cassette. However, we found that by using dbDNA as a cargo, we obtain comparable or higher transgene expression levels following non-viral gene delivery.

In addition, plasmid DNA carries bacterial genome sequences, such as antibacterial resistance genes, causing them to be much larger in size and potentially toxic and immunogenic. dbDNA eliminates all bacterial sequences and is therefore much smaller in size, resulting in a copy number advantage relative to plasmid that generally translates to high transgene expression. Thus, utilizing dbDNA makes non-viral gene delivery more efficient, less toxic, and potentially longer-lasting, too. dbDNA is a linear DNA with two capped ends, as compared to circular plasmid DNA. The capped ends ensure that there is very little chance of dbDNA integrating into the host genome and causing deleterious effects.

Compared to a viral platform, dbDNA harbors all the benefits of a non-viral platform: it is less immunogenic, less toxic, more consistent, and reduces cost. It also carries the potential for re-administration, which some of the viral vectors cannot offer, and it can avoid potential recombination events that yield unwanted by-products. Finally, dbDNA reduces any possible oncogenic events through random integration into the host genome, as integration is extremely rare. We have not observed any integration events when using either plasmid DNA or dbDNA.

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"I believe dbDNA will be a very competitive product for either non-viral gene delivery or gene editing. In particular, I believe it will be used for generating good manufacturing practice products for clinical trials because the cost will likely be reduced..."

Q Are there any challenges or considerations in employing dbDNA?

CM: The main challenge for me is that, because dbDNA is a new technology, it can't be made simply at the lab bench in the way that plasmid can for small scale research studies. However, once people realize the significant benefits of this platform, especially for non-viral delivery, then more people use it and it will become more adopted in the field. That is my hope. It is also important to emphasize that it is not expensive compared to viral delivery alternatives, and that the cost of dbDNA becomes progressively lower relative to plasmid as the scale of production is increased. This means that for larger clinical requirements, dbDNA can be less costly than a plasmid DNA equivalent.

Q What role do you expect dbDNA to play in the future of advanced therapies?

CM: I believe dbDNA will be a very competitive product for either non-viral gene delivery or gene editing. In particular, I believe it will be used for generating good manufacturing practice products for clinical trials because the cost will likely be reduced (again, particularly compared to viral vectors). The consistency of dbDNA will also contribute to its competitiveness. I believe this to be a product that people will prefer to use over plasmid DNA in the future.

Q What are some goals and key priorities that you have for the work in your lab over the next few years?

CM: We are very excited about our recent development in targeted delivery to hepatocytes or liver sinusoidal endothelial cells using either ultrasound-mediated gene delivery (UMGD) or lipid nanoparticles. In the next two years, we will continue to improve the efficiency and targeting capability of these tools for the delivery of the factor VIII gene as well as gene-editing platforms.

The reason we are very interested in targeting liver sinusoidal endothelial cells is because the factor VIII protein is predominantly made in that particular cell type, and not in hepatocytes. We are very excited that we were able to make efficient targeted deliveries, especially of gene editing strategies, to achieve the goal of therapeutic expression of FVIII. In order to make this strategy successful for therapeutic treatment moving forward however, it will be necessary to harness an optimized DNA platform such as dbDNA.

Q Can you tell me the most surprising thing that you have learned in your preclinical work to date?

CM: We learn new things every day, as long as we stay curious and open-minded. Sometimes, it is from the unexpected results that great discoveries are made. One example of this comes from our own preclinical study. Despite the common belief that plasmid DNA or dbDNA will disappear quickly following non-viral gene transfer, resulting in only transient transgene expression, we observed something quite different. Following non-viral delivery of either plasmid DNA or dbDNA, part of the unintegrated episomal DNA can be condensed by histones and protected from degradation. It can therefore remain stable inside the cells for a very long time, generating persistent expression. They are not permanent, like an integrated copy. However, non-viral delivery methods are less immunogenic than viral methods and can therefore be used to deliver cargoes repeatedly. For example, in hemophilia patients, non-viral gene delivery of factor VIII or factor IX genes every few years dramatically improves treatment options for these patients. Repeated treatment also allows for effective therapeutic treatment of many other genetic diseases.

It is amazing to me that we did not realize this earlier on. In the beginning, we were just using the non-viral strategy to test these constructs, but when we found that they are actually persistent, we got very excited. From there, we started trying to further improve the non-viral delivery technologies so that we could improve the efficiency of delivery.

Q What are you most excited about in terms of the Seattle Children's Research Institute's work over the next few years?

CM: Seattle Children's Research Institute has a lot of outstanding investigators. I am quite excited to work here, not only because of the excellent investigative team developing basic and translational research, but also because of its capacity to translate novel technologies into actual patient care. This is made possible because Seattle Children's is composed of three parts: the Hospital, the Research Institute, and the recently established Seattle Therapeutics.

Overall, one of our major goals in the next few years is to develop effective gene and cell therapy technology. Hopefully, we can also push forward more and more clinical trials to treat genetic diseases for patients.

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"I believe that the era of employing non-viral gene therapy to treat human diseases is approaching. The opportunities are immense, but challenges remain as well. So, be creative and persistent."

Q What would you do if you had a 2× increase in your R&D budget? How about a 10× increase?

CM: If I had two times my R&D budget, I would probably expand my team to develop non-viral gene delivery techniques and gene editing strategies. That is my focus in the lab already, but we are always restricted by the funding. We also have a lot of exciting projects that I would like to push forward and develop, which I could do with more funding.

If I had ten times my current funding, I would quickly push forward these new technologies into clinical trials, as some of them are nearly ready to make that step. However, with our current funding, we would have to wait and see if there were companies who might be interested in collaborating with us to push forward into clinical trials, since they do require a lot of funding.

Q What other research would you like to know more about?

CM: It would be great to find other collaborators on clinical trials using developed technologies. We recently had a company that was interested in helping us to target liver sinusoidal endothelial cells using their proprietary technology. Those are the kinds of things in which we are very interested; seeking collaborative opportunities.

Q What advice would you give other non-viral gene delivery researchers?

CM: Persistence is key. It took us many years to get to where we are today. However, I believe that the era of employing non-viral gene therapy to treat human diseases is approaching. The opportunities are immense, but challenges remain as well. So, be creative and persistent.

BIOGRAPHY

CAROL H MIAO is a principal investigator at Seattle Children's Research Institute, and a professor in the Department of Pediatrics at the University of Washington School of Medicine. Dr Miao's research focuses on the development of better treatment for hemophilia patients and gene therapy strategies for treating genetic diseases. Dr Miao has been awarded funding support by National Institutes of Health, National Hemophilia Foundation, and others. Her major research programs include: development of safe and efficient gene therapy approaches suitable for clinical applications including nonviral gene delivery via ultrasound-mediated gene delivery (UMGD) or lipid nanoparticles (LNPs); gene editing technology; Intraosseous delivery of lentiviral vectors targeting HSCs; and development of immunomodulation strategies to combat anti-factor VIII inhibitors; and study of the signature and impact of glycosylation on inducing anti-factor VIII immune responses.

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY PLATFORM EVOLUTION
PART 2: NON-VIRAL

SPOTLIGHT

INTERVIEW

Developing non-viral delivery & cell engineering technologies for *ex vivo* T cell immunotherapies

Lipid nanoparticles and electroporation are just two of the non-viral gene delivery technologies with potential to revolutionize the CAR-T cell immunotherapy field by removing issues of cost and complexity that come with virally transduced cell therapy manufacturing processes. **David McCall,** Senior Editor of BioInsights, asks Bristol Myers Squibb's **Julie Shi** (Scientific Associate Director, Immuno-Oncology and Cell Therapy Thematic Research Center) and **Nirveek Bhattacharjee** (Senior Principal Scientist) about the relative pros and cons and development status of the various non-viral cell engineering tools and technologies available today.

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Q What are you both working on right now?

JS: Nirveek and I are part of a team that specializes in non-viral delivery technologies for *ex vivo* applications, particularly in T cell therapy. Our team takes initial product concepts from research stage to the first iteration of a manufacturing process.

Q What do you regard as some of the key challenges and considerations as the cell and gene therapy space at large looks to apply lipid nanoparticle (LNP) technology?

JS: In the field of cell therapy, the integration of a chimeric antigen receptor (CAR) or engineered T cell Receptor (eTCR) into T cells is typically accomplished using a viral vector. The main challenge lies in finding a way to stably integrate a CAR using a non-viral vector, LNPs as an example. Currently, LNPs are used in *ex vivo* cell therapy for encapsulating gene editing components to induce a double-stranded break or for delivering mRNA that encodes the CAR cassette, which only leads to temporary CAR expression. With advancements in genome editing, there is growing interest in encapsulating mRNA-based 'gene writing' technologies, which can result in DNA being directly written into the T cell genome at specific genomic loci. Other technical hurdles include improving the delivery efficiency of LNPs compared to viral vectors, determining the appropriate payloads to encapsulate in LNPs, and assessing the impact of LNPs on cell health and function, separate from the effects on the payload.

Q Where is the cutting-edge within the field of electroporation and mechanoporation? What are the pros and cons compared to other viral and non-viral cell engineering technologies?

NB: Electroporation has been around for decades in research labs. However, the application of electroporation for cellular immunotherapy is a more recent development. There were two primary reasons for that. One is biological, which is that primary T cells are in general difficult to transfect. The second is technical, which is related to the scaling up of a primarily research-scale instrument and protocol to closed, good manufacturing practice (GMP)-compatible equipment and process. To address the delivery efficiency challenge, the applied electrical field, pulse characteristics, waveform, electrode configuration and buffers are some of the different parameters that have been optimized by different electroporation platforms to enable delivery of nucleic acids into T cells. In addition, significant instrument and consumable development, as well as improvements in processes adjacent to the electroporation coperation, have helped make it a more feasible option for cell and gene therapy. Conventional or bulk electroporation, where a large number of cells mixed with the molecular gene editing materials are electroporated in a batch-flow manner, is now a fairly mature technology. In contrast to batch-flow, other electroporation platforms that combine continuous fluid flow with electric fields promise a more seamless scaling from discovery to manufacturing.

The biggest advantage of viral vectors is that they enable highly efficient gene delivery. Electroporation is not able to reach these kinds of efficiencies, and it also causes the cells to

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"The genome editing field is also experiencing exciting advancements, such as finding alternatives to double-stranded breaks and exploring mRNA delivery methods for integrating DNA into the genome."

– Julie Shi

have lower recovery and survival rates. The impact on cell health and cell function that is part of the electroporation process necessitates strategies to mitigate those kinds of deleterious effects. The disadvantages of viral vectors revolve around their nature as a biologic, which results in production and supply chain complexities, long development time, cost of production, and an analytical release testing burden, all of which are less of a factor with non-viral delivery with electroporation.

Mechanoporation is a newer way of delivering exogenous material into cells. It uses mechanical shear or compression forces, typically generated in a microfluidic chip to disrupt the cell membrane and enable external cargo to enter the cells. The main advantage of mechanoporation is that it is gentler than electroporation. It does not lead to the kind of cell changes you see with electroporation, including at a transcriptomic level. People are excited about it, although the efficiencies achievable with mechanoporation do not yet reach those achieved with electroporation. So, there is a trade-off between cell health and delivery efficiency. Another key challenge with mechanoporation surrounds the actual integration of the operation into a drug product process, or into a process with upstream and downstream steps. It is still early days, but mechanoporation is certainly an interesting approach.

Q What do you see on the horizon for non-viral cell engineering that particularly excites you?

JS: I'm enthusiastic about the use of LNPs, especially considering the rapid growth of the field in recent years, primarily for *in vivo* applications. However, it would be beneficial for researchers to explore the technology's potential in *ex vivo* cell therapy manufacturing as well. The genome editing field is also experiencing exciting advancements, such as finding alternatives to double-stranded breaks and exploring mRNA delivery methods for integrating DNA into the genome.

Moreover, the overlooked aspect of cell-free production of essential raw materials like nucleic acids is worth considering. How can we develop methods for producing these raw materials without relying on *E. coli* or other cellular systems? Advances in cell-free protein

"The genome editing technologies beyond CRISPR are very exciting. The other piece I am keeping an eye on is the multiple technologies that are looking to more precisely perturb the cell membrane in order to have less harmful effects."

– Nirveek Bhattacharjee

and nucleic acid production can help support widespread adoption of non-viral engineering for cell therapy.

NB: The genome editing technologies beyond CRISPR are very exciting. The other piece I am keeping an eye on is the multiple technologies that are looking to more precisely perturb the cell membrane in order to have less harmful effects than electroporation or even mechanoporation. These are nascent technologies, but it will be interesting to see where they go.

Q Can you give us a comparative review of the non-viral tools with which you work with a particular focus on their translation into the clinical setting?

NB: Bulk electroporation is a mature technology and there are multiple options out there ready to be implemented in clinical manufacturing. The other technologies, like mechanoporation, are not there yet in terms of implementation in a clinical manufacturing process.

JS: LNPs are currently used in clinical manufacturing for at least one *ex vivo* cell therapy in the industry, specifically for gene editing. However, extending the use of LNPs to facilitate genomic integration of the CAR transgene has not yet been described in the published literature. The primary challenge lies in efficiently delivering the required payloads to replace viral vectors or electroporation in cell therapy.

Q What are the most important considerations in terms of critical raw materials?

JS: Since many of the raw materials used for non-viral delivery are relatively new to the field, there are limited contract manufacturing organizations prepared

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to produce fully GMP-compliant materials. To address this challenge, we collaborate with vendors early on to guide them through the process and determine the phase-appropriate integration of raw materials into our drug product processes. When engaging with different vendors for new raw materials, ensuring the materials meet the necessary quality standards for GMP manufacturing is crucial. Factors such as the vendor's manufacturing capacity and capability, technical expertise in the production process, experience with regulatory agencies, and their ability to scale up production must all be carefully considered during the evaluation of the supplier for these critical raw materials.

Q What is the most surprising thing you have learned during your research in the non-viral delivery space?

NB: The electroporation technologies that have been around for a while still seem to be at an early stage of development when considering clinical stage manufacturing, which is surprising.

In addition, T cells are a difficult primary cell type to transfect and to get gene delivery or editing materials into. The scale-up process from a research-based concept to something that is feasible and applicable for cell and gene therapy manufacturing, and how many associated factors come into play in realizing a technology, has definitely been surprising.

Q What are you most excited about in terms of the cell therapy field in general over the next few years?

JS: CAR-T therapy has revolutionized the treatment landscape for patients without viable alternatives, particularly for hematological malignancies. However, achieving the same clinical effectiveness as has been seen with liquid tumors in solid tumors presents significant challenges. The ongoing efforts to address these issues are highly promising, though. I am eagerly looking forward to witnessing the field's progress in the coming years, especially with regard to technological advancements in genome editing. Additionally, there are notable advancements in cell therapy processing, particularly in terms of automation, which are shaping the evolution of the cell therapy field.

Q Are there any specific technologies or research in academia or industry that you would like to know more about?

JS: An aspect that is frequently neglected is the production of the critical raw materials involved in non-viral cell manipulation. There is an emerging field of research

focusing on engineering the enzymes used in the production of these critical raw materials. Exploring this area would provide valuable insights and enable the development and implementation of non-viral delivery processes.

Q Finally, what parting advice would you each give other non-viral delivery researchers in the field?

NB: It is important to gain experience working in cell therapy. Understanding the integration of technologies and what scaling up means from a research scale to a manufacturing or clinical scale is key. Getting an understanding of what it actually means to scale up and what the different fac-tors are is helpful, as is knowledge of how to design research.

JS: Conducting experiments entails a technical aspect, but successfully developing a drug product by integrating these technologies in a drug product process requires additional expertise. Manufacturing, supply chain, and regulatory expertise are also important for advancing these innovative technologies and plat-forms into clinical applications.

BIOGRAPHIES

JULIE SHI is an Associate Director in Cell Therapy Process Research and Engineering at Bristol Myers Squibb working on developing non-viral gene delivery technologies for cell therapy applications. She completed her PhD in Bioengineering at the University of Washington followed by a post-doctoral fellowship in Biochemistry at the University of Toronto. Julie then joined Juno Therapeutics to establish technology capabilities for *ex vivo* non-viral and gene editing process development, and has had project leadership roles in lipid nanoparticle development and allogeneic platform technology innovation. Outside of work, she enjoys exploring her home city of Seattle with her spouse and three children.

NIRVEEK BHATTACHARJEE is a Senior Principal Scientist in Cell Therapy Process Research and Engineering at Bristol Myers Squibb working on non-viral technology and process development for cell therapy. He has a PhD in Biomedical Engineering from Johns Hopkins University, and a post-doctoral fellowship in Bioengineering at the University of Washington. As a research scientist at University of Washington, he developed microfluidic technology for cell-based research and clinical applications. He then joined Juno Therapeutics (Celgene) to work on non-viral gene delivery and editing technology and platform development, and its implementation in *ex vivo* clinical scale manufacturing. When not at work, he loves planning road-trips and exploring local Seattle food, parks and events with his partner and 3-year old.

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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

INTERVIEW

Exploring current shortfalls and potential improvements for non-viral gene delivery in cystic fibrosis

Within the last decade, the treatment options for patients with cystic fibrosis (CF) have greatly improved with the rise of genotype-specific therapies. However, there remain patients for whom these therapies do not work. **David McCall,** Senior Editor at BioInsights, speaks with **Uta Griesenbach,** Professor, Imperial College London, about her research with both viral and non-viral gene therapy approaches aimed at these treatment-resistant patients, and discusses next steps in the space for non-viral delivery in particular.

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Q Can you give us some background to your work with gene therapy for cystic fibrosis?

UG: I have worked in the cystic fibrosis (CF) space for more than 25 years now. My work started when I did my PhD at The Hospital for Sick Children in Toronto, Canada, in the lab of Lap-Chee Tsui, who was involved in cloning the cystic fibrosis gene. Very early on, I got interested in cystic fibrosis gene therapy.

"Over the last 5–10 years, the therapeutic options for patients with cystic fibrosis have vastly improved due to cystic fibrosis modulator therapies, which are very effective for many patients. These modulator therapies are genotype-specific and for about ~85% of cystic fibrosis patients, they have revolutionized treatment."

Following my PhD, I joined Imperial College London and Professor Eric Alton. We first worked on non-viral vectors for cystic fibrosis (CF) gene therapy, which we carried into a Phase 2B study. About 15 years ago, we developed a lentiviral vector specifically designed for airway gene therapy. In 2021, this vector was licensed to Boehringer Ingelheim and a first-in-human trial is in final preparation for regulatory submission.

Q How would you summarize the current picture in terms of existing therapeutic options for cystic fibrosis patents?

UG: Over the last 5–10 years, the therapeutic options for patients with CF have vastly improved due to CF modulator therapies, which are very effective for many patients. These modulator therapies are genotype-specific and for about ~85% of CF patients, they have revolutionized treatment.

The remaining 10–15% of patients are defined as modulator insensitive, which means they either do not have the right genotype to respond to modulators or they develop side effects to the modulators. These are the patients that we and others are developing genetic therapies for because they have no alternative treatment.

Q What have been the key recent challenges and breakthroughs in CF gene therapy R&D?

UG: The development of genetic therapies for CF has gained a lot of interest in the last few years. This is largely due to the fact that everybody now appreciates that these modulator-insensitive patients are being left behind. There are a number of companies as well as academic groups working in this space. For example, biotech companies such as 4D Molecular Therapeutics and Spirovant Sciences are developing AAV-based gene therapy vectors.

Following on from the success of the mRNA vaccines against COVID-19, utilizing mRNA therapeutics for CF has recently gained interest. There is more development now in non-viral formulation, again, mainly related to the delivery of mRNA. There is also our own

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pseudotyped lentiviral vector, which has a number of unique properties, namely a single dose supports long lasting and stable gene expression (2 years in mouse models) and in contrast to other viral vectors, our lentiviral vector retains efficacy when repeatedly administered—we are looking forward to the results of the upcoming first-in-human clinical trial.

Q You co-authored a paper in 2016 **[1]** with the Cystic Fibrosis Consortium—can you give us an update on the consortium's activities in this space since then, learnings that have been derived from this particular study, and what is still needed in the way of innovation and knowledge to tackle this disease with a gene therapy approach?

UG: This paper was really the first evidence that gene therapy to the lungs of CF patients can change what we call a clinically relevant endpoint, which is lung function, rather than changing 'just' molecular biomarkers. As part of this research, we have shown that, when we are comparing the actively-treated group to the placebo-treated control cohort, we can stabilize CF lung disease. At the end of a 12-month observation period, there was a significant difference in lung function between the two groups.

However, when we looked at the data, we had to admit to ourselves that this difference in lung function, which was around 4% comparing the active and the placebo groups, was not in a clinically meaningful range. Can a patient benefit from or feel a 4% change in lung function? The answer to that is probably 'no'. At the time, that decision was based on the more significant changes people had been seeing with the CF modulators. So, we concluded that non-viral gene transfer to the lungs was safe and could stabilize lung disease, but that better vectors are needed before we can move into Phase 3 studies and ultimately, licensing.

In parallel to our non-viral program, we had developed the novel lentiviral vector, which has surface proteins from the Sendai virus, which makes it very good at getting into airway epithelial cells. In preclinical models, we were able to show that we could get log-order higher gene transfer efficiency than with the non-viral formulation. Therefore, moving to the lentiviral vector was a logical progression in terms of moving forward with the CF program. That has recently paid off with Boehringer Ingelheim acquiring the license to this product.

Q You have gone ahead with a viral approach. How does the non-viral gene delivery space need to keep evolving in order to create new and better opportunities to advance the field?

UG: The lipid nanoparticles that are now developed by many companies to deliver mRNA to the lungs and the other organs are showing really good promise. I think one of the reasons why our non-viral formulation was not as optimal as we wanted it to be is because we delivered large plasmid DNAs. These large plasmids need to get into the nucleus of cells, which is very difficult in differentiated non-dividing cells. By contrast mRNA

complexed to lipid nanoparticles 'only' needs to get into the cytoplasm because in the cytoplasm, the mRNA is translated into protein; they do not have to overcome the nuclear membrane barrier. Time will tell, but I think there is a lot of promise for non-viral gene transfer agents in the context of mRNA delivery for CF and maybe other lung diseases. However, one has to keep in mind that mRNA is rather unstable and will require very frequent re-administration, which may impact on patient compliance.

I would also say that maybe CF was not the best target disease for non-viral gene therapy, because you need a fairly large number of cells transduced to express enough chloride channels to correct the ion transport. If there is a disease that could be treated with a secreted protein, for example, where it would not matter as much how many cells you are transducing to see a therapeutic effect, then non-viral approaches may be more efficient. We are actually working now on a disease that requires only very low levels of a secreted protein.

Q Finally, can you sum up some important next steps that you would like to see the advanced therapies community take to address unmet medical need in cystic fibrosis?

UG: Manufacturing needs to be improved because at the moment, the cost of goods for gene therapy-based treatments in general is far too high to be affordable to healthcare providers in the long run, particularly as more and more gene therapy-based medicines are being developed and licensed.

Another area for improvement is the training of allied healthcare professionals to support not only the clinical trials but also the delivery of licensed medicines. That has certainly been recognized as a big bottleneck in the field. For example, I recently went to a meeting of the European Clinical Trials Network, which a lot of research doctors and nurses attended. They told us, "We do not understand the differences between a lentivirus and an mRNA-based therapy and an antisense therapy—how do you expect us to communicate what the clinical trials are about, and the pros and cons of each of these approaches to our patient populations?" We need to spend more time and effort to develop learning and teaching materials for allied healthcare professionals to allow them to communicate treatment options to their patients.

BIOGRAPHY

UTA GRIESENBACH is a Professor in Molecular Medicine at Imperial College London, ex-President of the British Society for Gene and Cell Therapy (2017–2021), a director (non-executive) of the Cell and Gene Therapy Catapult and co-founder of AlveoGene (2023). Uta has over 25 years of experience in developing advanced therapeutic medicines. Her research interests are related to the development of gene and cell therapy-based treatments for cystic fibrosis and other lung diseases. As part of her translational research, Uta has overseen vector and biomarker development, toxicology studies, as well as GMP vector manufacturing. Uta is co-investigator on several gene therapy trials. Uta is a Strategy Group member of the UK Respiratory Gene Therapy Consortium and a member of the MRC/LifeArc Innovation Hub Co-ordinating Group. In addition, Uta is interested in teaching and work-force development. She is Deputy Director for post-graduate research at the National Heart and Lung Institute, Program Director for the MSc in Genes, Drugs and Stem Cells–Novel Therapies at Imperial College London and chairs the MRC/LifeArc Innovation Hub Skills and Training Committee.

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AUTHORSHIP & CONFLICT OF INTEREST

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UPSTREAM PROCESSING

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VECTOR CHAIN CHANNEL: UPSTREAM PROCESSING

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UPSTREAM PROCESSING

RESEARCH ARTICLE

Can novel bioreactors improve the cost of goods of viral vectors?

Chris Stamatis, Alex Chatel & Suzy Farid

Lentiviral and adeno-associated viral vectors make up the vast majority of gene therapy candidates for *in-vivo* and *in-vitro* applications. While effective for treating a range of debilitating diseases, they are also currently very expensive to produce, which hampers patient accessibility. While other biologics have been studied and optimized for several decades, viral vectors still suffer from relatively low titers, difficulty in scaling up and poor downstream recovery. A review of available technologies focusing on upstream solutions highlights that despite the development of randomly packed bed bioreactors for adherent cells and the move to suspension cell cultures in stirred tank bioreactors, technology design flaws hamper efforts to cost-effectively bring new therapies to the market. In this paper, the scale-X™ and NevoLine™ technologies are shown to provide conditions that support two to ten-fold increase in cell specific productivity for AAV and LVV relative to alternative technologies, which results in drug substance cost of goods reduction between −18% and −61%. Furthermore, increased titers, smaller footprint and reduced complexity could improve the efficacy of facility utilization.

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AAVS & LVVS WILL LIKELY DOMINATE THE CLINICAL & COMMERCIAL LANDSCAPE FOR YEARS

The cell and gene therapy field is one of the fastest growing fields in biopharmacy as it offers the potential to treat diseases that, up until now, had no cure. Together, Adeno-Associated viruses (AAVs) and Lentiviruses (LVVs) make up the vast majority of both marketed drugs and clinical candidates. Out of 489 known clinical trials covering both cell & gene therapies, a substantial

74% (364) are using either AAV (129) or LVVs (235) [1]. At the time of writing, 5 AAV (Luxturna® (Spark Therapeutics / Novartis), Zolgensma® (AveXis / Novartis), Roctavian® (BioMarin), Hemgenix® (CSL Behring), Upstaza® (PTC Therapeutics)) and 5 LVV therapies (Kymriah® (Novartis), Zynteglo® (Blubird Bio), Breyanzi® (Bristol Meyer Squibb), Abecma® (Bristol Meyer Squibb & Bluebird Bio) and Lipmeldy® (Orchard Therapeutics)), have been marketed. Growth forecasts for both vectors differ as a function of assumptions used, but sources agree that an annual market growth ~15–20% can be expected at least until 2030 [2–6] which is in line with the expected doubling in gene therapy market approvals in the next few years [1].

The popularity of AAVs and LVVs can be explained by their efficacy at delivering genes. AAV's low toxicity and the availability of several natural AAV serotypes offering broad tropism properties make them especially suited for *in-vivo* use. On the other hand, LVV's ability to deliver larger payloads and target immune and stem cells make them especially suited to *ex-vivo* cell therapy [7]. To this day, marketed AAV therapies are for *in-vivo* use only whereas marketed LVV therapies are for *ex-vivo* uses only, although several *in-vivo* applications are currently in the clinical pipeline with the latter (e.g., [8–10]).

PHYSICO-CHEMICAL PROPERTIES & SUPERNATANT EXPRESSION LEVEL DICTATES BIOPROCESSING CHOICES

AAVs and LVVs are fundamentally quite different from one another: AAVs are small (20–25 nm diameter), non-enveloped ssDNA viruses that carry a relatively small transgene (~4.7 kb) [11]. There are 11 known naturally-occurring serotypes, which do not cause any known human diseases. The degree of extra-cellular viral release depends on the serotype, but cell lysis is most of the time required to recover the viral vectors. Although relatively stable compared to other viruses at room temperature and physiological pH, active (full) AAVs are co-expressed during cell culture with non-functional AAV viral capsids (partially filled or empty) which must be removed during downstream processing.

Traditionally density gradient ultra-centrifugation has been employed to remove empty capsids, which is costly, time-consuming, and not currently scalable using single-use technologies. More recently, chromatographic techniques have demonstrated capability to remove empty capsids [12]. Additionally, AAV particles tend to stick to cell debris and some plastic materials which further adds to the recovery challenge [13–15]. Finally, they must be concentrated several-fold for final formulation, making the removal of impurities a particular challenge as they can co-concentrate with the product [11].

LVVs, on the other hand, are much larger (80–100 nm diameter) enveloped ssRNA viruses carrying a larger transgene (up to 10 kb) [16] which naturally buds from cells into the supernatant upon maturing, which means that lysis is not required to recover them. Compared to AAVs, LVVs display poor stability: they degrade quickly at room temperature and with exposure to shear forces, salt and pH gradients [7] which causes their downstream recovery yields to be typically in the range of 30% and lower.

CURRENT TECHNOLOGY CHOICES CANNOT FULLY MEET DEMAND

By far the most common production method both for AAVs and LVVs is additive-based triple-transfection (mediated by lipid (e.g., Lipofectamine™) or polymers (e.g., polyethyleneimine) complexes or simpler chemicals (calcium phosphate)) in HEK293 cell lines, although packaging (containing some of the required recombinant genetic material) or producer cell lines (containing all of the required recombinant genetic material) are being developed. The transfection process

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has drawbacks as Good Manufacturing Practice-grade plasmid DNA is very costly [17] and the process itself is delicate to execute and scale [18], but for the time being it is generally accepted as the most convenient solution available. For AAVs, production with baculovirus or herpes simplex virus (HSV) helpers is also possible but less exploited today [11]. The HEK293 cell line is originally adherent but growing it in this manner usually requires serum, which brings regulatory, supply and cost constraints. Furthermore, adherent culture requires scalable bioprocessing solutions for high demand applications. Manual adherent technology such as multi-tray dishes are acceptable for very low production scales (e.g., Leber congenital amaurosis (LCA) or aromatic l-amino acid decarboxylase deficiency (AADC)); yet developers are steadily moving to suspension processes using suspension HEK293 cell lines and single-use stirred-tank bioreactors (SUBs), as these are perceived as a more suitable solution to reach higher throughputs and economies of scale. However, high-volume bioreactors alone are not sufficient to meet the demands of high dose or high prevalence diseases such as spinal muscular atrophy (SMA), cystic fibrosis or hemophilia (Figure 1).

Though theoretical, this exercise uses real-world data and highlights that unless innovation brings significant improvements to existing processes and technologies, some therapies are doomed to remain excessively expensive or even impossible to produce in sufficient quantities to treat the patients who need it. Some relatively recent examples illustrate this: Kymriah (Novartis), a chimeric antigen receptor (CAR)-T autologous cell therapy against acute lymphoblastic lymphoma, was priced at $475,000 in the US and is estimated to have a cost of goods (CoG) per dose in the region of $100,000–$200,000 [19], or Zolgensma (Novartis), an AAV-9 systemic injection therapy against SMA, priced at $2.1 million (cost of goods unknown) [20].

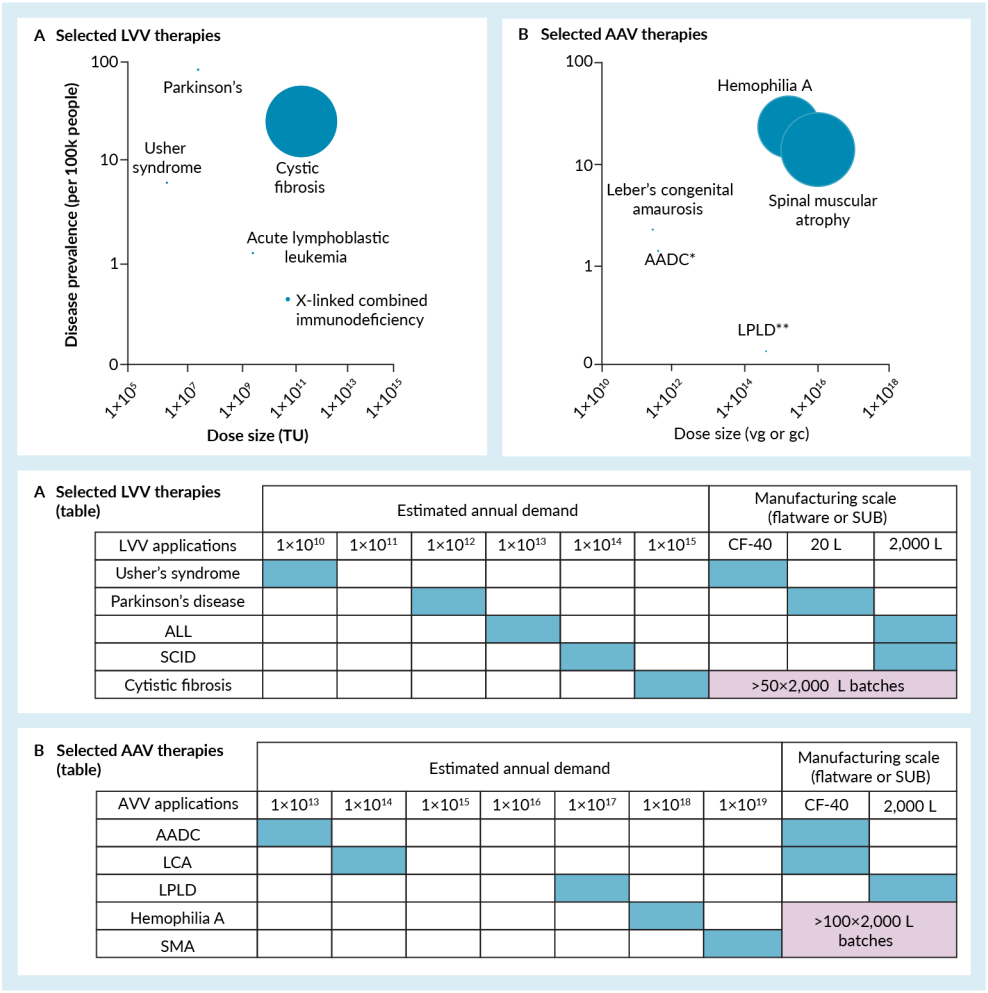
One of the basic assumptions in calculations, such as the ones used to generate the data in Figure 1, is the achievable production titer and the downstream yields of the target vector. While improving the latter is helpful, some of the more impactful ways to achieve the required throughput cost effectively are to significantly increase the viral productivity per unit volume either by increasing the cell-specific productivity (the antibody industry did it 100–200-fold over 40 years) or by increasing the total number of cells per unit volume.

THE MOVE TO SUSPENSION ALLEVIATES SOME PAINS BUT CREATES OTHERS

For this purpose, SUBs provide large scale options which help reduce production costs with scale and automation and enable the removal of serum from the process thanks to suspension cells. However, having little evolved since their introduction several decades ago, they also suffer from basic design limitations, detailed here below, which makes scale-up of the cell culture conditions, transfection and other process steps, such as lysis for AAV, challenging especially at scales above 200 L (Univercells Technologies' observation from individual conversations with contract development and manufacturing organizations (CDMOs) and developers in North America, Europe and Asia as well as cell and-gene therapy conference material in 2020–2022). Indeed, it is difficult but important to maintain homogeneous conditions during the scale-up above 200 L while minimizing shear, especially for shear sensitive processes such as during the transfection step (because DNA/polymer polyplexes are highly shear sensitive) and when producing LVVs which quickly degrade when exposed to higher shear forces [7]. Another limitation is that increasing the cell density is possible but requires an additional cell retention device, adding to cost and complexity. It is not currently possible to exceed cell concentrations above 2–4 million cells/mL without causing aggregation and therefore drops in cell-specific productivity,

FIGURE 1

Disease prevalence and dose size for selected (A) AAV- and (B) LVV-based therapies. In the bubble plot, the bubble size is proportional to vector quantity requirement relative to the disease prevalence. The annual demand is computed by multiplying the dose size with an estimation of the yearly number of patients to receive the therapy (1 dose per patient). To estimate the size and number of batches required to meet the demand, the following is used: for LVV, an average titer of 1×10^8 TU/mL and 30% DSP yield is assumed. For AAV, an average titer of 5×10^{10} vg/mL and 40% DSP is assumed. In both cases a cell density at transfection of 1×10^6 cells/mL is assumed. If available the yearly number of patients to receive therapy is used; elsewhere, it is estimated using the disease prevalence with a maximum of 10,000 patients per year.



A Selected LVV therapies (table)

	Estimated annual demand						Manufacturing scale (flatware or SUB)		
LVV applications	1×10^{10}	1×10^{11}	1×10^{12}	1×10^{13}	1×10^{14}	1×10^{15}	CF-40	20 L	2,000 L
Usher's syndrome	■						■		
Parkinson's disease			■					■	
ALL				■					■
SCID					■				■
Cytistic fibrosis						■	>50×2,000 L batches		

B Selected AAV therapies (table)

	Estimated annual demand							Manufacturing scale (flatware or SUB)	
AVV applications	1×10^{13}	1×10^{14}	1×10^{15}	1×10^{16}	1×10^{17}	1×10^{18}	1×10^{19}	CF-40	2,000 L
AADC	■							■	
LCA		■						■	
LPLD					■				■
Hemophilia A						■		>100×2,000 L batches	
SMA							■		

ALL: Acute lymphoblastic lymphoma; AADC: atic l-amino acid decarboxylase deficiency; LCA: Leber congenital amaurosis; SCID: Severe cross-linked combined immunodeficiency; LPLD: Lipoprotein lipase deficiency; SMA: Spinal muscular atrophy.

which is why most transfections are done at densities ~1–2 million cells/mL [22,23]. Additionally, STRs are not designed for easy media exchange and washing steps, which are desirable for transfection-based processes. STRs also generate one large bulk harvest volume that contains both the product and cell culture impurities, such as debris, DNA, Host Cell Proteins (HCPs), etc., especially for processes requiring *in-situ* lysis like most AAV serotypes. Consequently, downstream process unit operations must be sized accordingly to handle the large bulk harvest volume produced by STRs.

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CELL CULTURE NEEDS A PARADIGM SHIFT

As an alternative, single-use fixed-bed bioreactors embody a paradigm shift in bioprocessing by adapting the technology to the cells as opposed to the other way around. There are two types of fixed-bed bioreactors: the first generation packed (i.e., non-structured) bed bioreactors (PFBs), such as the iCELLis® from Pall, the BioBLU® with packed-bed Fibra-Cel® basket from Eppendorf and the TideXCell® from Cesco Bioengineering, and the novel second generation structured fixed-bed bioreactor (SFBs) such as the scale-X range. Originally developed to provide a scalable solution for adherent cells, PFB bioreactors have shown limitations probably due to the random (and variable) packing nature of the bed leading to inhomogeneities in cell and media distribution and resulting in difficulties in scale-up in dropping productivities at scale [24]. This scalability limitation is addressed with the structured fixed-bed, designed to provide a homogeneous environment for cell growth regardless of size, where cells and media are evenly distributed throughout the fixed-bed, leading to consistently higher productivity (up to ten-fold increases reported for LVV [25] compared to a packed bed, two-fold higher relative a SUB [26] , four-fold for AAV [27] compared to a packed-bed bioreactor, and three-fold compared to an SUB [28].

THE SCALE-X STRUCTURED FIXED-BED TECHNOLOGY IS A NOVEL SOLUTION FOR SUSPENSION & ADHERENT CELLS

The scale-X range features several scales for development, clinical and commercial production (scale-X hydro bioreactor is 2.4 m^2 of growth surface, scale-X carbo bioreactor is 10 or 30 m^2 and scale-X nitro bioreactor is 200 or 600 m^2) and the larger bioreactor can grow at least as many cells as a 2,000 L bioreactor but at a fraction of the working volume (the scale-X nitro bioreactor vessel is 60 L). An additional benefit of SFBs is their suitability to grow both adherent and suspension cell lines, which not only addresses the worries about reliance on serum but also enables the simplification of adherence-dependent seed trains, which can be operationally burdensome at larger scale. Nonetheless, for adherent processes, technologies exist today (e.g., the scale-X™ cell collect module) that enable the recovery of cells from a smaller SFB to seed a larger one, thereby also simplifying the process. Finally, the scale-X range further reduces the process footprint by integrating in-line clarification and concentration, resulting in an automated platform that combines several process steps in one.

For AAV production, where most processes require *in-situ* lysis, the combination of the low bioreactor volume and cell immobilization means that wash steps can be introduced in the process, enabling the development of advanced harvest procedures that retain many cell impurities (debris, DNA, proteins) while collecting the AAV product. Additionally, the product can be recovered highly concentrated as it is released via lysis within a bioreactor vessel volume, thereby simplifying and reducing the footprint of downstream processing (DSP) operations. For LVV, a budding virus, biomass immobilization enables the development of perfusion harvest protocol without the need of an external cell retention device and allows collection of the production fraction at conditions which prevent product degradation (e.g., at 4°C). Going one step further towards intensification, the NevoLine™ Upstream system integrates clarification and in-line concentration with the scale-X nitro bioreactor, in such a way that the harvest material containing the product can be clarified and concentrated further in-line without needing additional processing equipment. The result is a highly concentrated harvest, a fraction of the volume generated by an equivalent SUB process, and with less cell culture impurities such as HCP and DNA [29].

COST OF GOODS MODELLING HELPS INFORMED DECISION MAKING FOR BIOPROCESSING TECHNOLOGY SELECTION

The following case study aims to explore the impact of technology choice between SUBs, PFBs and SFBs on the drug substance cost of goods (COG) per dose. Evaluating the impact of different technology options on the cost of goods requires decision-support tools capable of simulating the process economics. The department of Biochemical Engineering at University College London has built decisional tools that offer valuable insights regarding the cost-competitiveness of different technologies, their cost drivers and process bottlenecks. They have been developed and used across a range of product modalities, from antibody-based biologics (e.g., [30]) to viral vectors (e.g., [17,31]), mesenchymal stem cells [32–35] , induced pluripotent stem cells [36], chimeric antigen receptor T cells [37] and bioartificial liver devices [38], but no study has yet compared the fixed-bed technology with traditional SUBs. The following details the method and assumptions used in the model and follows to demonstrate the impact of technology choice for the COG.

MATERIAL & METHODS

Material: cost of goods modelling tool overview

To perform a COG analysis for the different viral vectors (AAVs & LVVs), a process economics model developed in the Department of Biochemical Engineering, University College London [17,31], was extended to incorporate the scale-X technology platform. The modelling tool integrates bioprocessing costs (upstream and downstream reagents buffers, labor, consumables, QC testing and indirect costs) but for this study does not include fill-finish; the information given in this paper refers to drug substance. User inputs to the model are bioprocessing data (e.g., expression titers, downstream processing yields), equipment footprints and the cost of resources. A list of inputs and assumptions for the model is available in previously published work [17,31].

The model was developed in Python™ (v3.6) and operated through Jupyter Notebook (v5.2.2). A simulation started using the scenario inputs specified in Jupyter Notebook to perform the mass balance, equipment sizing and resources consumption calculations and to determine the COG per dose for each viral vector. The results were stored in a Microsoft Excel spreadsheet for further analysis and visualization. Figure 2 represents a schematic illustration of the basic structure of the process economics model.

Method: scenario analysis formulation

The main objective of this study was to compare the cost-effectiveness of different production platforms including a traditional single-use stirred-tank bioreactor (batch-mode operation), a first generation packed fixed-bed bioreactor and the combination of scale-X cell collect module with the Nevo-Line Upstream platform for the manufacture of viral vectors (batch mode for AAV, perfusion for LVV). Figure 3 shows the process flowsheets for LVVs and AAVs and across manufacturing platforms considered in this study. Additionally, key assumptions related to the manufacturing process in each different platform is summarized in Table 1.

The scenario analysis investigated a range of annual vector demands (driven by 500–10,000 patients per year) and dose sizes for both LVVs (10^8–10^{12} TU/dose) and AAVs (10^{11}–10^{15} vg/dose). For each demand-dose permutation, a scale analysis was performed for each manufacturing platform to identify the one with the lowest COG/dose. Furthermore, the scales leading to the minimum COG/dose for the three manufacturing platforms were compared to

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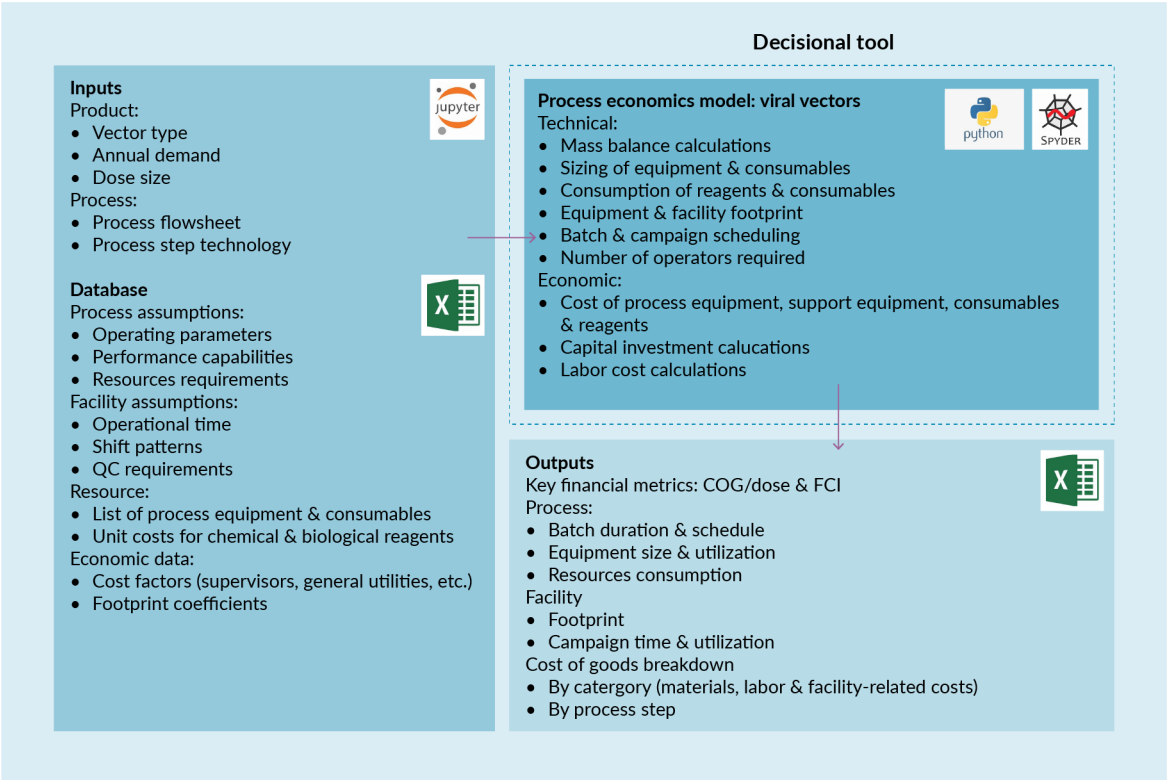
FIGURE 2

Schematic illustration of the process economics model.



COG: Cost of goods; FCI: Fixed capital investment; QC: Quality control.

determine the most cost-effective platform for each demand-dose permutation.

A key difference among the manufacturing platforms that were considered in this study is the cell-specific productivity, where the base case is a two-fold increase in the scale-X SFB compared to both the PFB and the SUB. This is a reasonable and conservative assumption based on results referenced in the first section of this paper. Additionally, to account for the cell retention achieved by design in the fixed-bed bioreactors (both the PFB and the SFB), a clarification filter capacity six times higher is assumed in both cases compared to the SUB. Finally, a sensitivity analysis was performed to evaluate the cost-competitiveness of scale-X bioreactors and NevoLine Upstream platform across a range of cell specific productivities.

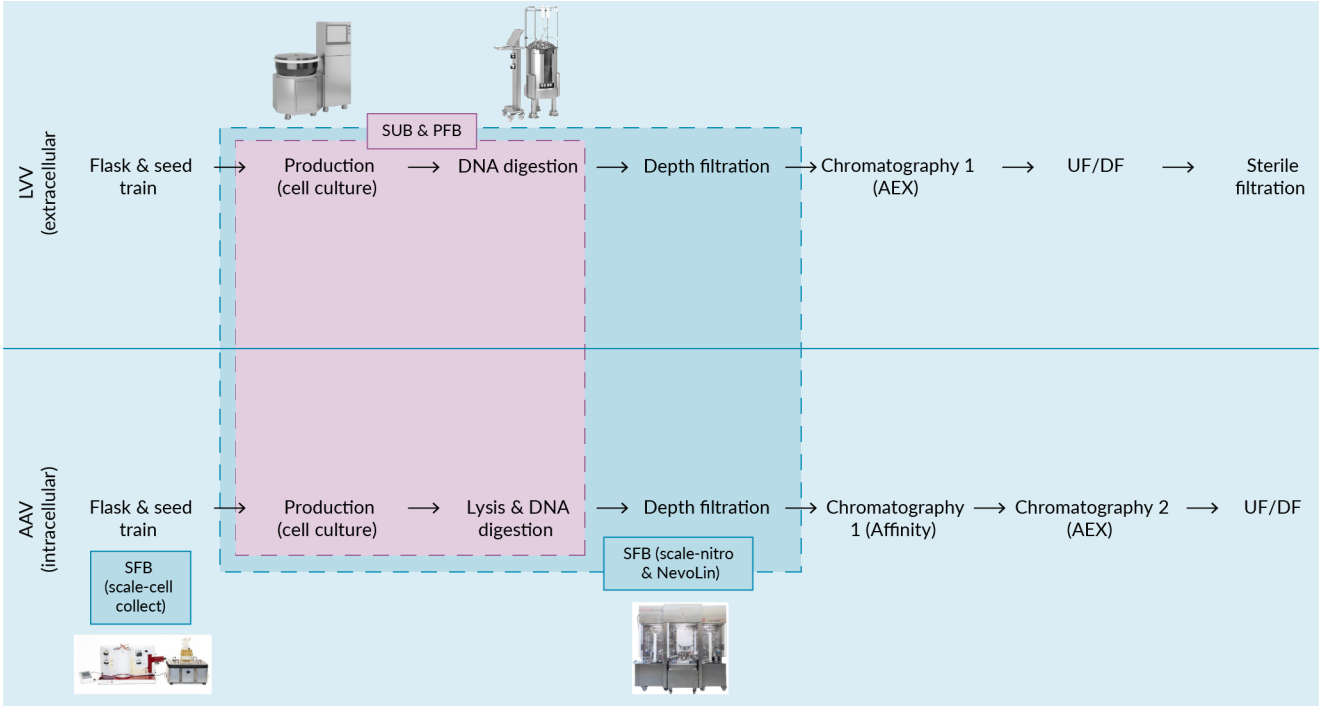
For all scenarios, the model is constrained to limit the number of parallel production lines to maximum 20, and over-production (i.e., the difference between viral vector quantity per batch and the requirement established at any given permutation of demand and dose size) to be lower than 30% in all cases. In the scenarios 'NS' is given as an output when no solution exists that meets the imposed batch number and over-production constraints.

RESULTS & DISCUSSION

Table 2 shows the outcome of the first scenario analysis that demonstrates which scale produces the lowest COG/dose for each dose size and demand permutation. Dictated by economies of scale as demand and dose size increase, the scale leading to the lower COG/dose increases as well. What is observed is that it is mostly the dose size, and not the demand, which drives the need for scale-up,

FIGURE 3

AAV and LVV process flow diagram. In the model presented in this paper, production (cell culture, virus production) and harvest treatment (DNA digestion) take place inside the production bioreactor in all cases. For the SFB, mid-stream clarification is an additional step that takes place in-line inside the NevoLine platform and the inoculum for the production bioreactor is prepared in the scale-X cell collect modules



which reflects the fact that dose sizes vary 10,000-fold across therapeutic target, whereas demand only varies 20-fold between high and low scenarios.

Table 3 shows the relative savings in COG/dose achieved if using the SFB compared to SUB and PFB for each dose/demand permutation. Firstly, looking at the design space,

TABLE 1

Summary of the key process assumptions for the AAV and LVV products COG model.

	AAV			LVV		
	SUB	PFB	SFB (scale-X™ nitro & NevoLine Upstream)	SUB	PFB	SFB (scale-X™ nitro & NevoLine Upstream)
Transfection cell density (10^6 cells/mL for SUB or cm^2 for PFB/SFB)	1	0.35	0.35	1	0.35	0.35
Cell specific productivity (vg/cell or TU/cell)	10,000	10,000	20,000	10	10	20
Media consumption (mL/10^6 cells)	1	1	1	1	1	1
DNA concentration (µg/10^6 cells)	2	2	2	2	2	2
Depth filter capacity (L/m^2)	50	300	300	50	300	300

AAV: Adeno-associated virus; COG: Cost of goods; LVV: Lentiviruses; PFB: packed (i.e., non-structured) bed bioreactors; SFB: structured fixed-bed bioreactor; SUB: stirred-tank bioreactors.

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TABLE 2

Scale analysis for SUB, PFB, SFB across demand and doses sizes for AAV and LVV. The number that follows the technology acronym signifies the scale (in L for SUB and in m^2 for the PFB & SFB). NS means that there is no solution within the technologies used here (either the technology is too large and leads to overproduction, or capacity cannot be met with less than 20 production lines parallel)

		AAV Dose size (vg)					LV Dose size (TU)				
		1×10^{11}	1×10^{12}	1×10^{13}	1×10^{14}	1×10^{15}	1×10^{8}	1×10^{9}	1×10^{10}	1×10^{11}	1×10^{12}
Demand (doses/y)	500	NS	SUB-50	SUB-50	SUB-500	SUB-2000	NS	SUB-50	SUB-250	SUB-1000	SUB-2000
	1,000	NS	SUB-50	SUB-250	SUB-1000	SUB-2000	NS	SUB-50	SUB-250	SUB-2000	SUB-2000
	2,000	NS	SUB-50	SUB-250	SUB-2000	SUB-2000	SUB-50	SUB-50	SUB-500	SUB-2000	SUB-2000
	5,000	SUB-50	SUB-50	SUB-500	SUB-2000	NS	SUB-50	SUB-250	SUB-1000	SUB-2000	NS
	10,000	SUB-50	SUB-250	SUB-1000	SUB-2000	NS	SUB-50	SUB-250	SUB-2000	SUB-2000	NS
	500	NS	PFB-66	PFB-100	PFB-500	PFB-500	NS	PFB-100	PFB-100	PFB-500	PFB-500
	1,000	NS	PFB-100	PFB-100	PFB-500	PFB-500	NS	PFB-66	PFB-100	PFB-500	PFB-500
	2,000	NS	PFB-100	PFB-100	PFB-500	PFB-500	NS	PFB-66	PFB-200	PFB-500	PFB-500
	5,000	PFB-66	PFB-100	PFB-500	PFB-500	NS	PFB-66	PFB-100	PFB-500	PFB-500	NS
	10,000	PFB-100	PFB-100	PFB-500	PFB-500	NS	PFB-66	PFB-100	PFB-500	PFB-500	NS
	500	NS	SFB-30	SFB-30	SFB-200	SFB-600	NS	SFB-10	SFB-30	SFB-600	SFB-600
	1,000	NS	SFB-30	SFB-30	SFB-600	SFB-600	SFB-10	SFB-30	SFB-30	SFB-600	SFB-600
	2,000	SFB-10	SFB-30	SFB-30	SFB-600	SFB-600	SFB-10	SFB-30	SFB-200	SFB-600	SFB-600
	5,000	SFB-10	SFB-30	SFB-200	SFB-600	SFB-600	SFB-10	SFB-30	SFB-600	SFB-600	SFB-600
	10,000	SFB-10	SFB-30	SFB-600	SFB-600	NS	SFB-10	SFB-30	SFB-600	SFB-600	NS

AAV: Adeno-associated virus; LVV: Lentiviruses; PFB: packed (i.e., non-structured) bed bioreactors; SFB: structured fixed-bed bioreactor; SUB: stirred-tank bioreactors.

TABLE 3

Analysis of the SFB COG/dose relative to SUB and PFB across demands and dose sizes and for AAV and LVV. In this figure, when 'SFB' appears in table, this indicates that this is the only technology that addresses the combination of dose and demand given the constraints. NS means no solution exists at the given conditions

Demand (doses/y)	SFB relative to SUB						SFB relative to PFB					
	AAV: Dose size (vg)											
	1×10^{11}	1×10^{12}	1×10^{13}	1×10^{14}	1×10^{15}	1×10^{11}	1×10^{12}	1×10^{13}	1×10^{14}	1×10^{15}		
500	NS	−30%	−31%	−25%	−46%	NS	−33%	−32%	−20%	−36%		
1,000	NS	−30%	−33%	−29%	−49%	NS	−33%	−32%	−21%	−42%		
2,000	SFB	−30%	−33%	−35%	−52%	SFB	−33%	−31%	−41%	−46%		
5,000	−30%	−31%	−25%	−46%	SFB	−33%	−32%	−20%	−36%	SFB		
10,000	−30%	−33%	−29%	−49%	NS	−33%	−32%	−21%	−42%	NS		
	LVV: Dose size (TU)											
	1×10^{8}	1×10^{9}	1×10^{10}	1×10^{11}	1×10^{12}	1×10^{8}	1×10^{9}	1×10^{10}	1×10^{11}	1×10^{12}		
500	NS	−25%	−32%	−35%	−60%	NS	−29%	−29%	−23%	−50%		
1,000	SFB	−26%	−33%	−45%	−61%	SFB	−29%	−29%	−31%	−49%		
2,000	−24%	−27%	−24%	−57%	−61%	SFB	−29%	−18%	−45%	−50%		
5,000	−24%	−32%	−35%	−60%	SFB	−34%	−29%	−23%	−50%	SFB		
10,000	−24%	−33%	−45%	−61%	NS	−31%	−30%	−31%	−49%	NS		

AAV: Adeno-associated virus; COG: Cost of goods; LVV: Lentiviruses; PFB: packed (i.e., non-structured) bed bioreactors; SFB: structured fixed-bed bioreactor; SUB: stirred-tank bioreactors.

at low demand and dose size permutations the technologies selected here do not offer a solution (in the model the smallest scale considered for the scale-X technology is scale-X carbo 10 m^2 bioreactor), or in other words the bioreactor is simply too large and over-production exceeds 30%. In such cases, the more economical solution is likely a number of multi-tray dishes, as shown in Figure 1. Likewise, at the edges of high demand and high dose sizes, no feasible solution exists as the number of parallel production lines would

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exceed 20 to deliver the required throughput. Nonetheless, the SFB addresses cost-effectively some of the high demand and high dose combinations relative to both SUB and PFB. Additionally, and interestingly the SFB is the only technology that can actually achieve the demand in some of these extremities. At the lower end of doses and demand, this is thanks to the smaller scale of the SFB (10 or 30 m^2) relative the smaller PFB available (66 m^2). At the higher end, this is because the SFB is the only technology able to deliver the throughput required.

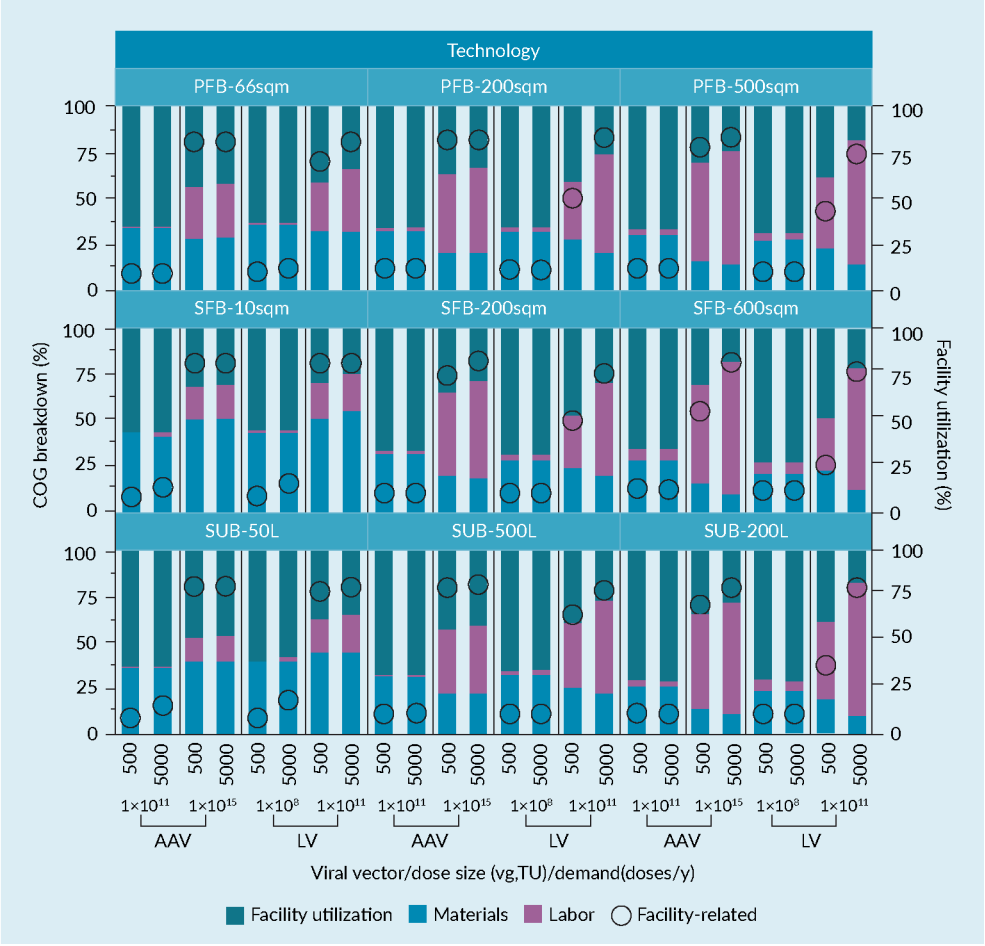
Secondly, looking at the extent of COG reduction, for AAV therapy, the SFB shows a COG/dose reduction ranging –25% to –52% relative to SUB and –20% to –46% relative to PFB. For LVV therapy, this ranges from –24% to –61% relative to SUB and –18% to –50% relative to PFB. Looking in more detail, at low doses (1×10^{11}–1×10^{12} vg/mL for AAV and 1×10^{8}–1×10^{9} TU/mL for LVV) increasing the demand has little to no impact on the COG/dose difference between technologies as, at this scale, all bioreactors have enough capacity to cover the demand. As the dose size increases, there starts to be a more significant impact of increasing demand on COG. The dip in COG/dose reduction observed at mid-dose sizes (1×10^{13}–1×10^{14} vg/mL for AAV and 1×10^{10}–1×10^{11} TU/mL for LVV) can be explained by the scale jump between the medium capacity scale-X carbo bioreactor (30 m^2 in this case) and the production size scale-X nitro bioreactor (200 m^2 in this instance), which is a 6.7-fold increase relative to the smaller gap between SUB sizes (maximum two-fold between sizes) and the PFB sizes (maximum 1.7-fold). While the SUB and PFBs offer smaller step increases in scale, the SFB still offers a significant COG/dose reduction for all permutations while offering scales that cover a wide range of demands. Importantly too, the scale-X bioreactor family is the only fixed-bed technology that offers production capacity at mid-scale with bioreactors in the 10–30 m^2 range.

Deep-diving into individual cost drivers for the COG, Figure 4 details the labor, materials and facility-related contributions as well as the facility utilization rate. Generally, it is observed that at low dose and low demand, the facility-related costs drive the COG, followed by labor. As demand (and therefore throughput) increases, the material cost contribution increases, too. The scale of production has a more significant impact on the ranking of COG contributor at high dose sizes, while at low dose sizes scaling-up would not change the ranking of the COG drivers. Conversely and as a consequence of the scale-effect at high doses, a scale-up would change the ranking of COG drivers with materials now driving the cost followed by facility-related contributors. Finally, the facility utilization rate is expectedly low at low demand and increases with the dose size, although it must be noted that increasing the dose size has a more significant impact on utilization rate. There is no significant difference in this trend across technologies, with marginal benefits for the SFB at high dose size and demand combination and at the largest scale.

As a final step in this study, a sensitivity analysis was conducted to determine the relative impact of productivity on the COG differences between technologies. For this, two simulations were run where the productivity assumptions for SFB (two-fold increase relative to other technologies–see material and methods) was either reduced to 1.5-fold increase or to no increase relative to the PFB and SUB. The key observation here is that even at reduced cell-specific productivity, the scale-X SFB remains the most cost-effective manufacturing platform. As shown in Table 4, starting with the 25% reduction in cell specific productivity (i.e., ×1.5), both the results and trends are similar to what has been observed in Table 3 with a small reduction in the COG benefit across both vectors, which is expected (for AAV: –23% to –40% relative to SUB and –20% to –36% relative to PFB; for LVV: –25% to –51% relative to SUB and –19% to -36% relative to PFB). Dropping the productivity by 50% (or if productivity is equal

FIGURE 4

Analysis of the COG/dose breakdown (in labour, material and facility related contributions) and facility utilization rate for the three production technologies, at low and high dose and demand combinations, all for both AAV and LVV products



across all platforms i.e., ×1), the COG reduction is still in the range of up to ~30% at a low dose and demand combination, but at the higher end of the spectrum, the differences are no longer significant across platforms. The conclusion from this sensitivity study is that cell-specific productivity is a key differentiator across technologies, but that even at equivalent performances fixed-bed technology provides cost advantages compared to SUB, although it must be considered that this will be sensitive to cell density at transfection as well as the ability to maintain titers during scale-up.

TRANSLATIONAL INSIGHTS

Univercells Technologies has developed and commercialized the scale-X and NevoLine product range, which is an intensified and integrated cell culture and mid-stream processing platform for the manufacture of viral products. The study presented in this paper focused on evaluating the cost-competitiveness of the scale-X bioreactor family and NevoLine platform compared to other adherent (packed fixed-bed bioreactor) and suspension (single-use stirred-tank bioreactors) cell culture technologies across a range of dose sizes, annual demands and production scales for

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TABLE 4

Sensitivity on cell specific productivity for the UTEC system. The ×1 and ×1.5 conditions refer to the relative increase in SFB cell–specific productivity relative to SUB or PFB. As for Table 3, NS means that no solution is available for the given conditions. SFB, SUB or PFB in a box means that the cited technology is the only one that can address the specific combination of dose and demand

				SFB relative to SUB					SFB relative to PFB				
				Dose size (vg)									
				1×10^{11}	1×10^{12}	1×10^{13}	1×10^{14}	1×10^{15}	1×10^{11}	1×10^{12}	1×10^{13}	1×10^{14}	1×10^{15}
Fold increase in SFB cell specific productivity relative to SUB & PFB	×1	Demand (doses/y)	500	NS	−31%	−29%	−18%	−13%	NS	−35%	−30%	−12%	4%
			1000	SFB	−31%	−28%	−17%	−10%	SFB	−34%	−26%	−7%	1%
			2000	SFB	−31%	−19%	−13%	−9%	SFB	−33%	−17%	−20%	2%
			5000	−31%	−29%	−18%	−13%	NS	−35%	−30%	−12%	4%	NS
			10000	−31%	−28%	−17%	−10%	NS	−34%	−26%	−7%	1%	NS
	×1.5		500	NS	−31%	−32%	−25%	−40%	NS	−34%	−33%	−20%	−28%
			1000	NS	−31%	−32%	−29%	−35%	NS	−34%	−30%	−21%	−27%
			2000	NS	−31%	−23%	−30%	−38%	NS	−34%	−21%	−36%	−30%
			5000	−31%	−31%	−25%	−40%	SFB	−35%	−33%	−20%	−28%	SFB
			10000	−31%	−32%	−29%	−35%	NS	−34%	−30%	−21%	−27%	NS
				SFB relative to SUB					SFB relative to PFB				
				Dose size (TU)									
				1×10^{8}	1×10^{9}	1×10^{10}	1×10^{11}	1×10^{12}	1×10^{8}	1×10^{9}	1×10^{10}	1×10^{11}	1×10^{12}
Fold increase in SFB cell specific productivity relative to SUB & PFB	×1	Demand (doses/y)	500	NS	−26%	−26%	−25%	−26%	NS	−31%	−24%	−11%	−8%
			1000	SFB	−26%	−18%	−26%	−26%	SFB	−30%	−14%	−7%	−4%
			2000	−26%	−26%	−19%	−26%	SUB	SFB	−28%	−12%	−4%	PFB
			5000	−26%	−27%	−25%	−26%	NS	−35%	−24%	−11%	−8%	NS
			10000	−25%	−17%	−26%	−26%	NS	−32%	−13%	−8%	−4%	NS
	×1.5		500	SFB	−27%	−30%	−35%	−48%	SFB	−31%	−28%	−23%	−35%
			1000	SFB	−27%	−29%	−40%	−51%	SFB	−31%	−25%	−24%	−36%
			2000	−27%	−28%	−25%	−51%	−51%	SFB	−30%	−19%	−37%	−36%
			5000	−26%	−30%	−34%	−48%	NS	−36%	−28%	−23%	−35%	NS
			10000	−26%	−29%	−40%	−51%	NS	−33%	−25%	−24%	−36%	NS

PFB: packed (i.e., non-structured) bed bioreactors; SFB: structured fixed-bed bioreactor; SUB: stirred-tank bioreactors.

both LVV and AAV viral vectors. The results demonstrate a competitive advantage for the Univercells Technologies platform over other technologies with cost of goods benefits of −18 to −61% depending on the demand/dose range, the scale of production and the viral vector. Additionally, as a result of its enhanced productivity, reduced footprint and reduced complexity, the Univercells Technologies platform could offer greater facility operational flexibility and a better throughput compared to other adherent and suspension technologies. This is because reducing the number of batches needed per product could allow for a greater number of products to be accommodated in the same facility and thus a better utilization and distribution of the facility's workforce and utilities. The affordability of cell and gene therapies is a problem that reduces access of life saving drugs to patients, but technological innovation such as the scale-X bioreactor family and NevoLine platform can help address the challenge by improving process efficiency and manufacturing throughput.

BIOGRAPHIES

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FASTFACTS

A large-scale lentivirus manufacturing platform to accelerate time to clinic

Brian Mullan, PhD, Chief Technical Officer, Yposkesi, an SK pharmteco company

To meet the requirement for safe and effective lentivirus production, LentiSure™ has been developed as an optimized solution for a plug and play lentivirus manufacturing platform.

Demand for lentivirus gene therapy vectors is higher than ever as more cell therapies enter pre-clinical and clinical studies globally. Thus, there is a requirement for safe, robust, and effective lentivirus production. Your clinical development and the commercial cell and gene therapy pipeline can be assured with the help of LentiSure™, a proven large-scale lentivirus production platform created by Yposkesi. This platform offers high titers, transduction efficiency, dependability, and the possibility to scale LVV production processes with improved purification methods.

OPTIMIZED UNIT OPERATIONS

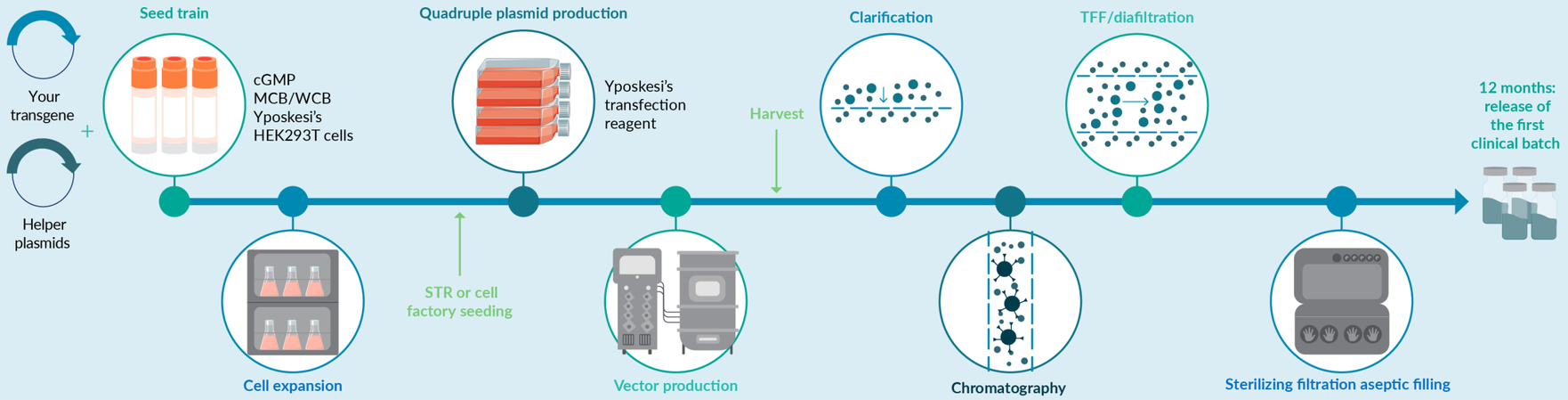
Developed by Yposkesi's experts, LentiSure is a safe and proven platform with a proven regulatory track record. The platform was developed with the goal to reduce time to market and can deliver the first batch release in 12 months. The optimization of unit operations, as detailed in **Figure 1**, is performed to increase efficiency and robustness for this plug and play platform.

LentiSure can provide both adherent and suspension processes, with the main differences highlighted in **Table 1**. Suspension methods offer greater scalability, providing lentiviral solutions at 50 and 200 L GMP bioreactor scales. The process yields achieved with both platforms are industry leading.

Table 1. Adherent versus suspension processing using LentiSure.

	Adherent	Suspension
Cell culture media	Serum-containing	Serum-free
Scalability	---	+++
Process yields	(Harvest>DP) ~ 15–30 %	

Figure 1. LentiSure optimized processing unit operations.



ROBUST & SCALABLE MANUFACTURING

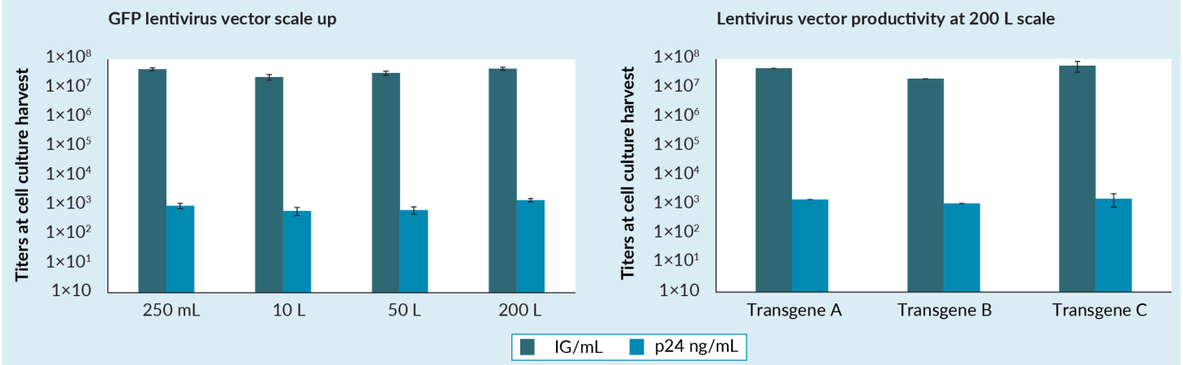
The scalable and robust nature of lentivirus manufacture using LentiSure is demonstrated in **Figure 2**. A number of scales are available for the manufacture of GFP lentivirus vector (250 mL, 10 L, 50 L, and 200 L bioreactor volumes) , with similar yields achieved at each. This allows optimization at smaller scales, and then easy scale-up for manufacturing at 50 or 200 L scales. No loss of titer is seen between 250 mL and 200 L scales.

Various transgenes have been shown to behave robustly at the 200 L scale. Yield at cell culture harvest ranges from 1×10^7 infectious genomes (IG)/mL to 5.9×10^7 IG/mL, depending on the transgene.

OPTIMIZED ANALYTICAL METHODS

A strong analytical capabilities suite supports a number of currently active clinical trials globally. These proven analytical capabilities are recognized by regulatory agencies. The majority of analytical tests are performed in-house, though some specialized testing is performed using a fully qualified external network. LentiSure offers the full package of a well-established manufacturing approach coupled with an analytical methods suite.

Figure 2. LentiSure: robust & scalable manufacturing for lentiviral gene therapy vectors.



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CELL & GENE THERAPY INSIGHTS

UPSTREAM PROCESSING

REVIEW

AAV production by transient transfection: strategies & challenges

Amanda Bretti & Stephanie Doong

AAV is the most common modality for gene therapy, with five approved drugs and over 200 in development. The most widely used method to manufacture AAV is by transient transfection of HEK293 cells. This review will describe strategies, challenges, and recent advances in the upstream cell culture and transfection operations of producing AAV. These strategies are categorized into four areas that approximate the primary raw materials of the process: transfection reagent, plasmids, medium, and cell line. Although we examine each category independently, we emphasize the importance of parallel development and combinatorial evaluation.

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Adeno-associated virus (AAV) is the most widely used delivery mechanism for genetic therapies, accounting for 82% of the viral vector-based gene therapy pipeline as of 2021 [1]. Several AAV-based therapies have been approved: Glybera (lipoprotein lipase deficiency), Luxturna (inherited retinal disease), Zolgensma (spinal muscular atrophy), Upstaza (aromatic L-amino acid decarboxylase deficiency), and most recently Hemgenix (hemophilia B). Over 200 AAV-based therapies are in development [2], and the AAV market is expected to grow 43% by 2030 [3].

AAV enjoys this popularity from its inherently attractive biological properties. A small and non-enveloped virus, AAV can transduce both dividing and non-dividing cells with long-term transgene expression, offering a broad range of therapeutic targets. The variety of AAV serotypes available also provides the ability to target desired tissues

with specificity. An additional advantage in safety stems from the inability of AAV to replicate independently.

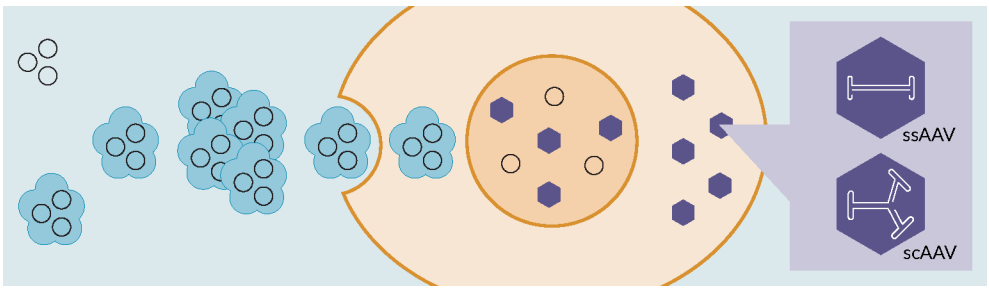
Initially discovered in 1965 in an adenovirus sample [4], AAV is a member of the *Dependoparvovirus* genus and requires infection with a helper virus for replication. The single-stranded DNA genome is approximately 4.7 kb in length and consists of two inverted terminal repeats (ITRs) flanking two genes. The rep gene encodes four rep proteins, which aid in replicating and packaging the viral genome. The cap gene encodes the structural proteins VP1, VP2, and VP3, as well as two non-structural proteins: assembly-activating protein (AAP) [5] and membrane-associated accessory protein (MAAP) [6]. For therapeutic gene delivery applications, recombinant AAV (rAAV) is generated by replacing the rep and cap genes with the desired gene of interest between the ITRs. The recombinant AAV payload can be single stranded or self-complementary DNA, where the genome self-anneals into a double-stranded configuration, bypassing the second strand synthesis step following transduction [7].

Several methods for manufacturing rAAV have been developed: adenovirus infection of HEK293 cells, herpes infection of HEK293 or BHK cells, baculovirus infection of Sf9 cells, and transient transfection of HEK293 cells [8–10]. A producer cell line expressing rep, cap, and the transgene of interest can be infected with wild-type adenovirus [11]. Alternately, baby hamster kidney (BHK) cells can be infected with two recombinant herpes simplex viruses (rHSVs), one containing rep and cap genes and the other containing the transgene [12]. A third method involves infecting Sf9 insect cells with one or more baculoviruses containing the required genes [13]. However, the most common option for producing rAAV is by transient transfection of HEK293 cells. This strategy, first described in 1998, utilizes plasmids containing the adenoviral helper genes, rep/cap genes, and transgene of interest [14,15]. Compared to other methods for manufacturing rAAV, transient transfection processes have the advantage of rapid initial development but are costly and challenging to scale-up [16,17].

Transient transfection involves a chemical reagent that delivers DNA encoding rAAV structural and synthesis genes into HEK293 cells (Figure 1). These genes are only temporarily expressed, as the viral and therapeutic genes are not integrated into the HEK293 genome, and each production run requires a transfection operation. The cell culture is expanded to the production scale prior to introduction of the DNA, which is commonly supplied on three plasmids with helper, rep/cap, and transgene on separate constructs [18], although two plasmid systems have been described as well [19]. Even though plasmid DNA is frequently biologically sourced,

FIGURE 1

Transient transfection. The plasmid DNA (black circles) and transfection reagent (blue) form a complex, which enters the cell through endocytosis. Following AAV capsid assembly and viral genome packaging in the nucleus, virions (purple) are secreted into the cytoplasm. Payloads can be designed as ssDNA or scDNA genomes.



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modifying the capsid or therapeutic transgene on a plasmid is simpler than in the mammalian cell line.

This review describes recent strategies and challenges of developing and optimizing the upstream transient transfection process for producing rAAV in suspension HEK293 cells. Although the transfection process was initially developed for adherent HEK293 cells, most processes today are suspension-based and more amenable to large-scale manufacturing. Four major areas, corresponding roughly to the raw materials of the process, contribute to both rAAV productivity and quality: the transfection reagent, plasmids, cell culture media, and the cell line. Although the strategies are grouped into these categories, there are interactions and inter-dependencies among them. We recognize that major gains can be made via bioreactor process optimization, and significant challenges lie in process scale-up, but these aspects of the upstream transfection process are not discussed here.

TRANSFECTION REAGENTS

The transfection process hinges on the ability to deliver plasmid DNA to the cell in an efficient manner. Mechanical delivery methods such as electroporation have been developed, but they often require additional equipment and further process steps such as buffer exchanges [20]. Instead, chemical delivery methods are the predominant choice for AAV production by transient transfection. Early techniques utilized calcium phosphate precipitation. However, this method requires precise pH control for optimal transfection efficiency, as well as media exchange post-transfection to counteract reagent cytotoxicity [21]. With the shift towards suspension-based processes that enabled more scalable cell culture operations [18], calcium phosphate was replaced. In the past few decades, a variety of reagents more amenable to manufacturing have been developed, which rely on the interaction between a cationic reagent and anionic DNA for cellular entry. These include cationic polymers such as polyethyleneimine, cationic lipid-based reagents such as Lipofectamine, and other cationic species such as FectoVIR.

Regardless of the reagent, the transfection procedure generally involves mixing of the reagent and DNA, followed by an incubation period for reagent-DNA complex formation and subsequent addition to the culture. The specifics of the procedure are determined by the reagent and frequently empirically optimized [17].

The cationic polymer polyethyleneimine (PEI) was one of the first transfection reagents used for AAV production in suspension-adapted HEK293 cells and continues to be routinely used today [22]. Several varieties of PEI are often employed, as this reagent is available in branched or linear form at multiple molecular weights. One common choice is PEI MAX, a 40 kDa linear PEI from Polysciences [18]. More recently, PEIpro (Polyplus-transfection) and PEI Prime (Serochem) have been found to produce slightly higher AAV vector genome titers compared to PEI MAX [23,24].

Because PEI is a widely used transfection reagent, the complexation and delivery mechanism has been studied, and many optimizations around the PEI transfection processes have been reported [18,30]. PEI condenses DNA into positively charged complexes, which then aggregate by hydrophobic interactions. After entering the cell via endocytosis, the PEI-DNA complex escapes the endosome [31,32]. While the exact mechanism is unknown, PEI is thought to facilitate the entry of the de-complexed DNA into the nucleus [33].

While PEI is affordable and well-established as a transfection reagent, the nature of PEI-DNA complexation presents a challenge at larger scales. Therefore, in addition to improving vector productivity, recent reagent development has focused on transfection scalability. In 2020, Polyplus-transfection released the novel transfection reagent FectoVIR-AAV, which was developed for improved vector productivity and scalability.

Polyplus-transfection demonstrated a ten-fold increase in infectious titers of AAV2 using FectoVIR-AAV compared to PEI MAX [25], and others have reported an increase in viral genome titer using FectoVIR-AAV compared to PEIpro and an unspecified market alternative [26]. Furthermore, FectoVIR-AAV-DNA complexes were found to remain stable for up to 6 h and could be concentrated 10 times to become only 1% of the final culture working volume without impacting vector genome titer. The longer incubation time and smaller volume offer the advantage of simplifying the large-scale AAV manufacturing process [25].

Cationic lipid-based reagents such as Lipofectamine have also been used for transient transfection. Shi *et al.* reported a transfection efficiency of nearly 100% in HEK293 cells transfected with Lipofectamine 3000 [27]. Another commercially available transfection reagent is AAV MAX, which was reported to produce a five-fold increase in viral genome titer compared to PEI MAX and PEIpro [23]. Other transfection reagents, such as TransIT-VirusGen, include both cationic lipids and polymers [28]. As with other categories of transfection reagents, there is much room for optimization of lipid-based reagents. Recently, Guan *et al.* synthesized cationic liposomes which resulted in significantly higher AAV productivity compared to PEI MAX. Optimizing the size of the liposomes was suggested to further improve transfection efficiency [29].

Several factors affect the stability of the PEI-DNA complex, including the ratio of PEI to DNA. Higher amounts of PEI have been found to stabilize the complex but are also linked to greater cell aggregation and cytotoxicity [18,30]. Consequently, many protocols use PEI:DNA ratios ranging from 2:1–4:1 [18,24,30]. Adding salts such as NaCl has also been shown to increase the stability [30]. Furthermore, the size of PEI-DNA complexes increases over time, reaching diameters of 3 µm or larger [34]. As a result, many transfection protocols involve incubating the PEI-DNA solution for only 10–15 minutes before adding it to the cell culture [18,24,35]. As a short incubation time complicates large-scale manufacturing, several methods for controlling PEI-DNA complexation have been proposed. These include adding a surfactant, decreasing the pH or temperature, or using a viscous cosolvent [36]. Hu *et al.* developed a method to halt PEI-DNA complexation at different particle sizes by adjusting the pH and salt concentration in a series of confined impinging jet mixers [37]. The technique was shown to produce greater lentiviral vector infectious titers at the 2 L scale compared to the standard protocol. Furthermore, the PEI-DNA complexes exhibited stability at room temperature for two days and at –80 ^{0}C for four months, providing flexibility in manufacturing [37].

Recently, a mechanistic model of the triple-transfection process was developed, describing the kinetics of plasmid delivery and rAAV biosynthesis [38]. Both the model and confirmation experiments found bottlenecks in the delivery of plasmid DNA into the nucleus, as well as mistimed ssDNA and capsid synthesis, leading to a large proportion of empty capsids. Identification of a transfection reagent that successfully targets the nucleus would significantly improve rAAV productivity.

Since the introduction of transfection for AAV production, many improvements to PEI and alternative reagents have been developed. Reagent selection and complexation optimization are strategies to improve productivity and manufacturability. However, the reagent still constrains transfection efficiency, and it is imperative to consider reagent and complexation conditions in the context of other variables impacting transfection, as discussed in the sections below.

PLASMIDS

Plasmid DNA is another key component of the transfection process. As the instructions for rAAV biosynthesis are provided on three separate constructs, the total amount of plasmid as well as relative plasmid ratios

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have been found to modulate rAAV productivity and quality. Plasmid DNA quantity is also linked to the cell density at transfection. Numerous studies have been conducted to optimize the cell density, DNA amount, and plasmid ratio for rAAV production.

DNA & plasmid ratio

Total plasmid DNA amounts are often reported at around 1.5 µg per 10^6 cells [18,35,29]. It has been found that higher quantities of DNA have not improved titers [24,29]. Furthermore, plasmid DNA represents a significant portion of manufacturing costs. The effect of each plasmid on infectious titer has been investigated as well—an early study characterizing plasmid ratios used a non-coding 'stuffer' plasmid to vary the amounts of the three rAAV plasmids independently of one other [40]. An equimolar ratio of the helper, rep/cap, and transgene plasmids was found to produce the highest infectious titer. Decreasing the amount of the rep/cap and transgene plasmids to 10% each while maintaining the amount of helper plasmid at 80% resulted in only a 33% reduction in infectious titer. However, decreasing the helper plasmid to 30% resulted in over a ten-fold reduction in infectious titer. This highlighted the importance of helper genes in vector production. Furthermore, decreasing the amount of rep/cap was also found to reduce infectious titer [40]. When using plasmid ratio to optimize rAAV productivity, it was found that higher proportions of helper and rep/cap plasmid increased the vector genome titer, reporting an optimal helper:rep/cap:transgene molar ratio of 2:1.5:1 [18].

Plasmid configuration

Construct design has the potential to impact vector productivity and particularly rAAV quality. The triple-transfection model described above [38] demonstrated that the timing of DNA and capsid synthesis are not aligned, leading to production of empty AAV capsids without therapeutic payloads. Plasmid ratio adjustments may aid in balancing synthesis rates, but it may be necessary to provide rep and cap on separate constructs for complete independent control of capsid and DNA synthesis. Indeed, a group recently engineered a doxycycline-responsive cap, with rep expressed from a different plasmid [41]. Controlling cap expression timing and rep protein ratios led to improved productivity and full capsid synthesis [39].

To simplify the transfection process and reduce cost of goods, two-plasmid systems have been developed, where the helper, rep, and cap genes are installed on one plasmid, and the transgene is supplied on the other [19]. The titers, packaging quality, and *in vivo* transduction efficiencies from the two-plasmid transfections were comparable to rAAVs manufactured by triple-transfection [19]. A different configuration with rep/cap and transgene on one plasmid and helper genes on the other has reported improved productivity and packaging quality compared to triple transfection [41].

Additional plasmid configurations have been used to modulate rAAV quality. Hybrid 'chimera' rep proteins improved the number of vector-containing capsids by two- to four-fold [42]. Promoter location adjustments on the rep/cap plasmid reduced contaminating backbone packaging events [43]. To avoid bacterial sequence contaminants altogether, modified plasmids and plasmid alternatives such as mini-circles and doggybone DNA (dbDNA) have been developed [44–47]. Mini-circles, plasmids that have been linearized and recombined to remove bacterial sequences, have been demonstrated to produce equivalent AAV titers with significant reduction in contaminant packaging [44,45]. Touchlight's dbDNA, manufactured *in vitro* with bacterial sequences removed enzymatically, has also demonstrated comparable AAV production to plasmid DNA [46,47]. While the details on genetic designs and alternative DNA manufacturing technologies are out of the scope of this review, DNA type and

design are considerations when optimizing ratios and total DNA quantities per cell.

Cell density

As cell density increases, the vector productivity per cell has been found to decrease, a phenomenon described as the 'cell density effect'. Therefore, densities between 1×10^6 and 2×10^6 cells/mL are commonly recommended [18,48]. This inverse relationship between density and specific productivity is thought to be a result of nutrient limitation and toxic by-product accumulation [49]. Indeed, a ten-fold decrease in specific vector productivity was observed when transfecting 5×10^6 cells/mL compared to 2×10^6 cells/mL. However, performing a media exchange before transfection increased specific vector genome titer approximately three-fold. As perfusion has been recommended as a method for improving nutrient availability and removing waste products, Mendes *et al.* implemented a perfusion process using either an alternating tangential flow or tangential flow depth filter device for cell retention [35]. Nevertheless, a limitation on cell density was still observed, with 10×10^6 cells/mL resulting in three- or four-fold lower specific vector genome titers compared to 5×10^6 cells/mL [35]. The disproportional increase in titer suggests that additional mechanisms are limiting transfection efficiency and rAAV productivity.

Historically, cell density, plasmid amount, and plasmid ratio have been investigated independently of one other. However, Zhao *et al.* described a design of experiments (DOE) approach for optimizing the three parameters simultaneously [50]. Two experiments were conducted, screening a total of 52 conditions. The results indicated that a cell density of 2.45×10^6 cells/mL, a plasmid amount of 1.5 µg/mL, and a helper:rep/cap:transgene ratio of 1:5:0.31 would produce the greatest vector genome titer. The proportion of rep/cap plasmid was higher than that determined from independent optimization experiments. The improvement in vector productivity was attributed to higher cap expression, leading to increased production of structural proteins. Conversely, the optimal proportion of helper plasmid was lower than expected. Reducing the helper plasmid may mitigate cytotoxicity from the adenoviral helper genes. Notably, these conditions produced 4.6-fold greater vector titers with 5.1-fold lower plasmid compared to conditions selected by a traditional univariate approach. This demonstrates the power of DOE methodology for optimizing process parameters [50].

MEDIA

Cell culture media, feeds, and additives are essential areas for development and optimization of HEK293-based production processes, including rAAV [51]. Many protein-free and animal-derived component free medias designed for suspension HEK293 cells are commercially available. Given the similarity of viral and transfection-based production processes, compositions and medium development efforts designed for production of other viral products or even transient protein expression in HEK293 can be adopted for rAAV production.

Basal medium & feeds

Several studies have evaluated multiple medium compositions for AAV and other viral-based therapy production in HEK293, finding strong effects of media on productivity [40,52–54]. In HEK293 cells producing virus-like particles, two of five commercial medias did not appear to support plasmid transfection-based virus production [55]. Similarly, a screen conducted with seven medias found three that produced negligible amounts of rAAV. The remaining four productive medias yielded titers ranging over three orders of magnitude, and different productivities were observed in the same media depending on the cell line [39]. These results demonstrate the presence of strong medium and cell line interactions and the potential for

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dramatic process improvements by medium screening and optimization.

Blending of different formulations has also been explored for improving vector genome titer [48]. A mixture of two medias (Ex-Cell and RPMI) produced two–five-fold higher vector genome titers than each medium alone [48]. Blending has been applied successfully in Chinese hamster ovary (CHO) platforms [56,57], and may be an effective strategy for growth rate and productivity improvement, as well as proprietary HEK293 medium development. Despite the many commercially available media for HEK293 cells, the compositions are proprietary, thus limiting our understanding of how the nutrients impact growth and productivity.

To better understand HEK293 physiology and aid basal medium development, HEK293 metabolism has been analyzed to identify nutrient limitations, waste accumulation, and pathway bottlenecks [58]. Metabolomics analysis of CHO cell cultures were able to identify several metabolic byproducts that inhibited growth and reduced protein production, and similar metabolites were found to be accumulated in HEK293 cells [59]. Metabolic models for suspension HEK293 cultures have been built and used to map the most efficient metabolic routes [60], which cells can be directed to use based on their medium composition.

HEK293 metabolism can also be re-directed with feeds. Generally, it is desirable to avoid excessive lactate production and thereby growth rate reduction by controlling glucose levels. Recently, glucose and lactate co-metabolism was observed in HEK293 cells at low pH (<6.75), marked by decreased glycolytic flux with minimal impact on growth rate [60]. Understanding and characterizing HEK293 physiology can assist in the design of glucose feeding and pH control strategies.

Additives

Due to the proprietary nature of medium development, studies reporting achievements in HEK293 medium optimization have largely focused on medium additives. Medium additives—compounds introduced to the culture around the time of transfection—are commonly employed to boost transfection efficiency and viral productivity. As there are many molecules spanning a diverse range of biochemical activity, these compounds are frequently screened via high-throughput methods and DOEs [61,62]. Some compounds, most notably histone-deacetylase inhibitors that have been found to increase transgene expression in CHO cells, have been successfully implemented in HEK293 for viral production processes [61,63]. These include sodium butyrate, valproic acid, and caffeine [62–65]. Other compounds that may increase membrane porosity, such as DMSO and ethanol, have been added to improve plasmid delivery and nuclear entry [63,66]. Cell cycle regulators such as nocodazole have also been tested to induce cell cycle synchronization and arrest cells in specific phases most beneficial to rAAV productivity [61,63]. The timing of compound addition is particularly critical for these molecules: nocodazole added 4 h post transfection was found to improve AAV8 titers by over two-fold, with a dampened effect when added 24 h post transfection [64].

As a greater understanding of transfection and AAV biology has emerged [38], molecules with functions for enhancing specific transfection mechanisms have been explored. A proteomic study of non-transfected versus transfected HEK293 cells producing AAV5 found transfected cells to be upregulated in cytoskeleton rearrangement, endocytosis, glycosylation, and apoptosis. The transfected cells were also downregulated in metabolism and cell cycle control and division [67]. Based on these findings, the authors added chloroquine to inhibit endocytosis, which led to a titer improvement of roughly 33%. A similar transcriptomics study has found significant upregulation of anti-viral response pathways post transfection, suggesting that repressing this response may be another avenue for productivity improvement [68]. Interestingly, a

proteomics study conducted on cells treated with ethanol for titer improvement found downregulated necroptosis pathways, which the authors attributed to a dampened immune and anti-viral response [66].

Cell culture medium and additives have been found to significantly affect AAV and viral productivity in transfected HEK293 cells. Successful additive-based perturbations of up- or down-regulated metabolic pathways, whose behavior is largely controlled by the (epi)genetic makeup of the cell line or clone, have been implemented [67]. In addition to the clear dependence of medium performance on cell lineage, these findings suggest that medium development should be conducted in concert with cell line selection and development.

CELL LINE DEVELOPMENT

Perhaps the most laborious area of transfection-based process development has the greatest potential to impact rAAV productivity. HEK293 cells are the most suitable rAAV production host because of their transformation with human adenovirus, providing endogenous helper functions [69]. However, using a GFP transgene plasmid and capsid antibody staining, Dash *et al.* observed that only 5–10% of cells in the culture produced AAV2, despite transfection efficiencies of ~60% [70], suggesting there is significant room for improvement. Furthermore, multiple HEK293 lines with vastly different phenotypes and capabilities for transfection and rAAV production are available. The selection and genetic modification of HEK293 cells are additional avenues to improve productivity.

Several HEK293 lineages have been derived from the cells first isolated and immortalized in the 1970s [69,71,72]. A major phenotypic change was the adaptation from adherent to suspension cells, often followed by or in conjunction with adaptation from serum-containing to serum-free medium. Along with developments in transfection reagents, these changes improved manufacturability by enabling the use of stirred tank bioreactors and removing animal-derived components [73]. The native adherent characteristic of HEK293 cells required cell stacks or microcarriers, limiting the options for large-scale AAV manufacturing [74]. Most AAV processes today utilize suspension adapted HEK293 cells.

More recently, omics studies have been conducted to better characterize the many HEK293 lineages available. The adaptation process was found to alter the gene expression and metabolic profiles of HEK293 cells [72,75]. For example, suspension lineages were found to have upregulated cholesterol biosynthesis and modified cell membrane and adhesion characteristics compared to adherent types [76]. Within suspension cell types, the lineage has been found to impact productivity. Comparison of the HEK293 E and F lineages found higher transfection efficiencies in the F lineage, but the magnitude of increase was dependent on the transfection reagent [62].

In addition to lineage selection, single cell cloning, and cell line development have been applied to improve productivity. Single cell cloning is a strategy to isolate and identify higher producing clones from a heterogenous population. Gu *et al.* initially selected a high-producing population via cell sorting after transfection by GFP. This was followed by clone isolation and expansion, which resulted in a nearly ten-fold titer improvement compared to the parent line [39]. One consideration in cell line and clone selection is stability with regards to AAV productivity. Decreased vector genome productivity was observed at higher passage numbers in a suspension adapted HEK293 cell line, despite no change in transfection efficiencies [18]. Thus, the stability of AAV production over several generations should be evaluated during clone selection.

With improved omics characterization and genetic engineering tools, targeted genetic modifications to HEK293 cells have also been implemented. Genome-wide activation screens targeting transcriptional activators have identified genes to improve rAAV

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production [77]. A proteomics analysis of cells transfected to produce VLPs found modifications to membrane remodeling proteins and lipid biosynthesis [78]. Subsequent metabolic engineering of HEK293 cells by overexpressing ESCRT (membrane remodeling) proteins and sphingolipid precursors plus knockdown of an anti-viral assembly gene led to improved VLP production by transfection [79]. Overexpression of the yeast pyruvate carboxylase (PYC2) gene in HEK293 cells has increased the growth rate and improved protein glycosylation quality [80]. Similar strategies could be applied for rAAV production.

HEK293 cells are endogenously advantageous for transfection-based rAAV production, and several lineages exist. Adaptation, clonal population development, and metabolic engineering of HEK293 cells have demonstrated the impact that cell line enhancements have on vector productivity.

TRANSLATION INSIGHT

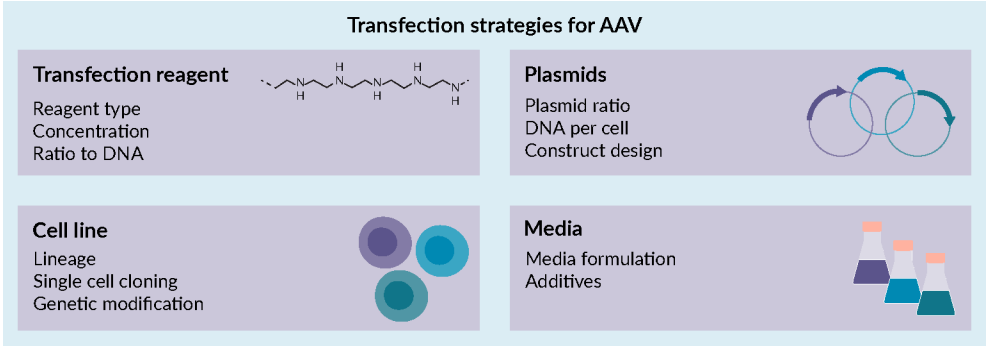
A summary of the strategies for developing and optimizing transfection-based rAAV production processes is shown in Figure 2. The strategic areas discussed here are closely intertwined, often requiring parallel development and 'stacking' of beneficial changes to yield significant improvements. Many examples presented here involved the serial implementation of multiple strategies to boost productivity. While it may be difficult to screen these aspects simultaneously in a combinatorial manner, multivariate designs can distinguish the most important variables and identify interactions between them.

Transient transfection offers the benefit of rapid development, particularly in the reagent, DNA, and medium additive areas of the processes. Cell culture medium design and cell line modifications often require longer term development efforts but can be worth the wait: these approaches for cell culture development have yielded dramatic improvements in protein production over the last few decades. While not discussed here, a major challenge in transfection-based processes is scale-up, which remains to be addressed [81].

Although new technology may eventually replace transient transfection, learnings from the development of transfection processes can be translated to next generation rAAV production platforms, which include the engineering of stable and inducible producer cell lines [82–84]. Transcriptomic and proteomics studies characterizing the behavior of AAV-producing cells offer insight to both transfection-based and producer cell line processes. Elucidating biosynthesis mechanisms and biological functions of rAAV will accelerate the development of improved and advanced manufacturing platforms, enabling the consistent delivery of AAV-based gene therapies.

FIGURE 2

A summary of strategies for AAV production by transient transfection.



BIOGRAPHIES

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CELL & GENE THERAPY INSIGHTS

LATEST ARTICLES:

INTERVIEW

Investing long-term in the cell & gene therapies of the future

David McCall, Senior Editor, *Cell & Gene Therapy Insights*, speaks to **Geeta Vemuri,** Founder and Managing Partner, Agent Capital about her VC company's portfolio of advanced therapy investments, and the keys to obtaining funding in a difficult environment. (From an interview conducted on Feb 22, 2023).

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Q What are you working on right now?

GV: We founded Agent Capital to invest in and work on four core areas: oncology, immunology, neuroscience, and rare disease. The latter two areas fall well within the gene therapy space, and there is a great deal of cell therapy activity in oncology.

We have been investing in these strategies since 2012. At that time, I was running Baxter Ventures Group. and these four core areas aligned with Baxter's strategic remit. Baxter Ventures Group then became Baxalta Ventures, but we continued working in these disease areas because we saw a lot of unmet medical need.

Investor returns in these four areas are also relatively strong because of the high mergers and acquisitions (M&A) activity. Between them, they account for almost 92% of total M&A in the advanced therapies space, including both large and small companies. So, we felt that this was the right strategy for us to both serve patients and to receive a good return on our investor capital. Over the past decade and more, we have backed a variety of novel transformative science initiatives through our funds.

Q What specific investments do you have in advanced therapies?

GV: We have made a variety of investments in the oncology cell therapy space, starting with companies with *ex vivo* autologous cell therapy approaches. We are also investing in *in vivo* cell therapies. One of our companies, Carisma Therapeutics, has a chimeric antigen receptor (CAR) macrophage therapy, which utilizes the underlying science of macrophages and monocytes as scavengers in our bodies to develop a cell therapy for solid tumors. Another company we invest in, Interius BioTherapeutics, is developing next-generation of CAR-T cells within the human body itself, as opposed to manipulating them outside the human body.

On the gene therapy side, we have invested in Carbon Biosciences, a company with a vector library designed to deliver large genes in a single capsid—for example, the entire cystic fibrosis gene can be put in a single vector and delivered. There are multiple other products behind that one which could also utilize this platform.

In gene editing, we have Metagenomi. They are coming up with novel enzymes for gene editing within the human body.

Q Can you expand on the key areas of innovation for cell and gene therapy at present, as you see them—why and how will they impact the field, and healthcare in general?

GV: The way we look at cell therapy is a continuum from *ex vivo* manipulation all the way to *in vivo* manipulation. Within that, on the *ex vivo* side, there are different types of cells to consider, including NK cells and macrophages. Different types of CAR constructs are becoming interesting, too, as they could improve the manufacturing of *ex vivo* autologous CAR-T cells. This is happening right now in front of our eyes. The next continuum on the *ex vivo* side is allogeneic CAR-T cells. They have certain advantages over autologous therapies, and there are many opportunities there. Finally, *in vivo* CAR-T cell therapy production is the 'holy grail' of the space.

On the gene therapy side, there are already approved products on the market, which is fantastic to see. Here, one thing we want to improve upon is dosing—specifically, being able to dose these patients more than once without seeing immunogenicity. We also want the ability to reduce the number of empty capsids in the manufacturing process. There is a lot happening in the gene therapy space currently, and Carbon Biosciences is one company behind this innovation.

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Q It continues to be a challenging time for cell and gene therapy biotechs seeking to raise finance. What's your view of the current landscape and its short to mid-term prospects?

GV: The ability of cell and gene therapy companies to raise capital has posed challenges recently, although this is not to say others are having a better time—the entire market is challenging for most biopharmas. One thing we have been able to do in past cycles is to at least tap the market with the cell and gene therapy companies that had some exciting programs to bring in investors that invest in public companies.

However, the capital being raised from public markets by cell and gene therapy companies has recently dropped sharply. In 2022, it fell by almost 96% from the previous year, from roughly $3.7 billion to only $162 million. In the overall biopharma market in 2022, there was a 72% decrease in terms of capital raised from the initial public offering (IPO) market compared to 2021. So, cell and gene therapies experienced a proportionally greater drop, but the whole biopharma industry is struggling in this regard.

In the mid-term, cell therapy companies are coming back in vogue, as some of the approved products in that space have shown nice market acceptance and an uptick in revenue. We are seeing some more life in these companies and the investors supporting them. We have our own portfolio companies going out to the market, and there has been good receptivity to them so far.

The hurdle for gene therapy companies is to show differentiation. No one wants to back a company that has a story they have heard before. You have to have a hook, either through unique indications or via another advantage—over existing AAV capsids, for example. If companies can show this, the probability of them being able to raise capital is not bad—they have good prospects of surviving long-term and achieving transformative therapies. Gene therapies are already out there in the market and saving lives—Zolgensma is a case in point.

Long-term, these therapies are not going to go away. Short-term, they might struggle a bit, but investment is not going to completely dry up. A lot of infrastructure has already been built, including by pharma companies, to support these technologies. There is certainly still capital available for strong and differentiated companies.

Q You mentioned the importance of differentiating to attract investment in what is a more competitive environment. What are the other keys to success, in the eyes of investors?

GV: In the end, it comes down to the management team that is going to develop these assets. If you have a management team that has shown prior success in knowing what it takes to get new compounds through US Food and Drug Administration regulatory process, then the dollars are available.

In 2021–2022, some of these companies became incredibly expensive for the stage they were at, which is one of the reasons why they were unable to acquire funding. In order to get the support these therapies require, a few areas of focus should be considered. One needs to look at management, but also at having the right value proposition and finding an unmet need that cannot be cured by a small molecule or any other modality.

Q What is your perspective on the remaining challenges in market access to cell and gene therapy products and what might be the likeliest pathway to their resolution?

GV: There is a new value-based payment model proposal by the Centers for Medicare & Medicaid Services (CMS) that is being put forth. This aim is to reduce the initial shock in terms of the cost of these therapies—a necessity, even if they are transformative and saving lives, and in the long-run, turn out to be neutral in terms of cost to the healthcare system.

We will simply have to wait and see how such new payment models work out. Products under the new CMS system are likely to be approved by 2026.

Q Lastly, can you sum up some key goals and priorities that you have for Agent Capital and its portfolio companies over the coming 12–24 months?

GV: Firstly, we are advising all of our existing portfolio companies to be cash-prudent—to invest in things that are the best value drivers: the 'need-to-haves' in their portfolios rather than the 'nice-to-haves'.

This is a good time for companies with cash to look into new and different things that they can either acquire or bring in through licensing and partnering. It is a good idea to look at the outside world and not just inside your own R&D shop. Additions are better than always looking inwards.

The other goal for us is to take care of our existing portfolio companies and maintain cash reserves for them to get through 2024. We are likely to see a lot of improvement in the investment environment in 2024, but things can change on a dime, so we are advising our companies to conserve money through the end of 2024 and the beginning of 2025.

I think it is a great time to be a biopharma investor. I have been investing in this space for a long time, and I feel like this is one of those unique moments where the innovation is truly remarkable. We simply must get through this period to see how many meaningfully life-changing new products we can get to the market.

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BIOGRAPHY

DR GEETA VEMURI is Founder and Managing Partner at Agent Capital. Geeta leads and oversees all Agent Capital activities, including portfolio management, new investments and operations activities. Prior to Agent Capital, Dr Vemuri was the Managing Partner of Baxter Ventures and the Managing Partner of Baxalta Ventures, where she led each corporate venture capital arm with more than US $ 300 million under management. Prior to Baxter, Dr Vemuri was a Partner at Quaker Partners where she focused on biotech and healthcare investments. Dr Vemuri has served on the board of a variety of companies, including Cempra (until its IPO and Dr Vemuri's departure in 2011), Covagen (acq. by J&J), Gadeta (acq. by Gilead), Naurex (acq. by Allergan), Ocular Therapeutix, Protez Pharmaceuticals (acq. by Novartis), Syntimmune (acq. by Alexion), and True North Therapeutics (acq. By Bioverativ/Sanofi), among others. Dr Vemuri received a PhD in biochemistry from Indian Institute of Sciences and an MBA from The Wharton School at the University of Pennsylvania.

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AUTHORSHIP & CONFLICT OF INTEREST

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

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CELL & GENE THERAPY INSIGHTS

EXPERT INSIGHT

A guided demonstration of the Counting Method Evaluation Tool (COMET) for implementing the ISO 20391-2 Cell Counting standard

Laura Pierce, David Newton, Steven P Lund, Firdavs Kurbanov & Sumona Sarkar

Cell counting methods are critical for the development, manufacturing, and release of cellular therapeutic products, where enumeration of total cells, viable cells, and/or defined subpopulations of cells is required as a part of release testing and is relied upon in manufacturing process control. The ISO 20391-2:2019 Cell Counting standard provides an experimental design and statistical analysis approach to quantify and compare counting method performance, supporting method development, selection, and validation. Here we demonstrate an open-source graphical user interface to execute the statistical procedures outlined in ISO 20391-2:2019. Counting Method Evaluation Tool (COMET) is a publicly available user interface that follows the statistical analysis and reporting structure of the ISO standard. The quality metrics of precision (% coefficient of variation), proportionality index, and goodness-of-fit R^2 are computed, and convenient plots and tables can be visualized and downloaded using COMET. Additional analyses are available in COMET to evaluate experiment integrity and cell viability results. Here, we demonstrate the utility of COMET in comparing cell counting methods through a series of studies on a nutrient deprived Jurkat cell model system. Using the ISO standard and COMET, we illustrate the evaluation of counting method performance and selection of a fit-for-purpose cell counting method in the model system.

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Cell count is a fundamental measurement in the biosciences. For cell-based products, cell count underpins key decision making in all stages of product development from research

to manufacturing control and product release testing. Additionally, cell count is an important parameter used in evaluating the biological activity per cell in bioassays that measure cell activity and product potency [1,2]. Advances in cell counting measurement platforms have provided increased measurement throughput and improved precision; however, discrepancies exist between cell counts acquired via various measurement processes [3–6]. Stakeholders in the cell therapy field may seek to move away from manual methods, which are prone to user error, bias and low precision. Others may find it necessary to switch methods due to sample constraints in manufacturing or to align methods with those of a contract research organization (CRO) manufacturing the final product. In addition, the biotechnology industry has recognized that complex biological properties, including variability in cell source and donor, as well as variations in operator, equipment, and procedures can greatly affect the cell count measurement quality [7,8]. Due to the wide diversity of cell types and cell counting applications, a fit-for-purpose approach is needed when designing cell counting assays [9,10]. A two-part ISO (International Organization of Standards) standard on cell counting has been recently published to support common understanding of cell counting related concepts and terminology and to support the development and evaluation of cell counting methods that are fit-for purpose [11–13].

ISO 20391-2: 2019 'Biotechnology – Cell counting – Part 2: Experimental design and statistical analysis to quantify counting method performance' provides a method to evaluate the quality of a counting method or to compare the quality of two or more methods [12]. These evaluations focus on the precision and proportionality of the counting measurement process(es) under investigation. Precision of a cell counting measurement process is critical to assure repeatability and reduce uncertainty in the measurement process. A proportional response to a dilution series is a fundamental property of a cell counting method; deviation from proportionality would indicate systematic measurement error that would reduce the accuracy of the method across the dilution range [14]. The ISO Cell Counting Part 2 Standard requires the reporting of several metrics calculated from data generated from a carefully designed dilution series study. These metrics include the mean concentration for each dilution fraction (DF) in the series, coefficient of variation percentage (%CV) across replicate observations for each dilution in the series, goodness of the proportional model fit (R^2), and a proportionality index (PI) that is calculated from smoothed residuals from the proportional model fit. These metrics can help support method selection, optimization, and validation. The standard's principles have been demonstrated by stakeholders to evaluate counting method performance and to compare methods [15].

The statistical analysis recommended in the standard considers the many levels of replication incorporated into the experimental design, as well as statistical considerations when working with count-based data. Some aspects of this analysis are not easily implemented manually and may be unfamiliar to many users of the standard. Additionally, there are extensive reporting requirements within the standard to assure that results of the analysis can be independently evaluated (i.e., information regarding experimental design, statistical equations, etc.) To support the broad implementation of ISO 20391-2: 2019, we have developed an open-source web-based tool to import data collected following the prescribed experimental design and automatically model the data and calculate the cell counting quality metrics as well as to provide easy access to meta-data required for reporting.

The Counting Method Evaluation Tool (COMET), is written using the R programming language [16] and developed into a graphical user interface (GUI) via R Shiny [17]. COMET provides a user-friendly tool that allows users of the ISO standard to evaluate and compare cell counting methods

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following the requirements of the standard. Using COMET facilitates the advanced statistical analysis required by the standard, which is not typically available in common statistical software packages without extensive coding. In addition, COMET accepts metadata associated with experimental design, sample stability, and viability, providing useful output for evaluating the integrity of the study and other aspects of cell counting that may be of interest for cell therapy manufacturers. Information on how to access and use COMET along with the source code can be found at the official COMET GitHub page. We provide a public website for using COMET, along with instructions for users on how to run the application on their own servers using Docker, if desired.

Through GitHub, COMET has version control, which allows for tracking of any necessary changes to code, and its code is open source, thus allowing users to adopt and implement the source code with their individual software control procedures (e.g., to meet regulatory requirements). Additionally, to verify that COMET is working reliably and as expected, the ISO Cell counting Part 2 document contains a reference dataset and analysis that can be run through the COMET application as a quality control check.

In this study, we introduce the COMET tool through two use case examples. The first use case compares the quality of four counting methods (including both manual and automated methods) in an individual study (Study 1) and across four identical, independent experiments (Study 2) using a nutrient starved Jurkat cell population. An additional demonstration (Study 3) uses COMET to compare the results of three Trypan blue viability based counting methods. We use COMET to process the data following the analysis recommended in the Cell Counting Part 2 standard. We examine differences in proportionality index (PI), R^2, precision (%CV), and bias (absolute counts) between counting methods.

In the absence of a reference material or reference method for cell counting, the COMET application can be used to provide quantitative metrics to assess the quality of a single cell counting method, as well as to compare cell counting performance between methods using specific cell preparations.

MATERIALS & METHODS

Materials

Jurkat Clone E6-1 (acute T cell leukemia) cells used in these studies were obtained from ATCCR, Manassas, VA (Cat #TIB-152TM). Complete Jurkat cell culture media RPMI1640 without L-glutamine was purchased from HyClone GE Life Sciences (Catalog #SH30096). Other reagents and consumables used were as follows: Glutamax (Gibco, Catalog #35050061); Fetal Bovine Serum (Gibco, Catalog #26-140-079), Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS) (Gibco Catalog #14190250). Acridine Orange/DAPI (AO/DAPI) (Solution 13, Catalog #SKU:910-3013) and NC-Slides A8 (Catalog #942-0003) were from ChemoMetec (Lillerod, Denmark). Acridine orange/Propidium Iodide (AO/PI) staining solution (Cat. No. CS2-0106-5mL) and SD-100 slides (Catalog # CHT4-SD100-002) were from Nexcelom/Perkin Elmer (Lawrence, MA). Coulter counter ISOTON II electrolyte diluent (Catalog #8546719) and Vi-Cell XR reagents (Quad Pack Reagent Kit, Catalog #383722) were from Beckman Coulter (Brea, CA). Trypan blue used in manual hemocytometer counting and CellDrop (DeNovix, Wilmington, DE) studies was from Gibco (Catalog #15250061).

Cell count & viability analytical methods

The following demonstrations of the Cell Counting Part 2 standard using the COMET application for data analysis consist of six

total counting methods. The first comparison uses four methods including an AO/PI method (Method 1, Cellometer Auto 2000, Nexcelom/Perkin Elmer, Lawrence, MA), a Coulter counter method of impedance-based particle counting (Method 2, Multisizer-4, Beckman Coulter, Brea, CA), an AO/DAPI method (Method 3, NucleoCounter NC-3000, Chemometec, Lillerod, Denmark), and a Trypan blue dye exclusion method (Method 4, Vi-CELL XR, Beckman Coulter, Brea, CA). A second demonstration compares three Trypan blue-based methods: a fully automated method (Method 4, Vi-CELL XR), a semi-automated method (Method 5, CellDrop, DeNovix, Wilmington, DE), and a manual hemocytometer counting method (Method 6). Further details describing the workflows of these methods can be found in Supplementary Section 1.

Cell culture

Jurkat cells were seeded at a concentration of 150,000 cells/mL in a total volume of 15 mL in a T-75 flask with complete Jurkat culture media consisting of RPMI 1640, 10% FBS, and 1% Glutamax. The culture was allowed to grow in a 37°C 5% CO_2 humidified incubator for 8 days without replenishment of media with the aim of generating a cell population with heterogeneous morphology and reduced viability. Initial flask counts were performed in duplicate using the Vi-Cell XR to measure cell concentration and viability. An average of the duplicate counts and viability measurements was used for calculations. For the study comparing four counting methods, the initial flask was at passage 51 with 3.93 million cells/mL and 80.7% viability (automated Trypan blue count and viability method). For the study comparing three Trypan blue methods, the initial flask count was at passage 28 with 4.5 million cells/mL and 71.0% viability (automated Trypan blue count and viability method). Cultures were resuspended in media to an approximate concentration of 1.5 million cells/mL for starting stock solution, and the dilution fraction scheme shown in Figure 1 was prepared from the stock solution. In alignment with the Cell Counting Standard Part 2 (Section 5.3.2), a single stock cell solution was used in each study to generate all representative test samples, and the stock cell concentration was chosen to facilitate the generation of test samples over the concentration range of intended use of the methods under investigation. Dilutions were prepared in complete Jurkat cell culture media.

Experimental design

The dilution fraction scheme used for this study is shown in Figure 1. A stock cell solution of nutrient- starved Jurkat cells was created at 1.5 million cells/mL by diluting the cells in the flask with complete Jurkat media. Dilution fractions were prepared using weighted pipetting and a gravimetric scale (Model XS-R205DU, Mettler Toledo, Columbus, OH). Three independent replicate test samples were created at each of the five dilution fractions shown shown in Figure 1 (see Table 1).

Measurements began immediately following dilution fraction preparation, and all tubes and measurements were held at ambient room temperature for the duration of the study (23±1°C). Tubes were labeled randomly and blinded, and timestamps were recorded for each measurement. Blinding of samples reduces potential operator bias, and random ordering of samples along with recording of timestamps enables monitoring for systematic temporal effects on measurements, in alignment with the Cell Counting Part 2 standard (Section 5.5).

Data analysis using COMET

COMET is an R Shiny App that accepts data in a specific template based on a specified experimental design, conducts statistical analysis, and outputs quality indicators for the cell counting methods as well as additional meta-data important for reporting of results [17].

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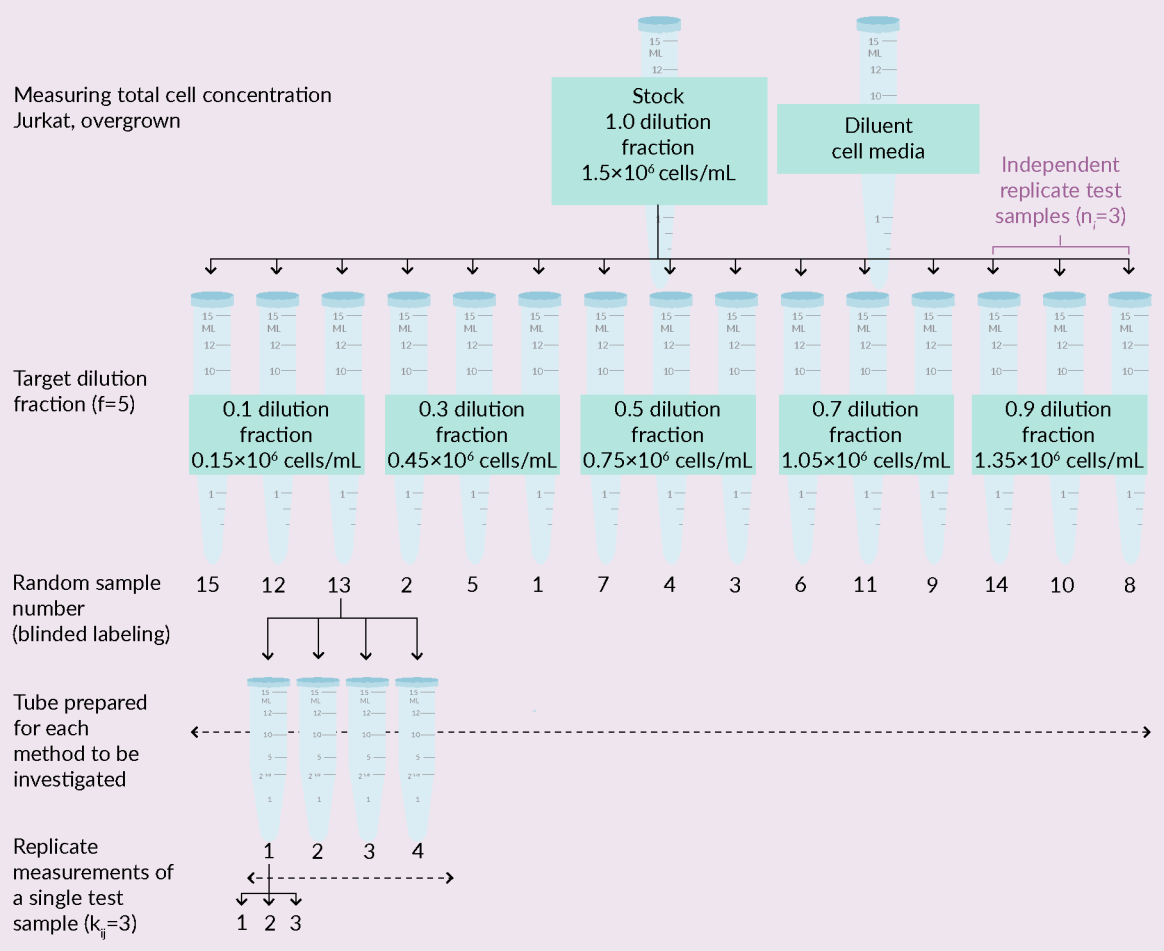
FIGURE 1

Dilution fraction scheme indicating the ISO Cell Counting Part II experimental design implemented in this study. Stock cell solution was diluted with cell media into the indicated replicate test sample tubes (3) at each of 5 independent dilution fractions. Samples were then split into four tubes (one for each method). Three replicate measurements were acquired for each sample using each counting method.



COMET is designed to accept data from experimental designs that follow the principles outlined in the ISO standard, 'ISO 20391-2:2019 Biotechnology – Cell counting – Part 2: Experimental design and statistical analysis to quantify counting method performance'. The outputs of this app are designed to follow the reporting requirements and recommendations of the standard, including the required reporting of the quality indicators R^2, PI, and %CV.

The recommended experimental design described in the standard includes a minimum of four target dilution fractions, three replicate test samples per dilution fraction, and three replicate observations for each test sample. Data sets that do not meet these minimum requirements may not be suitable for analysis using the COMET tool, and any analysis of such data should be interpreted with caution. This is for several reasons: the appropriateness of the bootstrapping uncertainty approximations has not been fully tested with more minimal experimental designs, and some aspects of the statistical analysis cannot be calculated with fewer replicates.

TABLE 1

Table of dilution fractions and weights of cells and media used to prepare tubes.

Tube	Dilution fraction	Order of pipetting	Random sample number	Weight media (g)	Weight of cells (g)	Weight of diluent + cells (g)	Measured dilution fraction (weight cells / weight (diluent + cells))	Expected cell concentration (cells/mL)	Working stock conc. (cells/mL)	Volume media (µL)	Volume cells (µL)
DF 1-1 (0.9)	0.9	1	15	0.268	2.320	2.587	0.896	1.35×10^{6}	1.36×10^{6}	250	2250
DF 1-2 (0.9)	0.9	2	11	0.266	2.326	2.592	0.897	1.35×10^{6}	1.36×10^{6}	250	2250
DF 1-3 (0.9)	0.9	3	2	0.270	2.299	2.569	0.895	1.35×10^{6}	1.36×10^{6}	250	2250
DF 2-1 (0.7)	0.7	4	1	0.781	1.801	2.582	0.697	1.05×10^{6}	1.36×10^{6}	750	1750
DF 2-2 (0.7)	0.7	5	7	0.785	1.802	2.587	0.697	1.05×10^{6}	1.36×10^{6}	750	1750
DF 2-3 (0.7)	0.7	6	4	0.779	1.790	2.569	0.697	1.05×10^{6}	1.36×10^{6}	750	1750
DF 3-1 (0.5)	0.5	7	12	1.293	1.273	2.566	0.496	7.50×10^{6}	1.36×10^{6}	1250	1250
DF 3-2 (0.5)	0.5	8	3	1.299	1.277	2.576	0.496	7.50×10^{5}	1.36×10^{6}	1250	1250
DF 3-3 (0.5)	0.5	9	14	1.294	1.273	2.568	0.496	7.50×10^{5}	1.36×10^{6}	1250	1250
DF 4-1 (0.3)	0.3	10	10	1.803	0.761	2.568	0.296	4.50×10^{5}	1.36×10^{6}	1750	750
DF 4-2 (0.3)	0.3	11	6	1.802	0.708	2.564	0.276	4.50×10^{5}	1.36×10^{6}	1750	750
DF 4-3 (0.3)	0.3	12	13	1.744	0.821	2.509	0.327	4.50×10^{5}	1.36×10^{6}	1750	750
DF 5-1 (0.1)	0.1	13	8	2.308	0.260	2.565	0.101	1.50×10^{5}	1.36×10^{6}	2250	250
DF 5-2 (0.1)	0.1	14	5	2.310	0.255	2.567	0.099	1.50×10^{5}	1.36×10^{6}	2250	250
DF 5-3 (0.1)	0.1	15	9	2.305	0.255	2.565	0.099	1.50×10^{5}	1.36×10^{6}	2250	250

For example, if only one replicate observation was collected per sample, %CV cannot be calculated.

Upon launching the COMET application, users will find a brief description of the process of analysis and features of the site and will have the option of downloading a 'README' file. The README file is an Excel file which contains 11 tabs, the first of which contains instructions including a description of the contents of the README file and a brief description of the ISO Part 2 experimental design, as well as a list of additional resources to guide the user in implementing the Cell Counting Part 2 ISO standard.

Tab 2 contains a Full Data Template followed by Tab 3 with Full Data Template Descriptions. The remaining tabs include a Simple Data Template (Tab 4), a description of the COMET input parameters (Tab 5), a description of the plots and metrics displayed on the COMET Output tabs (Tab 6), a tab describing the output file of the 'Download Results' tab (Tab 7), and finally, three full template examples which can be used to verify the function of COMET by the user (Tabs 8–10) and a simple template example (Tab 11).

Additional files are available for download including templates for data entry, and example data sets. 'Full Template Example' 1, 2, and 3 correspond to the data presented in the ISO Cell Counting Part 2 annex sections and can be helpful in verifying the COMET analysis by comparison to the results in the standard.

Description of the COMET 'Full data template' & 'simple data template

To use the COMET application, users must upload data for analysis in one of two possible formats: the 'Full Data Template' or the 'Simple Data Template'.

The Full Data Template is a comprehensive data template which users can implement to input a comprehensive set of data for each counting method, including raw count and concentration data, sample numbering and order, timepoint, and meta-data such as analyst, stock solution number, and time elapsed. The required data fields for the Full Template are noted here: counting method, target dilution fraction, replicate observation, and (measured) cell concentration. All other fields are optional; however, entering data such as time elapsed, viability, and measured dilution fraction facilitates monitoring aspects of experiment integrity such as sample stability over time with respect to concentration and viability, and allow for accounting of pipetting error.

The application also accepts a minimal set of data in a simple format that can be uploaded to COMET using the 'Simple Data Template'. COMET will transcribe the data into a format that can be analyzed by the app and the new data file (now in the 'Full Data Template' format) can be downloaded post-processing to add more meta-data (e.g., measured dilution fraction, sample extraction order, time elapsed, etc.) if desired. Users may add data to the template where appropriate and leave all other fields blank. Method labels (e.g., 'Method 1, Method 2, Method N') can be replaced with more descriptive cell counting method label information. Analysis conducted directly from this template assumes: 1) the user-defined criteria for pre-evaluation of dilution integrity has been met (i.e., target dilution fraction will be used in the data analysis with the assumption that the user has completed preliminary studies to ensure dilution integrity under similar experimental conditions, as described in the Cell Counting Part 2 standard) and 2) a single stock cell solution was used to generate all samples. Analysis using this template does not include monitoring of experiment integrity (e.g., sample stability over time, pipetting error, etc.) or cell viability.

Each of these templates can be individually downloaded from COMET.

COMET input parameters

In this example data demonstration, we upload a Full Template file containing a comparison of four counting methods with all associated metadata described in the COMET README files (raw count, percent viable cells, time elapsed, stock solution.

The raw data sets used in these studies are publicly available here and can be run from the GitHub page to visualize and explore the tables and results discussed here [18,19].

The 'COMET Input Parameters' tab highlights each of the user-defined input parameters which contribute to loading data and the resulting statistical analysis. In this example, missing values are denoted by 'NA'.

Next, we are asked to choose a 'Variance assumption', an important parameter for the statistical evaluation of measurement precision. Cell counts would ideally be modelled using a Poisson assumption for the mean-variance relationship (variance is equal to the mean) instead of a constant mean-variance relationship (ordinary least squares); however, in biological studies, count data are often over dispersed. We apply a model in which the variance of the cell concentration is assumed to be proportional to the mean cell concentration (quasi-Poisson mean-variance relationship). This specification, which coincides with the common observation that higher counts result in larger spreads in measurement observations, will reduce the influence of the measurements from the highest dilution fraction on the proportional model fit. A goodness-of-fit test can be used to support the choice of a specific mean to variance relationship.

We then choose the 'Order of smoothing polynomial'. We use the default order of smoothing polynomial, where the total number of coefficients to be fit in the flexible model, including the intercept, is equal to the number of unique target dilution fractions, as required by the standard when using measured dilution fractions. This setting also meets the requirements of the standard when using the target dilution fraction, where smoothing is performed by calculating the average cell count across all replicate test samples at each unique target dilution fraction. COMET allows flexibility in selecting the order of the smoothing polynomial as a research tool to evaluate the effects of the smoothing polynomial on the calculation of PI.

The next input parameter asks us to choose a level of bootstrap analysis. Bootstrapping is a tool for estimating the variability in statistics based on repeated simulated experiments, which are used as proxies for performing repeated experiments. Each simulated experiment produces data by randomly resampling with replacement from the observed data of the actual experiment (non-parametric bootstrap analysis). Statistics of interest are recomputed for each simulated experiment, and the observed variability of their values across the simulated experiments is used as an estimate of the uncertainty in the statistics computed for the actual experiment. We launch an analysis using 1,000 bootstrap iterations for our final analysis.

Choosing how many bootstrap iterations is a tradeoff between computational time and obtaining stable characterizations of uncertainty. We have found 1,000 iterations to be a reasonable balance between these considerations. For this example, we choose a 95% confidence level. Increasing the level of confidence will result in wider uncertainty intervals. The choice of confidence level is restricted to be between 80% and 99%, which is based on ranges of practical interest.

We also select all four proportionality indices for our analysis output. Each index views the data slightly differently, so it can be reaffirming to see that all indices provide a similar interpretation, such as when all metrics produce the same method rankings when comparing counting methods. If the interpretation varies from one metric to another, it is important to recognize that performance assessments depend on subtle choices on how to measure performance. Further information regarding

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the calculation of the four proportionality indices can be found in the Discussion section.

RESULTS

After running the analysis, the COMET page displays results across 8 individual tabs.

A comprehensive *.csv file containing tabular results can be downloaded using the 'Download Results' button on the main page of COMET with all quality indicators (statistical metrics) and a comparison of all metrics for all pairwise combinations of methods relevant data for the experimental design and analysis results. The input parameters and the experimental design are also included in the resulting file. The format of the data within this download file can be helpful for generating custom plots and conducting further analysis. In the 'Metrics' section of the 'Download Results' file, the mean value as well as the upper and lower confidence bound are shown for each statistical metric. In the 'Comparing Metrics' section of the 'Download Results' file, the ratio of each metric for each pairwise combination is displayed, as well as the upper and lower confidence interval bounds. When comparing metrics, if the value of '1' does not fall between the ratio's upper and lower confidence bounds, the pairwise combination is determined to be significantly different. By examining metrics that indicate whether a method has statistically significantly better proportionality or precision for a given sample type, users can start to determine which method may be more suitable for their particular intended use.

Study 1 & detailed description of COMET outputs: comparison of four cell counting analytical methods with varying measurement principles

Data overview

The 'Data Overview' tab contains plots of the raw data (cell concentration versus dilution fraction), Mean Cell Concentration versus. Dilution Fraction, and a Residual Plot for Model Fits. Raw data for each replicate observation and replicate sample is plotted in an individual plot for each specific counting method.

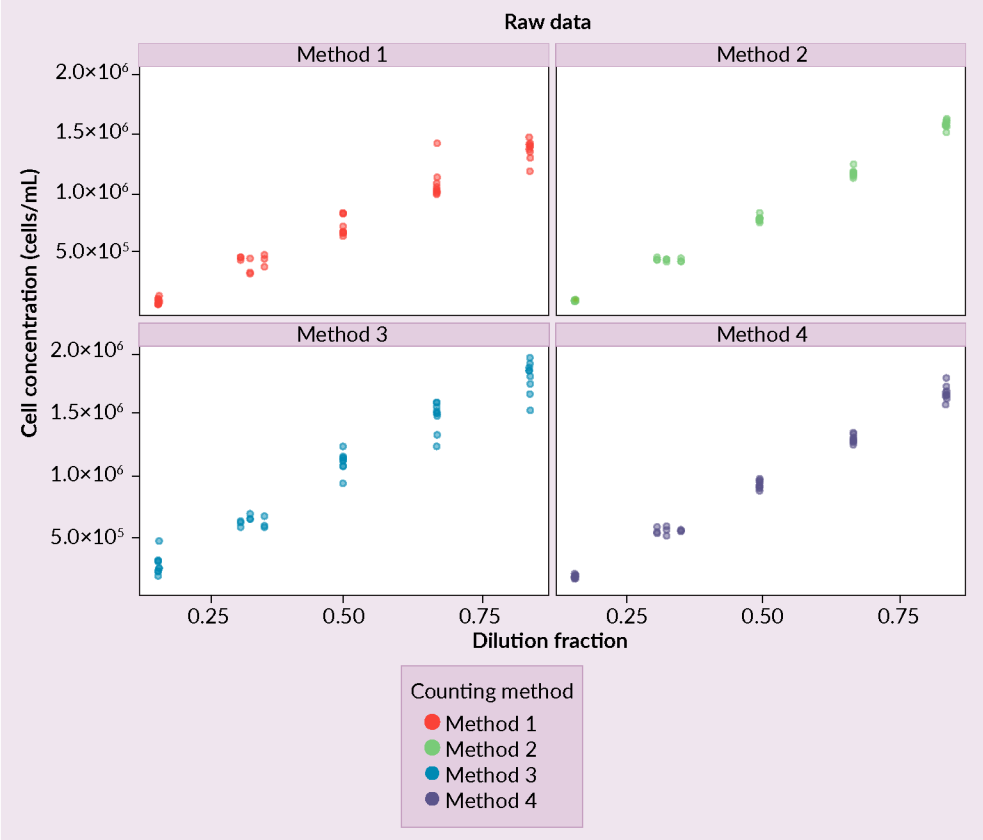
The raw data plot (Figure 2) provides a display of the raw data values uploaded by the user. Users may inspect the plot for any obvious data anomalies or errors, as well as to get an initial sense of each method's measurement response to dilution fraction. For this example, observing the spread in replicate points on the plot suggests that Method 1 and Method 3 have higher variability between replicate observations than do Method 2 and Method 4. Additionally, since our example uses a measured dilution fraction, we can see the distribution of points along the x-axis (for example, points near a dilution fraction of 0.3 have a wider spread along the x axis, indicating greater differences in pipetting between replicate samples).

Figure 3 displays the Mean Cell Concentration (average of replicate observations) for each replicate sample at each dilution fraction and method. Here, we see that relative bias in overall concentration may exist between methods (bias is further investigated in Results Section [Quality indicators], Figure 5C). In this example study, Method 3 tends to produce the highest overall counts and Method 1 tends to produce the lowest counts. We can also use the toggle button 'Show Prediction Intervals' to visualize the predicted range of mean cell concentration values for a new replicate sample based on the fitted polynomial model.

The final plot on the Data Overview tab shows the residuals from each replicate sample to the proportional model fit (Figure 3B). Residuals represent the difference between the predicted values of y (based on the flexible model fit) and the observed values of y. This visualization allows the user to observe the deviation from the proportional model fit of each replicate sample, for each method at each dilution fraction. A pattern where the points do not appear to be randomly

FIGURE 2

Cell concentration raw data is plotted versus dilution fraction for each method. All replicate observations from each replicate sample are shown.



scattered about the y=0 horizontal line suggests potential systematic deviation from proportionality. For Method 3 data in this experiment, the residuals at a dilution fraction of 0.1 all fall above the y=0 line and at a dilution fraction of 0.9 the residuals all fall below the line. This may suggest a systematic deviation from proportionality, such as could occur if non-cell components of media were counted as cells or if an instrument experiences saturation effects. This plot also allows the user to begin to observe method precision, with large vertical spreads indicating higher variability (less precision). Smoothed residuals are computed to better accentuate potential disproportionalities in measured cell concentrations. When measured dilution fraction is provided, smoothed residuals are computed at each measured dilution fraction as the difference between the fitted polynomial model and the proportional model. When measured dilution fraction is not provided, smoothed residuals are computed at each target dilution fraction as the difference between the average cell concentration of replicate samples and the value of the proportional model. Raw residuals, smoothed residuals, and scaled smoothed residuals (smoothed residuals normalized by the dilution fraction value) are shown (Figure 3B).

Quality indicators

The next tab, 'Quality Indicators', provides a visualization of the R^2 value along with the selected proportionality indices for each method and corresponding bootstrap confidence intervals (Figure 4).

The results shown in Figure 4A indicate a high R^2 value for Method 4 with a very

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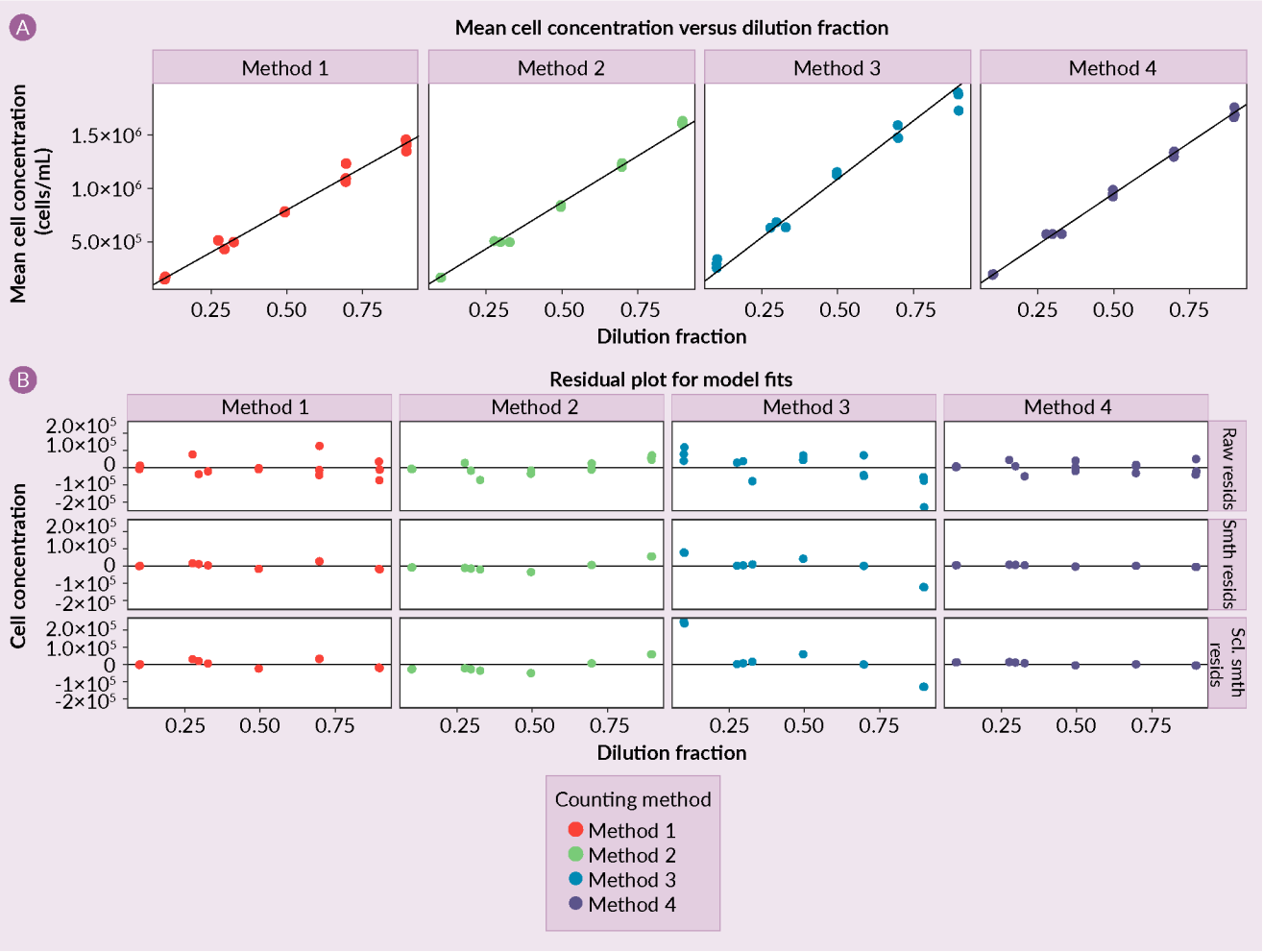
FIGURE 3

(A) mean cell concentration versus dilution fraction indicating average of the triplicate observations for each sample with error bars indicating the prediction intervals computed using the fitted flexible polynomial model. (B) raw, smoothed, and scaled smoothed residuals from the proportional model fit for each of 4 methods at each dilution fraction sample.



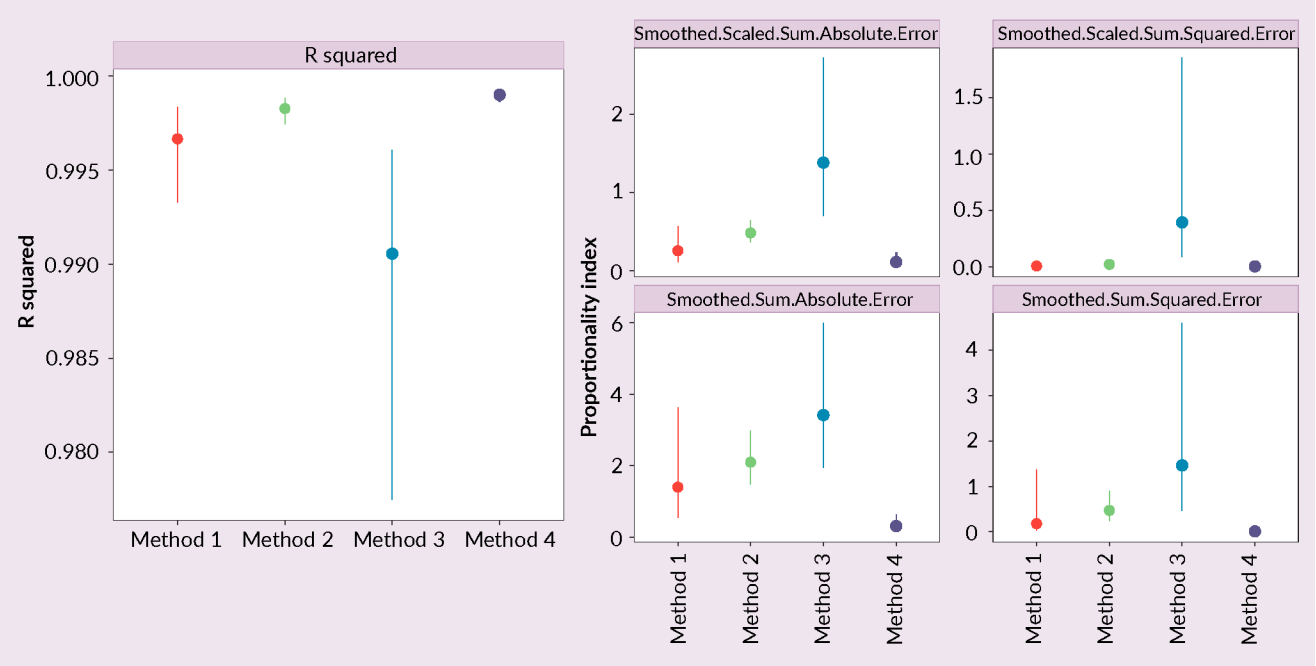
narrow confidence interval range, indicating a high level of confidence in the calculated R^2 value. Method 3 has a lower R^2 value and a much broader confidence interval. Large uncertainties from bootstrap analyses can often be explained by the variability across replicate samples. Looking at the Method 3 data, we see that one of the replicate samples at a dilution fraction of 0.9 had a particularly low count, which could explain this result.

The Proportionality Index (PI) plots allow the user to compare the proportionality indices for each method, using several different calculations for PI. PI metrics measure the deviation of each method from proportionality, therefore a larger value for PI indicates a higher deviation from proportionality.

Here, Method 3 demonstrates the highest deviation from proportionality across all four PI metrics, with wider confidence intervals in each case. Method 4 has the smallest deviation from proportionality for each of the calculated PI values. PI metric calculations, as well as the equation for the flexible model fit and the smoothing approach, can be found on the 'Stat Analysis' tab. All forms of the PI metrics are based on the smoothed residuals of the measured data from the flexible model fit. They vary by whether residuals are scaled (i.e., scaling is when residuals

FIGURE 4

(A) R^2 value of each flexible model for each counting method. The vertical bars represent bootstrap confidence intervals computed at the requested 95% confidence level. (B) Proportionality Index (PI) value for each counting method, computed using four different calculations for the sum of the residuals from the flexible model. The vertical bars represent bootstrap confidence intervals computed at the requested confidence level. Four metrics for calculated PI are shown in the four sub-plots.



are divided by the dilution fraction to increase the influence of measurements at low dilution fractions) and whether absolute or squared value of the residuals are used, where a squared value focuses most heavily on a method's most extreme residuals and the absolute value considers residuals more evenly. Each of these metrics provides a slightly different perspective of the data, so users may feel greater confidence in which method appears most proportional when all four metrics produce the same ranking, as in this case.

Following the R^2 and PI metrics, COMET outputs a plot of mean concentration per dilution fraction for each method, allowing a visualization of bias between methods and the error associated with each method at each dilution fraction at the confidence level chosen by the user. Mean concentration for each dilution fraction is a required reporting element of the ISO cell counting Part 2 standard. In Figure 5A, we see that the ordering of the methods' measured concentrations is consistent across each dilution fraction. From smallest to largest, this ordering was Method 1, Method 2, Method 4, and Method 3.

The mean concentration plot is followed by a plot of the calculated Mean %CV at each dilution fraction for each method (Figure 5B), which gives a measure of variability (or inversely, precision) and is another quality indicator that must be reported for the ISO cell counting Part 2 standard. In this example, we observe the overall %CV tends to be lowest for Method 2 and highest for Method 1 or Method 3. We also observe an atypically high %CV for Method 3 at the 0.1 dilution fraction, which may indicate that the instrument performed poorly at that concentration for this cell preparation.

COMET next plots the estimated Proportionality Constant (the value β1 from the proportional model fit equation

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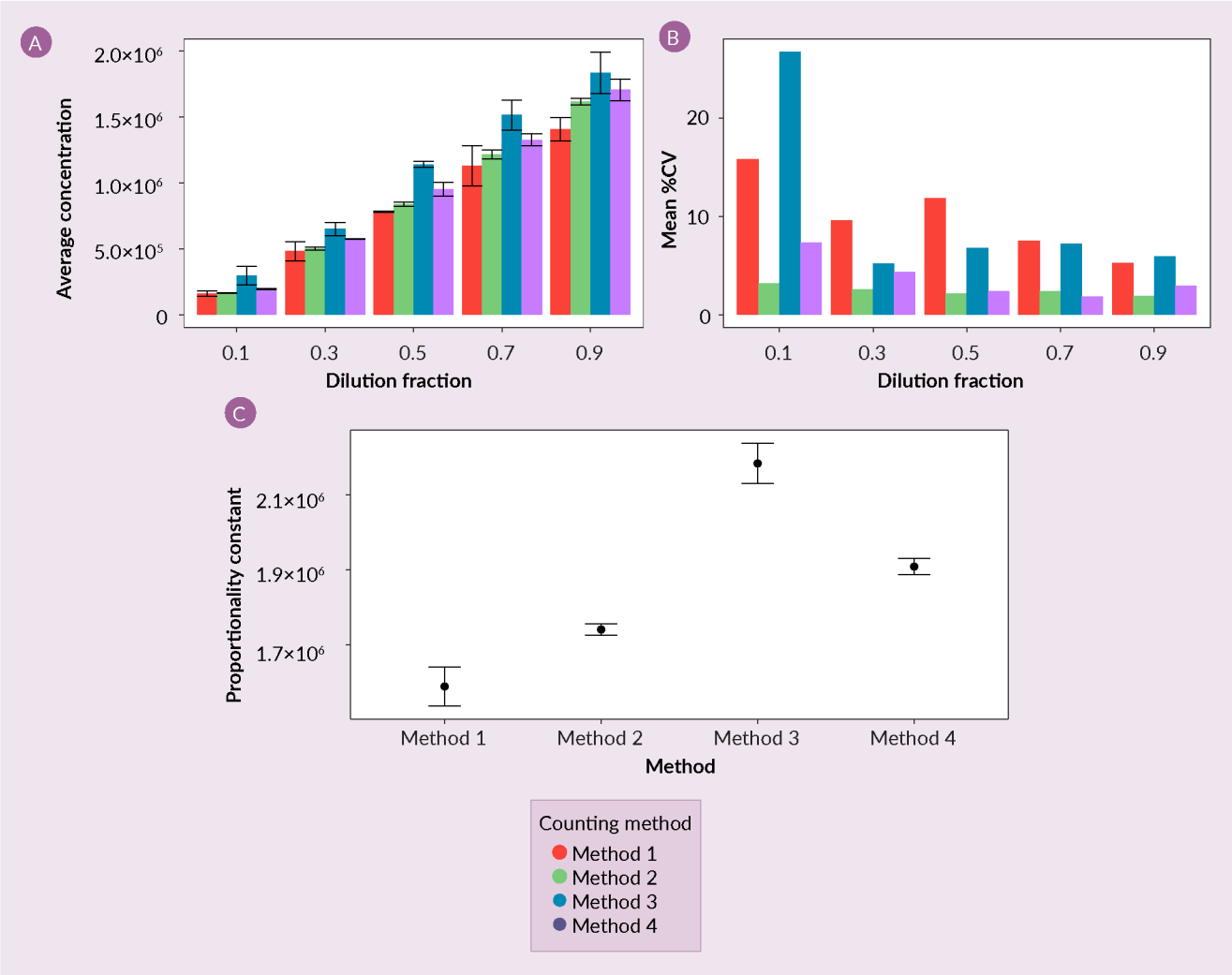
FIGURE 5

A) Mean concentration versus dilution fraction for each method. B) Mean %CV for each method at each dilution fraction. C) Proportionality constant (slope estimate) for each method with bootstrap confidence intervals. Non-overlapping intervals suggest that differences between proportionality constants across the counting methods are statistically significant.



$\lambda_{ij}=\beta 1 DF_{measuredij}$) for each cell counting method, along with the corresponding confidence interval (Figure 5C). Under the proportional model, the proportionality constant represents the cell concentration of the undiluted stock solution. The β1 from the proportional model fit also needs to be reported when following the ISO cell counting part 2 standard.

Here, we observe that Method 3 has the highest estimated proportionality constant compared to other methods, followed by Method 4, with Method 1 having the lowest value for the cell sample tested. This implies that Method 3 systematically produced higher counts than the other methods, and Method 1 systematically produced lower counts than the other methods. Moreover, there is no overlap between the confidence intervals for the proportionality constants, indicating statistically significant differences between the counting methods. We note that the proportionality constant does not provide information on the performance of a cell counting method, only the slope of the fitted proportional model. We also note that if a cell counting method seems to deviate significantly from a proportional model, the

proportionality constant may not be an appropriate metric by which to summarize or compare method outputs.

Metrics tables, experimental design & stat analysis

The next three tabs found on the COMET page are the 'Metrics Tables', 'Experimental Design', and 'Stat Analysis' tabs. The 'Metrics Tables' tab presents analysis results for each method in table form, allowing the user to see the mean concentration and %CV at each target dilution fraction, as well as R^2 and PI, with the upper and lower confidence levels for each metric. For convenience, these tables can be downloaded into a spreadsheet format with the 'Download Tables' function. Data from these tables can allow users to re-create the plots presented in graphical format within the other tabs (e.g., the quality indicator plots).

The 'Experimental Design' tab offers a quick check to determine if all data has been entered in the proper format. Here, we can check for the correct number of dilution fractions, replicate samples and observations, as well as for pipetting error if a measured dilution fraction was obtained. These tables can also be easily downloaded into spreadsheet form. These tables can be useful in preparing the reports described in the standard. The standard requires that the experimental design, including the target dilution fractions, numbers of replicate samples, and numbers of replicate observations, be reported. In some cases, the study design may not be perfectly balanced due to errors with individual samples or observations that may have need to be excluded. These tables help to track and report that information.

The 'Stat Analysis' tab provides details on the equations, models and assumptions used in the analysis that was conducted which was based on user inputs. These statistical details, for example, the proportional model assumption, the proportionality constants for the proportional model fits, the equations for calculating PI, the smoothing approach, and the number of bootstrap iterations, are all required reporting elements in the ISO standard.

Compare methods

The 'Compare Methods' tab shows the computed PI metrics in tabular form and calculates the ratio of values for comparisons between each pair of methods as well as gives the upper and lower bootstrap interval limits for each ratio (Table 2). This table also indicates whether the difference in PI is statistically significant (when the value '1' falls outside the range between the upper and lower bounds of the bootstrap interval, the difference between methods is considered statistically significant).

The discrimination bands plot (Figure 6) is intended to illustrate the range of input cell concentrations (dilution fractions) that can produce a given measured value for each measurement method. Since there is variability among measurements from a single sample (i.e., a fixed cell concentration), there is a range of input cell concentrations that will result in a specific measured value. For a given measured value, we estimate this range by looking at all the cell concentrations for which the prediction interval from the flexible model includes the measured value. The discrimination plot conveys this information using dilution fraction in place of cell concentration to enable direct comparison between methods that may exhibit substantial bias in cell concentrations relative to one another. For example, at the x-axis value of 0.50, the vertical range on the y-axis gives the range of dilution fractions that could have generated the cell count predicted by the flexible model for a dilution fraction of 0.50. In Figure 6, we see that for Methods 1 and 3, the estimated cell concentration for a dilution fraction of 0.5 could reasonably occur from any sample with an underlying dilution fraction between about 0.4 and 0.6. Methods 2 and 4 produced narrower ranges of roughly from 0.45

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TABLE 2

(A) Comparison of PI values for each pair of methods, using the Smoothed Sum of Absolute Error PI metric. (B) Comparison of Proportionality Constant values for each pair of methods. For both tables, upper and lower limits of the bootstrap interval for the ratio are provided and a 'yes/no' indicates whether pairwise differences in reported metrics are statistically significant.

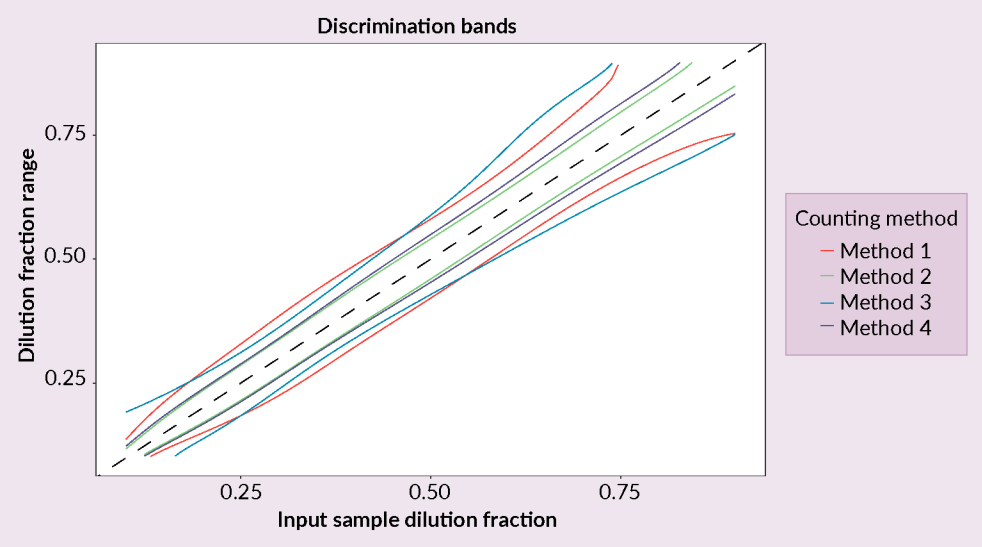
A		Metric	Method 1	Method 2	Ratio	Bootstrap lower CL	Bootstrap upper CL	Significant
	1	Smoothed. Sum. Absolute. Error	Method 1	Method 2	0.66662	0.36957	1.7312	No
	2	Smoothed. Sum. Absolute. Error	Method 1	Method 3	0.40926	0.20238	1.0967	No
	3	Smoothed. Sum. Absolute. Error	Method 1	Method 4	4.4875	0.94236	6.7763	No
	4	Smoothed. Sum. Absolute. Error	Method 2	Method 3	0.61394	0.35497	0.89205	Yes
	5	Smoothed. Sum. Absolute. Error	Method 2	Method 4	6.7318	1.6495	6.3161	Yes
	6	Smoothed. Sum. Absolute. Error	Method 3	Method 4	10.965	2.7142	12.728	Yes
B		**Metric**	**Method 1**	**Method 2**	**Ratio**	**Bootstrap lower CL**	**Bootstrap upper CL**	**Significant**
	1	Prop. Const.x	Method 1	Method 2	0.91291	0.89002	0.93514	Yes
	2	Prop. Const.x	Method 1	Method 3	0.72792	0.70772	0.7474	Yes
	3	Prop. Const.x	Method 1	Method 4	0.83269	0.81041	0.85391	Yes
	4	Prop. Const.x	Method 2	Method 3	0.79736	0.7817	0.8131	Yes
	5	Prop. Const.x	Method 2	Method 4	0.91213	0.90513	0.92147	Yes
	6	Prop. Const.x	Method 3	Method 4	1.1439	1.124	1.681	Yes

to 0.55. This suggests that measurements from Methods 2 and Method 4 are more discriminating or more informative about the underlying cell concentration than are measurements from Methods 1 and 3 in this use case. Note that because we use dilution fraction in place of cell concentrations, these assessments are not affected by the substantial relative bias in concentration measurements across the four methods.

The discrimination bands plot repeats this analysis for each dilution fraction in turn, gathering all target (or measured) dilution fraction values whose prediction intervals contain the estimated cell concentration for the considered dilution fraction.

FIGURE 6

Discrimination bands plot.



Vertical range on the y-axis gives the range of dilution fractions that could have generated the cell count observed (or predicted) at any given x input dilution fraction value. The plot uses the fitted flexible model to estimate the range of instrument readings that could plausibly have generated the observed reading at the dilution fraction on the x-axis. The range is computed by gathering all target (or measured) DF values whose prediction intervals contain the cell count corresponding to the desired dilution fraction. The dotted line indicates a perfect response where the input sample dilution fraction and the dilution fraction range is equivalent.

Discrimination band plots are sensitive to both non-linear measurement trends (such as saturation effects at higher cell concentrations) and method precision. Among counting methods that exhibit proportional (or linear) behavior across the considered range of concentrations, having narrower discrimination bands is essentially equivalent to having a lower %CV.

In this example, the discrimination band is narrowest for Method 2, demonstrating that in this use case a measured result from Method 2 would be expected to be most informative about the input dilution fractions. At the lower end of the input sample dilution fraction axis, the Method 4 band overlaps with that of Method 2, but widens slightly when approaching higher dilution fractions, indicating that the informativeness of a Method 4 measurement starts to diminish compared to a Method 2 measurement when approaching higher dilution fractions; this is also observed more dramatically for Methods 1 and 3.

Experiment integrity

The ISO standard recommends that the time at which each observation is made during the experimental design be documented to monitor for unexpected temporal effects over the course of the study. The 'Experiment Integrity' tab includes plots of cell count and when available cell viability over time, if the 'time_elapsed' variable is included in the Full Data Template.

The 'Experiment Integrity' tab allows the user to investigate aspects of sample stability with respect to time. **Figure 7** displays the scaled difference (i.e., (observed-predicted)/predicted) of the cell concentration from the expected concentration (estimated by the proportional model), where agreement with the expected concentration is indicated by the horizontal black lines. Separate subplots are displayed for each method, and different shapes, sizes, and colors are used to distinguish the different analysts, target dilution

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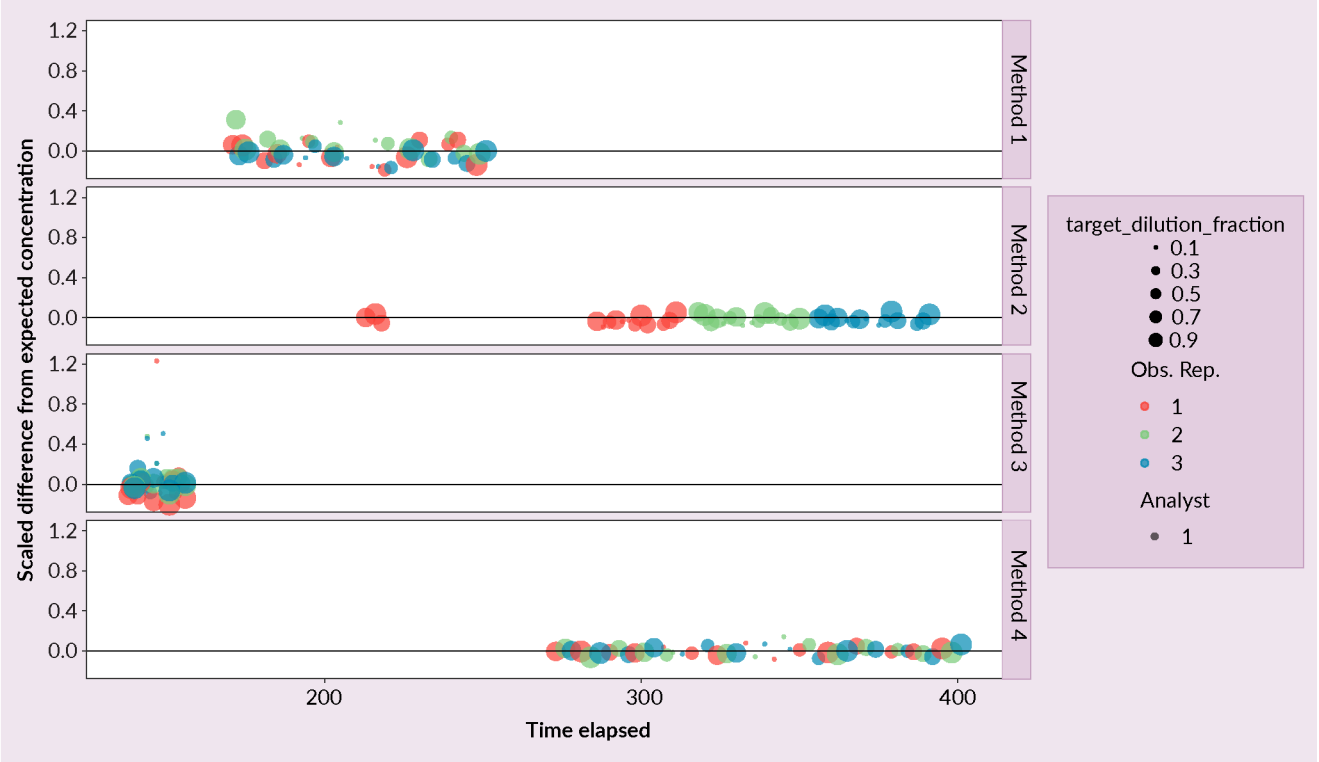
FIGURE 7

Sample integrity over time. Scaled difference from expected concentration is calculated and displayed versus time elapsed for each method. Visualization includes different size points for each dilution fraction and different colors for each replicate.



fractions, and replicate observations, respectively. There is a dropdown menu where the user may choose to focus on one counting method or may view them simultaneously (as shown in Figure 7). Systematic differences from the horizontal black line over time may suggest instability in the cell samples or changes in the counting method over time. For instance, a downward or upward trend over time could indicate that the sample is unstable, which could potentially be due to aggregation over time or settling of cells over time. In this example, we observe minimal changes over time from the horizontal line suggesting that the experimental systems (i.e., test samples and method) are stable over the course of the study for cell concentration measurements.

There are individual observations in the Method 3 plot at the 0.1 target dilution fraction that have larger differences from the expected concentration than other observations for that method. This result is also reflected in the %CV plot, where we observe higher deviation for Method 3 at the 0.1 dilution fraction. No other prominent outliers are observed here.

This visualization allows us to easily observe that there are more pink dots (Obs. Rep. 1) falling under the line for Method 3, which may indicate a potential replicate effect for the first observation of each replicate. This could point to a sample handling effect or chamber slide issue. This is a potential area of measurement error for this method that could be further evaluated and may allow for improvement in the method's PI based on correcting this variation at one or more dilution fractions.

If cell viability data is included in the Full Data Template, COMET will display a plot showing Viability Over Time Comparison between methods within the 'Experiment Integrity' tab. If a measured dilution

fraction data was included in the Full Data Template, the final plot on the 'Experiment Integrity' tab displays a measured dilution fraction versus target dilution fraction plot to illustrate pipetting error. See Supplementary Sections 2.1 and 2.2 for further discussion of these results.

Viability analysis

The final 'Viability Analysis' tab includes analysis for differences in cell viability and comparison of cell viability results when the 'percent_viable_cells' data is included in the uploaded template. The first plot, titled 'Viability Comparison', provides a histogram with probability densities of the observed percentage of viable cells for each observation, where different colors indicate the different cell counting methods (Figure 8A). Theoretically, it is expected that cell viability should remain constant even as cell concentration changes at each dilution fraction. Therefore, it is expected in an ideal scenario, that the distribution in cell viability results across all of the observations in the study be narrowly distributed. In this study, we observed a relatively narrow distribution in the viability observations for Method 4 (ranging from approximately 80% to 95% viability across observations), followed by a slightly wider distribution for Method 3 (65% to 85%) and the widest distribution in viability for observations from Method 1 (55% to 80%).

The plot also provides visualization of potential bias in % viability results between methods. The remaining plots and tables in the 'Viability Analysis' tab provide statistical analysis on whether observed differences in viability among the methods appear statistically significant. The analysis looks for potential differences in both variability and mean of the viability values. First, the absolute residuals of each method's viability percentages (i.e., |viability % – average viability %|) are analyzed to look for differences in variability. Empirical Cumulative Distribution Functions (CDFs) and histograms of the absolute residuals are presented for each counting method's viability percentages (Figures 8B & 8C). The results of a Kolmogorov-Smirnov test are computed between each pair of methods, indicating whether the data show statistically significant differences in variability between methods. In Figure 8D, we see that Method 1 appears to have a distribution that is statistically significantly different than that of Method 4 ($p < 0.05$), with the Method 4 data showing a statistically narrower distribution in viability measurements than Method 1. No other statistically significant differences were observed at the 0.05 significance level.

Lastly, two-sample t-tests are carried out between each pair of methods to investigate whether differences in the mean percent viability are statistically significant between each of the methods. In Figure 8E, we see that the mean % viability is statistically significantly different between each of the methods, as the reported p-values are all less than 0.05. Moreover, Method 1 and Method 4 appear to have the largest difference in viability, as the confidence interval for the difference is furthest from zero among the three pairwise comparisons (and is also visually evident in the Viability Comparison plot).

Study 2: evaluation of studies & comparison to non-parametric bootstrap analysis

When replicate experiments are available, statistical methods can be applied to provide consensus estimates and uncertainty intervals that aggregate information from each of the experiments. Completing replicate studies can provide a better estimate of PI and reduce error bars associated with the statistical metrics, allowing the user to establish a more robust understanding of the PI associated with a particular sample and method. The experimental design involving the analysis of 4 methods was repeated in 3 additional studies,

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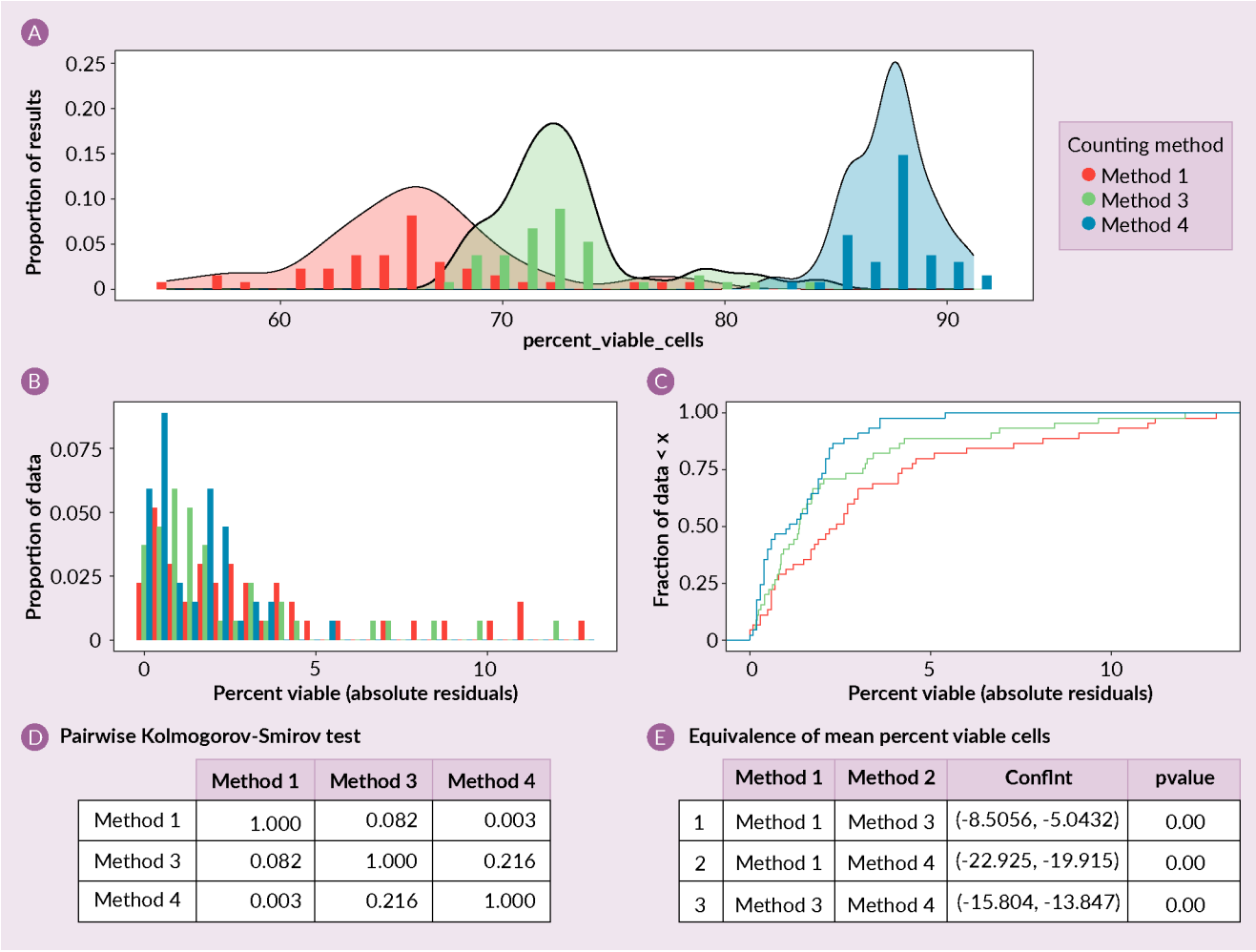
FIGURE 8

(A) Histogram showing % viable cells for each method and the proportion of results at each measured viability bin. (B) Fraction of data < each absolute residual from percent viable. (C) Proportion of data at each absolute residual value from the % viable cell data. (D) Pairwise Kolmogorov-Smirnov test for each pair of methods indicating statistical significance in differences in variability between Methods 1 and 4 ($p < 0.05$). (E) Pairwise equivalence of mean % viable cells, indicating statistical significance in differences in viability between each pair of methods.



D Pairwise Kolmogorov-Smirov test

	Method 1	Method 3	Method 4
Method 1	1.000	0.082	0.003
Method 3	0.082	1.000	0.216
Method 4	0.003	0.216	1.000

E Equivalence of mean percent viable cells

	Method 1	Method 2	ConfInt	pvalue
1	Method 1	Method 3	(-8.5056, -5.0432)	0.00
2	Method 1	Method 4	(-22.925, -19.915)	0.00
3	Method 3	Method 4	(-15.804, -13.847)	0.00

for a total of 4 studies with similar conditions using the same four measurement methods and a nutrient starved Jurkat population with nominally the same starting concentration (see Supplementary Table 1).

In the plots below, we use results from the COMET analysis across four repeated experiments to provide consensus estimates and uncertainty for PI across four cell counting methods (see annex for similar analysis on other metrics such as proportionality constant and R^2). We use the DerSimonian-Laird procedure to provide the consensus estimates as recommended in the National Institute of Standards and Technology (NIST) consensus builder user's manual [20] and implemented in an open-source web tool, the NIST Consensus Builder. Prior to performing the procedure, we log-transform the proportionality index values. The standard error of the individual estimates (which is needed for the DerSimonian-Laird procedure) is computed by using the bootstrap 95% intervals and approximating the bootstrap distribution as Gaussian.

Study 3: comparison of three Trypan blue-based cell counting analytical methods

In a follow-up study, we used COMET to compare three counting methods which all utilize the Trypan blue nuclear stain to differentiate live and dead cells: Method 4 (an automated Trypan blue based counting method), Method 5 (a semi-automated trypan blue counting method), and Method 6 (a manual Trypan blue based counting method).

Using an experimental design dilution scheme like the one shown in the Methods section, we compared these three methods (Supplementary Table 2 & Supplementary Figure 3). Using the COMET templates and resulting statistical analysis enables an in-depth comparison of these three methods.

Comparing the raw data in this example, we observe a substantially larger spread in the replicate observations for Methods 5 and 6 compared to Method 4 (Supplementary Figure 4). The plot suggests that cell concentration measurements from Method 4 method were less variable and had a lower average cell concentration than Methods 5 and 6 measurements across the range of considered dilution fractions.

The Mean Cell Concentration plot suggests that Method 5 trends toward higher overall cell concentration than the other two methods, and that Method 4 has the highest reproducibility between measurements at the sample replicate level (Figure 10A).

In the 'Quality Indicators' tab, the plots comparing R^2 and PI values between the three methods suggest that all three methods have strong linearity, and that Method 4 has the highest R^2 value with a confidence interval that does not overlap with those from the other two methods. This suggests that that the Method 4 R^2 value is statistically significantly higher than those from Methods 5 or 6.

The Proportionality Index plot (Figure 10C) suggests that the three counting methods have similar levels of proportionality. Although Method 4 has the best point estimates for proportionality (lowest calculated PI values), the confidence intervals are overlapping with the other two methods. This is consistent with visual inspection of the Raw Data and Mean Concentration versus Dilution Fraction plots in that no method exhibits clear disproportionalities.

Note that in this example, Method 4 exhibits the strongest proportionality. Additionally, these data do not suggest a meaningful difference in proportionality between Methods 5 and 6, given that their PI confidence intervals overlap. The Average concentration versus Dilution Fraction plot shows a consistent relative difference in overall concentration between Method 5 (highest absolute counts), Method 6, and Method 4 (lowest absolute counts) (Supplementary Figure 5). Biases between absolute counts for each method can be further observed in the Proportionality Constant plot and the bias tables (Supplementary Figure 4 & Supplementary Table 3).

The Mean %CV plot (Supplementary Figure 6) suggests that Method 4 had the greatest precision at each dilution fraction. The non-overlapping error bars suggest that the difference between the precision of Method 4 and the other two methods is statistically significant at each dilution fraction. Overall, Method 5 and Method 6 had similar precision levels to one another, with Method 5 having the highest estimated %CV for three of the five dilution fractions, and the Method 6 having the highest %CV in the other two (Supplementary Figure 6).

The Proportionality Constant plot indicates bias between methods in the overall total cell concentration. Non-overlapping error bars suggest these differences are statistically significant, which is further confirmed in the Proportionality Constant Comparison table (Supplementary Table 3). As initially observed in the Raw Data (Supplementary Figure 4) and the Mean Concentration versus Dilution Fraction plots (Figure 10A), Method 5 produces the highest measurements of cell concentration for this sample, followed by Method 6

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and then Method 4. We cannot assess which of the methods is most accurate from this plot; we can only observe relative bias between the concentration measurements provided by the different counting methods.

The discrimination plot (Figure 10D) shows that across the full range of considered cell concentrations a measurement from the Method 4 corresponds to the narrowest range of underlying cell concentrations. That is, the Method 4 measurements are most informative or most discriminating for these data. This is not surprising given that Method 4 had the greatest precision among the three methods and all three methods had similar levels of proportionality for these data.

DISCUSSION

Cell counting is a fundamental measurement in biotechnology and is especially crucial for the development, manufacture, and release of cell-based products [13,21]. Recently published ISO standards for cell counting have established terminology and required practices for conducting cell counting measurements and processes for evaluating the quality of a cell counting measurement process [11,12,15]. The open-source COMET was developed to facilitate implementation of ISO 20391-2:2019 [22,23]. This tool is a graphical user interface that accepts cell counting data generated via the experimental design outlined in the ISO standard in pre-defined templates ('Simple' and 'Full'). COMET outputs graphical and tabular representations of the statistical analysis outlined in the standard as well as additional information relevant to the reporting requirements and recommendations in the standard (i.e., experimental design, experiment integrity, and pipetting integrity). Additional analysis is also available in COMET to compare quality indicators and, when applicable, evaluate cell viability results. COMET also facilitates interpreting results through the Discrimination Bands Plot (Figure 6) and statistical comparisons in the 'Compare Methods' tab.

The use cases presented here compare different counting methods using the metrics outlined in the standard (PI, %CV, R^2) as well as a comparison of proportionality constants to evaluate bias between methods, and in some cases, comparison of the quality of viability results.

We illustrated the use of COMET to evaluate and compared four counting methods that use different measurement principles (biological markers and techniques) to determine count and viability of a nutrient starved Jurkat cell population (Study 1). The nutrient starved Jurkat cells provide an example of a cell material that is health compromised with reduced viability and increased complexity with regards to cell shapes, sizes, and cellular debris (see images in Supplement Figures 9–11). Complex samples such as these are a useful test material when evaluating cell count and cell viability methods as they can present challenges to the measurement system as opposed to fully healthy cell samples, which can be relatively simple to count even when the measurement method is not optimized [13]. Nutrient starved cells generated by overgrowth are particularly useful as they represent cell conditions that can occur during the expansion phase of a cell manufacturing process. Other treatments of cells can also be relevant based on the bioprocessing steps that may be conducted prior to the cell count assay (pre-analytical processes).

In Study 1, the analysis indicated that there was a significant bias between all of the methods (i.e., there was a significant difference in the proportionality constant used to model the different method responses) (Figure 5A & 5C & Table 2B). This indicated that it was reasonable to expect that each method would produce a different cell count result for the same sample. In this analysis, we did not make any assumptions as to the true cell count, and none of the methods were considered a reference method. Therefore, analysis of bias does not provide any information on the accuracy or quality of each method. Instead, we look to precision and proportionality analysis (%CV,

R^2, and PI) to evaluate method quality (note that evaluation of the quality indicators also does not provide information on true accuracy of the methods).

Evaluating precision (mean %CV from replicate observations) across replicate samples at each DF, we observed that Method 2 had consistently low %CV across all dilution fractions (%CV less than approximately 3%) (Figure 5B). Method 4 also had relatively low %CV at all dilution fractions except for the lowest cell concentration at DF 0.1 (% CV less than approximately 5%). For Method 1 and Method 3 challenges with precision were particularly pronounced at lower cell concentrations (DF 0.1) and %CV was greater than 5% at all other DFs. Obtaining more replicate observations during routine use, when low cell concentrations are expected, could help to improve the quality of cell count analysis for Methods 1 and 3 by reducing the variability of the average.

Evaluating proportionality, we observed that the proportional model is a reasonable fit for all methods (Figure 4A, goodness of fit, $R^2 > 0.99$). The R^2 value was greatest for Methods 2 and 4, followed by Methods 1 and 3 (Figure 4A) while uncertainty around the R^2 value was particularly large for Method 3 compared to the other methods. Since the evaluation of R^2 is dependent on both systematic deviation from proportionality and random variability of the data (e.g., variability between replicate samples) this metric cannot distinguish the source of deviations from the proportional model as arising from random variability or systematic disproportionality. To further evaluate systematic deviation from proportionality, the proportionality index (PI) is calculated.

PI, a cell counting quality indicator established in ISO 20391-2:2019, is used to evaluate systematic deviation from proportionality by reducing the influence of random variability on the analysis (i.e., through smoothing of the data). PI is evaluated by summarizing the smoothed residual and can be calculated in numerous ways. COMET provides the user with a choice of four different approaches to calculate PI. The user can choose to evaluate PI based on scaling by dilution fraction ('scaled') and can choose whether to use the absolute value ('Abs') or squaring of raw residuals ('Sq'). Because counts with higher averages tend to have greater variability, PI based on unscaled residuals will tend to emphasize behavior at higher concentrations. Scaling residuals by dilution fraction reverses this effect and leads to PI characterizations that tend to emphasize behavior at lower concentrations (at least when variances are presumed to be proportional to dilution fractions, as in an idealized system following a Poisson distribution). To evenly weigh each dilution fraction's expected influence on the PI measure, one could use a variance-stabilizing transformation (i.e., divide each residual by the square root of its estimated variance). This approach is illustrated in Supplementary Section 5. PI metrics based on the squared value of residuals will emphasize behavior of the largest residuals, while PI metrics based on the absolute value of residuals will more evenly reflect the behavior of all residuals. For Study 1, we found that the 4 different PIs calculated by COMET followed the same trend where Method 4 had the lowest PI (indicating the least deviation from proportionality, i.e., the most proportional method), followed by Method 1, then Method 2, then Method 3 (Figure 4B). Focusing on PI based on the smoothed scaled sum of absolute error (Table 2A), the non-parametric bootstrap analysis further indicates that PI is significantly different between Method 2 and Methods 3 and 4, and PI for Method 3 is also significantly different from Method 4. Due to the confidence interval for Method 1, it is not found to be statistically different from the other methods with regards to PI.

Combining analysis from R^2 and PI, Method 4 had the strongest performance across each metric. Method 2 had the second-best R^2 value, and Method 1 had the second-best PI metrics. This reaffirms observations from the raw data that Method 2 had lower noise

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than Method 3 (which is confirmed by Method 2 having lower %CV values than Method 3) but also appeared to be less proportional than Method 1 (smoothed scaled residuals for Method 2 had an apparent parabolic trend in Figure 3B). Though its R^2 was still above 0.99, Method 3 had the poorest performance among the methods considered in this example.

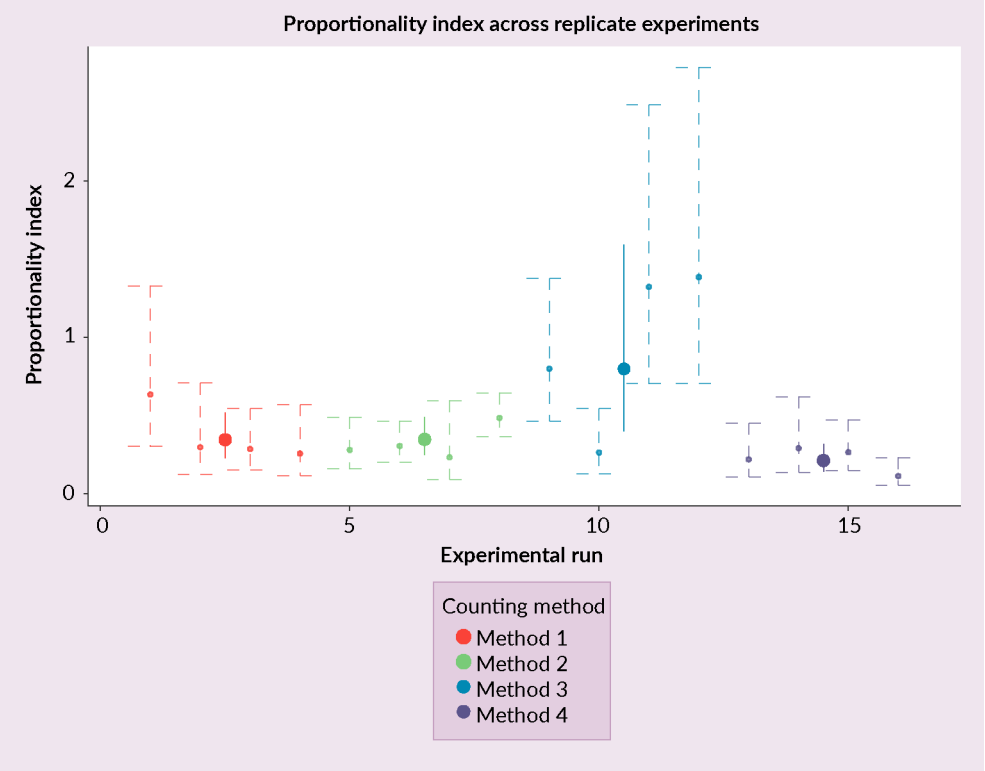
To gain a wholistic view of the entire data set derived from the experimental design, and to support interpretation of the quality indicators, we designed the 'Discrimination Bands' plots (Figure 6). As an example, based on the discrimination bands for Method 2, at an input sample DF of 0.5, it is possible that any sample that has a true DF between approximately 0.45 and 0.55 could have resulted in a DF of 0.5. The discrimination band plot is presented as a function of dilution fraction to put all methods on the same scale (since each method would have a different range of cell concentrations due to the bias between methods). When looking at each method individually, we can consider this plot on a concentration scale, for ease of interpretation. If it is assumed, based on the proportionality constant for Method 2, that the stock cell solution concentration for Method 2 was approximately 1.75×10^6 cells/mL, this would indicate that for a sample that is measured at 8.75×10^5 cells/mL (i.e., 0.5 DF), it is possible that any sample between the concentration range of 7.88×10^5 and 9.63×10^5 cells/mL could have generated that result. Therefore, the plausible range of cell concentrations associated with the measurement of 8.75×10^5 cells/mL could actually be somewhere between 7.88×10^5 and 9.63×10^5 cells/mL. By examining these ranges, a user can determine if these margins of error are acceptable for their intended purpose and can also evaluate their instrument's ability to discriminate between different cell concentrations for their test samples.

A key feature of COMET is the inclusion of non-parametric bootstrap analysis to facilitate the comparison of quality metrics between cell counting methods. COMET allows users to input the number of bootstrap iterations and the level of confidence for the metrics (e.g., a 0.95 level of confidence means that a user may be roughly '95% confident' that the true metric lies within the presented 95% confidence interval). This bootstrap analysis is particularly useful when the experimental design cannot be replicated due to time, resource, or sample availability constraints. The experimental design can take several hours to execute and can require the availability of numerous operators, especially when several methods will be compared. The bootstrap analysis provides an alternative approach to estimating uncertainties that does not require deriving replicate values for quality indicators (i.e., conducting replicate studies). The bootstrap approach also has the advantage that it requires relatively few assumptions on the data-generating process, which allows users to proceed without needing to verify, for example, distributional assumptions for each replicate study. In Study 2 we compare the bootstrap approach to the approach of replicating the experimental design four times and find that for this study, the consensus estimates for each method generally fall within the bootstrap intervals for each individual study (Figure 9).

At a high level, this demonstrates that for these methods and data, the bootstrap approach to estimating confidence intervals produced a reasonable characterization of the uncertainty in the average metric value. In the long run, the bootstrap intervals are intended to capture the true average value 95% of the time. Methods 2 and 3 each have one bootstrap interval that does not include the consensus estimate (which still has uncertainty) (Figure 9). Missing the respective consensus estimates for two out of 16 intervals could potentially suggest that the bootstrap intervals are slightly underestimating the uncertainty, but 16 tests are not enough for this difference (12.5% observed versus 5% targeted) to be statistically significant (p-value of 0.189,

FIGURE 9

Consensus estimates and intervals from the combined analysis of repeated studies are shown in large points and bold lines respectively. Raw data and corresponding bootstrap intervals from individual studies 1–4, respectively, indicated with the smaller points and dashed lines. Proportionality index represents the sum of residual calculation based on the smoothed scaled sum of the absolute error.



evaluated from binomial distribution). The consensus intervals benefit from four studies worth of information, which generally leads to narrower uncertainties than the bootstrap intervals from individual studies. In general, the individual bootstrap intervals appear consistent with one another within each method. One exception occurs for Method 3, where in one of the four studies, a low PI is observed with more narrow bootstrap confidence bands than the other studies. If this was the only study conducted, the PI analysis may have led to different observations on the quality of the method. This serves as an important reminder that additional data can always help facilitate deeper and more robust insight into the quality of our methods. In many cases, a single execution of the dilution fraction design with bootstrap analysis can give meaningful insight into the quality of the method; however, where cost and time permit, there are benefits to repeating the experiment, especially when hoping to identify small differences between methods or when a counting method appears to have high variability.

In addition to calculating and comparing quality metrics, COMET can provide an analysis of the quality of % cell viability data, if it is included in the data set. Many cell counting methods count live and dead cell populations in addition to the total cell count. If % cell viability is recorded in the 'Full Data Template', the robustness of the % cell viability measurement to concentration, sample to sample variability and observation to observation variability can be evaluated. Bias between % cell viability measurements can also be evaluated. Theoretically, % cell

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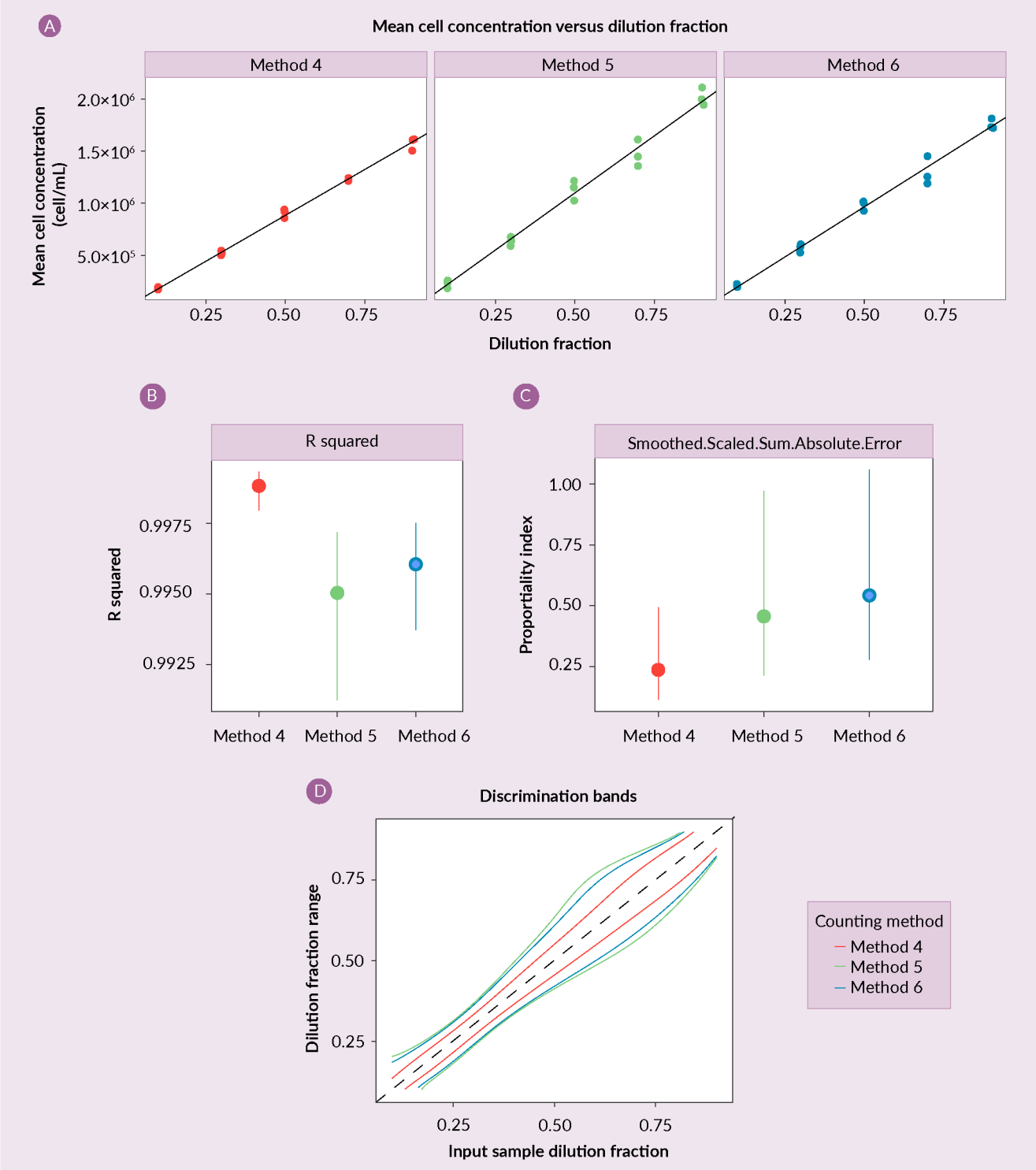
FIGURE 10

(A) Mean cell concentration at each dilution fraction for the three methods evaluated. Error bars represent the prediction intervals based on the fitted flexible model. (B) R^2 value for each of the three Trypan blue based methods. Error bars are based on the 95% confidence interval from a 1000 iteration bootstrap analysis. (C) PI values for one calculated PI metric for three methods. Error bars are based on the 95% confidence interval from a 1,000 iteration bootstrap analysis. (D) Discrimination plot for the three Trypan blue based methods, indicating the range of input dilution fractions that could product a target dilution fraction measurement result.



viability should have a narrow distribution even across samples with varying cell concentration. The analysis provided by COMET allows users to evaluate their % cell viability method against this theoretical, ideal outcome. In Study 1 we observed that the Mean % Viability was significantly different between Methods 1, 3, and 4 (Method 2 was not included in this analysis as it does not evaluate cell viability). Previous studies have also observed that cell viability assays based on different measurement principles (e.g., using different dye-exclusion membrane permeability dyes) can produce different cell viability results [13]. These differences can arise from different artifacts introduced in the measurement process (e.g., dye toxicity, signal to noise ratio etc.) or if there are differences in the measurement principles that may be probing different stages of cell health [24]. We also observed that, for this use case, Method 4 had the lowest variability in viability results across all observations compared to Methods 1 and 3.

ISO 20391-2:2019 and COMET can be used to aid the selection of fit-for-purpose cell counting methods through the comparison of quantitative metrics of method quality. This selection process can be done in the absence of a cell count reference material and without comparison of cell counting results from a historical or 'gold standard' method for evaluating accuracy. Using a nutrient deprived Jurkat cell population provided a sample with varying cell morphology and size characteristics (see images in **Supplementary Figures 9–11**), potentially adding additional complexities to the method comparison. For this specific cell sample, although morphology was complex, the three distinct methods of counting (fluorescent dye exclusion, Trypan blue dye exclusion, and Coulter based counting) all resulted in strong proportionality. However, we observed significant differences in viability distributions between the different methods. In this specific set of studies for a nutrient-starved Jurkat cell population, Method 4 yielded the highest R^2 value and the lowest PI across all four calculated PI indices, while maintaining a low overall % CV. Method 2 produced a high R^2 and low PI values (with slightly wider confidence intervals) and maintained the lowest overall % CV. These results allowed us to identify and select Method 4 for this particular cell system, while keeping in mind that Method 2 may have performed as a suitable alternative (although Method 2 did not provide viability information). Methods 1 and 3 had lower R^2 values, indicating less linearity and more random variability in measurements, and this result can also be observed in the higher % CV values for Methods 1 and 3. Based on the cell samples analyzed and the resulting data and statistical metrics generated, as well as the ability to analyze viability, Method 4 had the strongest performance metrics for this specific set of studies. COMET users should be cautioned that this result applies only to this particular cell sample and measurement process (including sample handling, data collection settings and data analysis settings), and any changes made to the cell sample type or its treatment or the measurement process may result in a different ranking of methods.

In order to have confidence in the quality indicators calculated from the data generated in the experimental design, it is critical to have confidence in the integrity of the experimental design. This includes 1) assuring that the materials used in the study are relevant for the intended use (i.e., cell concentrations within the range of the dilution fractions tested, and cell materials/diluent having similar composition to expected test samples), 2) having confidence in the dilution fractions generated (i.e., verifying pipetting integrity, and keeping samples adequately mixed and homogeneous before sampling and 3) maintaining sample stability over the course of the study execution. COMET provides tools to visualize and monitor aspects of experiment integrity including sample/measurement process stability over time and pipetting integrity. The 'Experiment Integrity' tab provides a convenient way to look for flags

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that something unexpected may be affecting analysis results such as potential replicate effects, temporal changes in count or viability, or effects at specific dilution fractions. In the current study, no significant trends in cell concentration over time were observed for any method. Interestingly, by observing the counting results as a function of time elapsed, we can see that Methods 2 and 4 took longer to execute than Methods 1 and 3. These practical considerations may be important for some applications, and a counting method with slightly lower but adequate proportionality and precision may be more appropriate. For example, Method 2 may be appropriate in Study 1, in the case that time constraints are a critical factor. The discrimination bands can also be reviewed to understand the consequence, in terms of measurement uncertainty, of selecting Method 2 over Method 4.

In Study 3, comparing three Trypan blue methods with different levels of automation ranging from fully manual to fully automated, we see that difference in counting method quality can arise even when the same measurement principle is utilized. In Study 3 we found that the fully automated Trypan blue method, Method 4, had better precision and proportionality compared to the semi-automated and manual approaches. This is not an unexpected finding as automation can reduce operator bias by automating aspects such as sample handling and data analysis and improve measurement precision by increasing the number of cells sampled [14]. Interestingly, we observed significant biases in cell viability results between methods (Supplementary Figure 8A and 8E). Through observations of the brightfield images from the Trypan blue methods of counting (Supplementary Figures 9–11), we observed that many of the identified objects may have been mis-classified as live or dead, resulting in differences in viability results between methods (but not necessarily affecting total cell counts). Further optimization of image analysis algorithms may reduce the biases between methods observed in Study 3.

In each study, observing the differences in statistical metrics through COMET's visual plots and tables allowed for the detection of significant differences in counting performance across the methods, and aided in the identification and selection of a fit-for-purpose cell counting assay for each individual use case.

CONCLUSIONS & TRANSLATIONAL INSIGHT

Cell counting is a critical measurement in the characterization and testing of cell-based products. ISO 20391-2:2019 provides an experimental design and statistical analysis approach to quantify counting method performance in the absence of a reference material. The Counting Method Evaluation Tool, COMET, is designed to support the implementation of the standard, reducing barriers to use such as inexperience with the statistical approaches and reporting burden. Here we demonstrate the utility of COMET in evaluating and comparing the quality of several cell counting methods. The quality of each of the different methods is compared by evaluating the metrics required by the standard (i.e., PI, R^2, %CV, Mean, Proportionality Constant), and specific methods are identified that better meet the fit-for-purpose needs for cell counting. The ISO cell counting standards and the COMET application respectively provide critical framework and tools for developing, evaluating, and comparing cell counting methods for biotechnology applications.

NIST DISCLAIMER

Certain commercial equipment, instruments or materials are identified in this paper to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are

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CODE & DATA AVAILABILITY:

Information on how to access and use COMET along with the source code can be found at the official COMET GitHub page.

Data citation: data sets used in these studies are publicly available here.

A video guide demonstration of the COMET interface is publicly available here.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: Sarkar S and Lund SP contributed to the preliminary concept, experimental design, and design of the statistical analysis. Sarkar S, Lund SP, Pierce L and Newton D contributed to experimental design for the studies described herein. Pierce L, Sarkar S, Newton D, and Lund SP contributed to manuscript writing. Pierce L and Kurbanov F acquired the data and contributed to data analysis. Newton D, Lund SP, and Sarkar S made substantial contributions to the concepts and to data interpretation. Lund SP and Newton D provided significant insights to the statistical metric interpretations. Newton D continues to maintain the COMET application and GitHub site.

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FASTFACTS

Efficient, scalable purification of VSV-G lentivirus by novel affinity chromatography

Frank Detmers, Director of Ligand Application for CaptureSelect, Thermo Fisher Scientific

Lentiviral vectors have emerged as a long-term stable gene expression tool for cell and gene therapies. However, large-scale production of purified clinical-grade lentiviral vectors remains a challenge because of the complex feedstock and its sensitivity to changes in temperature, ionic strength, pH, and other environmental factors. This poster presents the chromatography conditions and performance of a recently developed affinity chromatography resin for the purification of lentivirus particles.

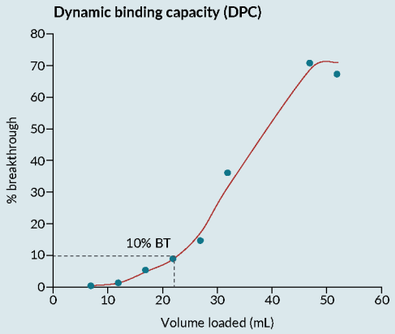
LENTIVIRUS PURIFICATION CHALLENGES

Lentiviral vectors (LVV) have limited stability, requiring a narrow range of pH, temperature, shear stress, salt concentration, and osmolarity. Because of this, traditional methods of purification suffer from difficulties relating to yield, purity, and scalability. With these methods, general recoveries in the field are not higher than 25–30% for the overall process, with a significant part of the losses being in the final filtration step utilizing a sterilizing-grade filter. Thermo Fisher Scientific recently developed an affinity chromatography resin, CaptureSelect™ Lenti VSVG Affinity Matrix, as a solution to these challenges.

DYNAMIC BINDING CAPACITY OF CAPTURESELECT™ LENTI VESICULAR STOMATITIS VIRUS G (VSV-G) AFFINITY MATRIX

Based on CaptureSelect™ technology, the immobilized ligand is developed to specifically bind to the VSV-G envelope protein present in the vast majority of recombinant lentiviral pseudotypes. Lentivirus produced in HEK-293 cells in suspension is loaded on 0.66 x 3 cm column containing 1 mL of CaptureSelect™ Lenti VSV-G resin, equilibrated in 50 mM HEPES buffer solution, 150 mM NaCl pH 7.5.

Figure 1. Dynamic binding capacity (1 mL column).



DBC is determined by P24 total particle ELISA

Fraction	Volume (mL)	TP/mL	C/C$_0$	
Start		3.98E9		
1	7.1	1.55E7	0.39	
2	12.1	5.24E7	1.32	
3	17.1	2.14E8	5.39	
4	22.1	3.53E8	8.88	24.6
5	27.1	5.85E8	14.7	
6	32.1	1.44E9	36.09	
7	47.1	2.82E9	70.84	
8	52.1	2.68E9	67.33	

- 10% breakthrough (C/C$_0$=10%) estimated from the curve at 24.6 mL loading
- This relates to 9.78E10 total particles/mL resin (=1E11)

Dynamic binding capacity at 10% breakthrough is 1E11 total particles/mL resin

Figure 2. Chromatography conditions of the CaptureSelect™ Lenti VSVG affinity matrix.

Endonuclease treatment

↓

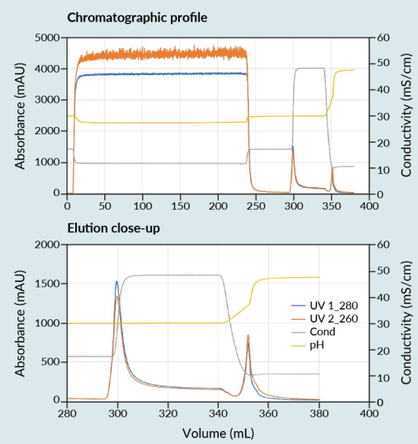
LV clarification using microfiltration (0.4 µm)

↓

CaptureSelect Lenti VSVG affinity matrix

- Column=1.6 × 5 cm (10 mL)
- Flow rate=150 cm/h
- Contact time=2 cm

Binding/equilibration buffer: 50 mM HEPES, 150 mM NaCL pH 7.5
Elution buffer: 50 mM HEPES, 150 mM NaCL, 0.8 M arginine pH 7.5
Strip buffer: 50 mM sodium phosphate pH 12



The CaptureSelect Lenti VSVG affinity matrix demonstrates an efficient elution profile

As shown in **Figure 1**, 10% breakthrough of the lentivirus particles is reached after loading 24.6 mL of the feed material, resulting in a dynamic binding capacity of the resin of 1x10^{11} total particles/ml of resin. C_0 is the titer of the feedstock (3.89×10^9 particles/mL), and C is the titer measured in the flow through fractions. The 10% breakthrough point is interpolated from the breakthrough curve.

CHROMATOGRAPHY CONDITIONS

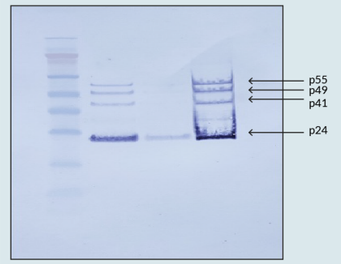
Figure 2 illustrates that the elution with 50 mM HEPES, 150 mM NaCl, 0.8 M arginine pH 7.5 is efficient and has good compatibility with the enveloped virus particles, resulting in high concentrations of infectious particles in the elution fraction. Depending on the feed and application, optimization of the elution buffer might be needed with adjustments of the arginine concentration, pH, or combinations thereof.

COMPARISON OF TOTAL PARTICLES TO INFECTIOUS PARTICLE RATIOS

The total concentration of infectious particles increases after purification **(Figure 3)**. Total particles are determined by p24 ELISA and infectious particles are determined through a cell infectivity assay. In the first run, 1 in every 100 particles is infectious in the elution fraction, while in the feedstock it is 1 in every 138 particles. In the second run, this ratio becomes 1 in 165 particles in the feed to 1 in 70 particles in the elution fraction.

Figure 3. Concentration of infectious particles in the elution fraction.

P24–WB pattern



Total particle (TP) and infectious particle (IP) ratio

Sample	TP/mL	IP/mL	TP/IP ratio
1. Feed	1.10E10	7.98E7	138
1. Flow through	3.25E8	8.30E5	392
1. Elution	4.44E10	4.42E8	100
2. Feed	1.11E10	9.00E7	165
2. Flow through	1.28E9	5.45E6	245
2. Elution	2.6E10	4.66E8	71

(n=2)

- The eluted fractions show a more than 5-fold increase of the infectious particle concentration compared to the load

The concentration of infectious particles in the elution fraction has been enriched through purificaton using the Lenti-VSVG resin

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In partnership with: ThermoFisher SCIENTIFIC

CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHTS

Environmental monitoring: optimizing microbial control in cell & gene therapy workflows

Nico Chow

Microbial control within biopharmaceutical production is critical to help ensure drug products are free from viable microorganisms. Implementation of an effective environmental monitoring program is required as part of the good manufacturing practice (GMP) guidelines, where minimally, the program should require the microorganism identification at the species level, or genus level where appropriate. Although various techniques exist, genotypic identification is considered to be the industry standard for microbial identification, because a DNA sequence potentially offers an unambiguous result.
In this article, the Applied Biosystems™ MicroSEQ™ Rapid Microbial Identification System, designed to support the recommended qualification guidelines by regulatory agencies worldwide, is discussed. Used by major pharmaceutical companies worldwide, the MicroSEQ Microbial Identification System is an end-to-end rapid genotypic sequencing solution based on ribosomal gene sequencing for bacterial and fungal species-level identification. The solution can generate accurate results in less than 5 hours.

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PHARMACEUTICAL MICROBIAL CONTROL

Pharmaceutical microbial control helps ensure that drug products are free from viable microorganisms that may ultimately harm the patients and compromise product quality. Finished and final drug products should be free from endotoxins (toxins that are produced by microorganisms). They can compromise product quality and safety, and have potential negative implications to the patient—notably, causing anaphylactic shock, and in worst-case scenarios, even death. These toxins can be difficult to remove if present. Therefore, it is crucial that both the products and the facility in which they are manufactured are kept free from harmful microorganisms. Products and product preparations need to be both sterile and endotoxin-free.

Due to the possible harmful effects, regulatory agencies expect an effective and robust

environmental monitoring program to be implemented to comply with good manufacturing practice (GMP) guidelines for the biopharmaceutical manufacturing. Recent updates to the EU GMP Annex 1 outline the implementation of effective quality risk management and contamination control strategies, such as requirements to identify microorganisms to species level for cleanroom grades A and B, and recommended microbial identification for grades C and D.

With the implementation of an effective environmental monitoring program, alert levels are set for each microbial technique. Microbial identification is an important tool for a contamination control strategy via environmental monitoring, enabling product sterility by identifying corrective and preventative action for microbial control failures. To implement microbial controls, manufacturers use different microbial techniques to track possible contaminants. For example, bioburden tests can be carried out to test raw materials. Continuous environmental monitoring programs in production areas of the facility should be considered to ensure the sterility of the manufactured products. Finally, sterility testing for final product release is used to reconfirm the sterility status of the drug product itself.

CHOOSING A SUITABLE METHOD FOR MICROBIAL IDENTIFICATION

When choosing an identification method or technology to adopt, there are three main parameters to consider: data quality, technology, and cost. When looking for accuracy in identification, the size of the database and the resources required to achieve specific identification must be considered. The ease of use, time to result, and throughput of the technology must also be taken into account. It is beneficial to explore the return on investment, not only reviewing the capital expenditure for the system, but total operational expenses.

The MicroSEQ Rapid Microbial Identification System is a full, end-to-end workflow solution, which includes all of the reagents for both bacterial and fungal identification, the instrumentation, and the software to enable progression from DNA extraction to automated data analysis of microbial species-level identification results. The MicroSEQ System is based on sequence analysis of ribosomal genes in bacteria and fungi. It is a well-established genotypic system that has been adopted by nine out of ten of the world's largest pharma companies, with 20 out of the 25 largest pharma companies having multisite adoption. Genetic sequencing has been considered a gold standard for the phylogenetic classification of bacteria and fungi and thus, the system has been designed to support the recommended regulatory guidance.

There are two main methods for microbial identification: phenotypic and genotypic approaches. Phenotypic methods are biochemical tests—for example, matrix-assisted laser desorption ionization (MALDI) or a phenotypical microarray. These methods are based on the expression of proteins, metabolites, enzymatic reactions, and responses to environmental conditions such as pH and temperature. Genotypic methods are based on the identification of the microorganism's DNA. Examples include sequencing or ribosomal strain typing. With phenotypic methods, which are dependent on culture conditions, the expression of proteins and enzymatic reactions can be variable depending on the culture conditions provided. With genotypic methods, either viable or non-viable cultures (including older cultures) can be used for accurate identification. Therefore, the genotypic method is the gold standard for microbial identification.

MICROSEQ ID SYSTEM

The MicroSEQ ID System core technologies utilize both PCR and sequencing. After DNA extraction, DNA is amplified using PCR kits. Then, DNA sequencing is completed involving the labeling of nucleotides and amplified

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regions with fluorescent dyes, and sequences are generated by the genetic analyzers. Sequence quality analysis and comparison of the generated sequences against the reference databases is performed using the MicroSEQ ID software. Further details of the MicroSEQ ID System components are shown in Figure 1.

MicroSEQ ID assays target the ribosomal RNA gene (rDNA) genes universally present in all bacterial and fungal species. The PCR primer designs of the kits are targeted to the conserved regions of the 16S gene for bacteria and the D2 LSU gene for fungi. The divergent regions within these genes allow identification between genera and even species. With this, a genotypic approach has a higher discriminatory power for microbial identification than other methods.

Most of the currently available microbial technologies offer similar times to results. The technology is either for routine species identification or strain typing, as these are two different functionalities. Each technology differs primarily in the size of the databases, which will determine the rate of accuracy of identification. The MicroSEQ ID system has validated databases focused on sequencing technology and validated libraries with >12,000 species, with a particular emphasis on environmental species. Other phenotypic methods can have extensive libraries, but their focus is on clinical species. The MicroSEQ ID System workflow has five easy steps, shown in Figure 2, and can take 5 h from isolated colony to identification.

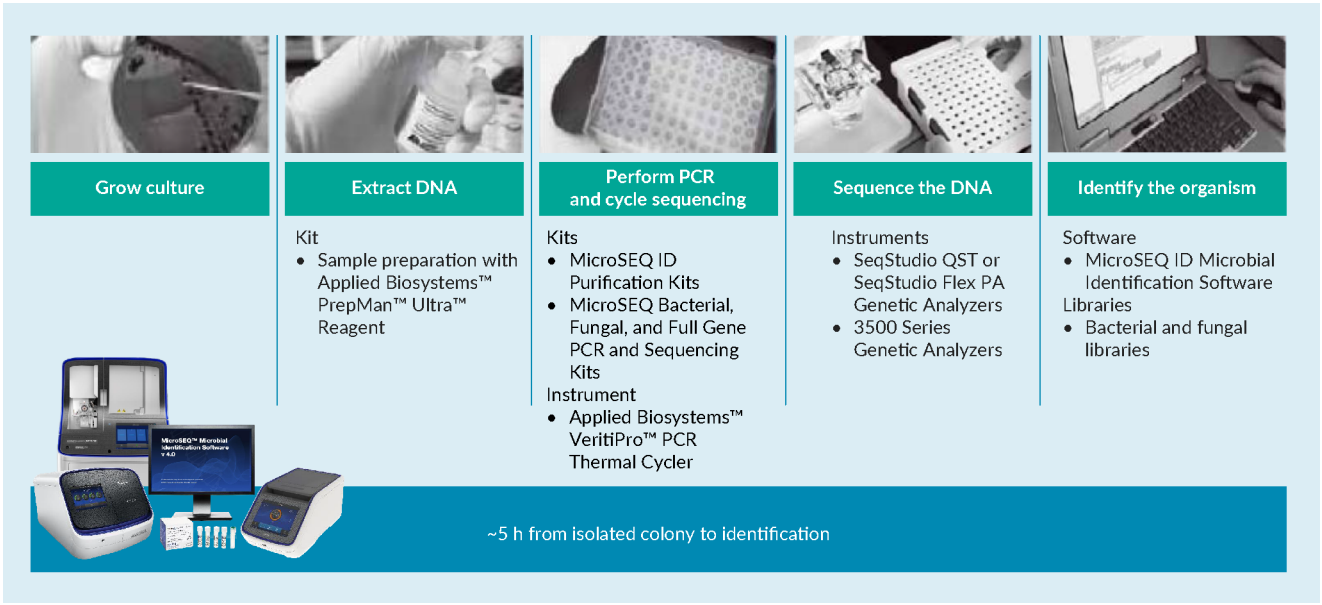
The MicroSEQ ID software is used to collect, analyze, and compare the sequenced data to the library that generates the identification. The software also has features to assist with 21 CFR part 11 compliance and has auto-analysis capabilities for sequence quality analysis, and subsequently, microbial, bacterial, fungal, or yeast identification to the species level. The MicroSEQ ID System has validated libraries for bacteria with 2,100 entries, and 1,700 entries in fungal libraries. The bacterial 16 Full Gene Kit has its own library of around 1,300 entries. An additional bacterial supplementary library includes a further 7,645 entries. This makes the MicroSEQ ID database the largest and most comprehensive, commercially available, validated library for bacteria and fungi.

FIGURE 1

MicroSEQ ID system: a high throughput comparative DNA sequencing for identification of bacteria, fungi and yeast.

FIGURE 2

The MicroSEQ rapid microbial ID System workflow.



SOLUTIONS FOR MID- & LOW THROUGHPUT

The Applied Biosystems™ SeqStudio™ Flex System is the newest addition to the SeqStudio family of genetic anayzers and has both an 8-cap and 24-cap configuration, designed to be a flexible, easy-to-use, mid-throughput solution. For ease of use, the system is simplified, streamlined, and includes auto-calibration functions. The SeqStudio Flex PA system, as a function of the MicroSEQ ID system, has a security audits and e-signature (SAE) module designed to aid compliance with GMP and 21 CFR Part 11. This instrument also has a 4-plate loading capacity to allow for continuous plate loading and increased throughput per run. The system is validated and integrated with MicroSEQ ID version 4.0 software, making it a fully integrated microbial identification solution that works with both the SeqStudio family of instruments and MicroSEQ ID assays.

The SeqStudio QST Genetic Analyzer is a simple, low-throughput solution in an all-in-one cartridge. The SeqStudio instrument offers alternative features and potential benefits to the workflow. This plug-in-and-go system is a 4-capillary instrument with auto-calibration features and compatible MicroSEQ ID software. It has unified SAE features to aid GMP 21 CFR Part 11 compliance.

The innovative cartridge system of the SeqStudio family of genetic analyzers integrates all consumables to allow plug-and-play sequencing, and the system utilizes a universal polymer to allow Sanger sequencing, in addition to other applications such as fragment analysis. Regardless of the system chosen, the full end-to-end workflows have been validated on both the SeqStudio QST and SeqStudio Flex PA systems.

The run rate by sample throughput per hour for the SeqStudio QST and the SeqStudio Flex PA for the 8-capillary and the 24-capillary is shown in Table 1.

Service support offerings provided include full Installation and Operational Qualification (IQ/OQ) and Computer System Validation (CSV) services for method validation as part of the installation servicing. A full implementation program is provided, covering

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TABLE 1

Throughput of the SeqStudio systems.

	SeqStudio QST	SeqStudio Flex PA	
# of capillaries	4	8	24
IDs/ 8*h	10	6	22
IDs/ 24*h	46	34	94

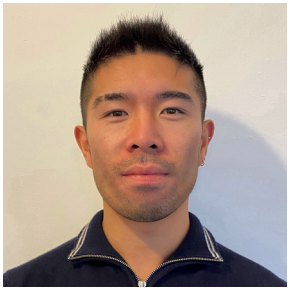
*All workflows include a sample preparation time of 3 h.

extensive theoretical and physical product application training, and providing assistance on laboratory design and other implementation needs. This includes assistance with any type of process qualification design required for the MicroSEQ ID system.

SUMMARY

Due to regulatory requirements and expectations with respect to biopharmaceutical GMP production, accurate microbial identification is crucial to ensure product and patient safety. By implementing an appropriate method, corrective and preventative actions can be taken to limit any future potential microbial control failures. The MicroSEQ ID System is a genotypic method based on DNA sequencing, offering the largest validated database and enabling accurate results from DNA extraction to identification in approximately 5 h.

Q&A



Here, **Nico Chow,** Field Application Specialist, Pharma Analytics Business Unit, Thermo Fisher Scientific (pictured) answers your questions about Environmental monitoring: optimizing microbial control in cell & gene therapy workflows

Q I saw that there are two kits available for bacterial identification: the 16S 500 and Full Gene. Could you explain the differences between the two?

NC: The bacterial 16S 500 kit sequences 500 base pairs (bp) of the bacterial 16S gene, whereas our Full Gene kit sequences the full gene, approximately 1,500 bp.

We recommend and expect that for routine identification, the 16S 500 kit should be suitable for 99% of samples. Only in the event that a greater discriminatory power or longer genetic sequence is required for the identification should the Full Gene kit be considered. For example, the Full Gene kit should be considered if you have an interest in discrimination between very closely related species or subspecies.

Q What is the curated bacterial and fungal database for this system?

NC: For the MicroSEQ ID system, we have validated databases in the form of MicroSEQ ID libraries. For bacterial identification, we have the 16S 500 library and the 16S supplementary library. Together, they include around 9,500 species. We have one fungal kit: the fungal library. We have a separate bacterial library for the Full Gene kits as well.

Q Could you explain the differences between the classic libraries and the supplementary libraries?

NC: The classic libraries are the 16S 500 and the fungal libraries. We recommend using these two kits for routine analysis and identification. They have been validated in accordance with ISO 9001 and 2000 quality systems requirements. Specifically, we took type strains or genomic DNA sequences obtained from preeminent culture collections such as controlled sources from the American Type Culture Collection (ATCC). Then, we generated specific sequences by using our MicroSEQ ID PCR and sequencing kits and ultimately verified that the sequence that was generated was an accurate sequence identification of the species that we were testing. The measurements and the quality of the sequence generated were as expected. The supplementary library, which contains an additional 7,645 species, is only applicable for bacterial species analysis.

Q Could you give more information on the specific software that is used in this application for experimental setup and data analysis?

NC: The latest MicroSEQ ID software version that is compatible with the SeqStudio Flex and SeqStudio is version 4.0. This is seamlessly integrated as part of the MicroSEQ ID workflow system. The software is used to control the system itself, and to create and analyze experiments. Once the sequence has been generated, we use the exact same software to perform the sequence quality analysis and ultimately, the sequence match to get our identification results. The results can also be presented in a report format, which can be generated with the software.

Q Could you tell us more about the SAE software features?

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NC: The MicroSEQ ID system does have the SAE module, which contains features for GMP 21 CFR Part 11 compliance. These include the ability to create users who have different access levels to the software, with unique usernames and passwords. This comprises an audit trail for both software and experiment levels to allow full traceability of all actions performed on the system, as well as the ability to allow individual users to sign an e-signature on both the experiment file level and reports, providing unsigned and signed status traceability.

BIOGRAPHY

NICO CHOW is a Field Application Specialist for the Pharma Analytics business unit, part of the Thermo Fisher Scientific Bioproduction Group, supporting customers in BeNelux/Nordics, SEE, Turkey, Middle East and Africa. He has been in the role for 3 years specialising and supporting with the implementation and validation of rapid analytical QC methods, based on real-time PCR and sequencing technologies, focused on ensuring product quality and product safety of various biotherapeutic modalities and associated manufacturing workflows. During this time, he has taken on the role as MicroSEQ ID subject matter expert.

AFFILIATION

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AUTHORSHIP & CONFLICT OF INTEREST

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Analyzing full & empty AAV capsid ratio in less than 5 minutes

Åsa Hagner-McWhirter

In gene therapy, there is a need for quick, robust, and cost-efficient full and empty capsid analytical methods during adeno-associated virus (AAV) process development. The gold standard for full and empty capsid ratio is analytical ultracentrifugation (AUC). However, AUC is costly, time-intensive, and poorly suited for high-throughput screening of conditions. Several other methods are used with varying performance and cost; most require relatively pure and concentrated samples in large quantities to give accurate results. Separation of full and empty capsids can be achieved with ion exchange by using a small difference in charge, as full capsids have a lower pI compared to empty capsids, on average. This article will explore the critical parameters for high-performance separation, and how Capto™ Q (HiTrap™ column, 1 mL) run on an ÄKTA pure™ 25 chromatography system can be used to determine percent full capsids of AAV8 and AAV9 in less than 5 minutes with low sample consumption.

The top AAV processing challenges in the polishing stage include incomplete separation of full and empty capsids with overlapping peaks and the need to optimize to maximize separation for each serotype, often leading to poor enrichment of full capsids and low viral genome (VG) recoveries. Cytiva has sought to overcome these challenges with a one-resin, one-protocol method for all serotypes tested: AAV2, AAV5, AAV8, and AAV9. In the analytical stage, challenges remain around confirming the full and empty capsid ratio in the separated peaks, which is required to optimize the polishing step. During process development, there is a need for quick analysis to access the proportion of full capsids, and for the optimized process several analytic methods are required to confirm the percent full capsids using qPCR:ELISA ratio, analytical ultracentrifugation, transmission electron cryomicroscopy (CryoTEM), or other often time-consuming and costly analytics.

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FULL & EMPTY CAPSID ANALYTICS

The accuracy of full and empty capsid analytics varies depending on the method you use. For process development, a faster method that is still accurate enough to enable selection at the optimization stage is needed. The first thing to start with is looking at the peak UV 260:280 ratio in chromatograms to give an indication of the percent full capsids, provided that the samples are pure. To ensure that the buffers used are not giving UV background signals, it is important to run a blank.

Then, qPCR:ELISA ratio can be used; however, these independent assays have their variations. In addition to the UV and qPCR:ELISA, you need orthogonal methods, such as analytical ion exchange, isoelectric focusing (IEF)-CE, AUC, size exclusion and multi-angle light scattering (SEC-MALS), or charge detection mass spectrometry. A balance between quality, time, and cost must be found. Using several methods (at least one in addition to qPCR:ELISA) to confirm percent full capsids is highly recommended. With less pure samples, qPCR:ELISA is the main analytical method that will give trustworthy results. Most orthogonal methods require highly pure samples to be able to get accurate results.

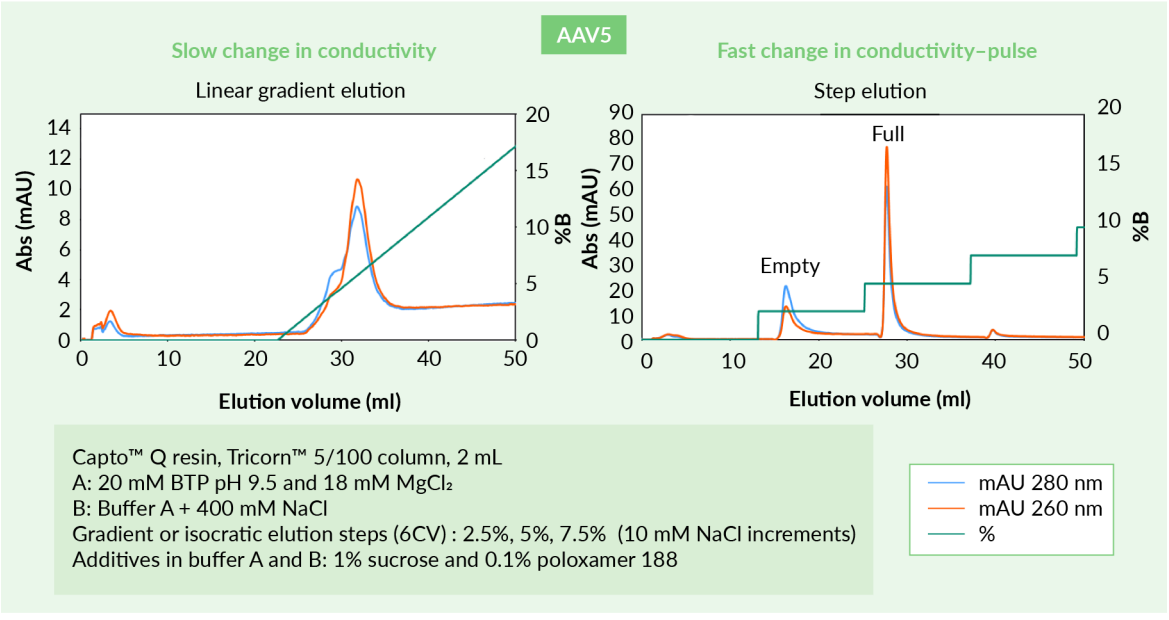
OPTIMIZING FULL & EMPTY CAPSID SEPARATION

The principle of ion exchange separation for the full and empty capsids can pose difficulties, as the average pI difference between full and empty capsids is small. Full capsids have an average pI of 5.9, and empty capsids have a slightly higher average pI of 6.3. Cation exchange with a low pH buffer will give less positively charged full capsids than empty. Anion exchange requires a higher pH buffer and gives more negatively charged full capsids than empty. If anion exchange is performed with a salt gradient, empty capsids will elute before full at a lower salt concentration. In our hands, cation exchange did not perform well for separating full capsids from empty. With anion exchange chromatography we achieved high separation performance.

Dextran surface extenders enhance the full and empty capsid separation and reduce overlapping peaks to near baseline separation

FIGURE 1

Linear gradient elution versus step elution effects on AAV5 separation.



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using optimal conditions. Capto Q anion exchange resin, which has dextran surface extenders, shows dramatic improvement in separation performance compared to a resin without them.

$MgCl_2$ (2–18 mM) in the buffer also helps to enhance full and empty capsid separation performance. A higher constant concentration of $MgCl_2$ has been shown to give an earlier elution and baseline separation.

Step elution also is shown to improve separation, as demonstrated in Figure 1. This is particularly important for AAV5, which has a full and empty capsid separation that is more challenging than that of AAV8.

POLISHING WITH CAPTO Q RESIN

A Capto Q resin protocol using high $MgCl_2$ and NaCl worked well for AAV2, AAV5, and AAV8; however, this needed to be adjusted to work for AAV9. AAV9 behaves differently, with weaker binding to the anion exchange, so the protocol was adjusted to use a softer elution salt, Na-acetate instead of NaCl. The $MgCl_2$ was reduced to 2 mM.

There are several alternative protocols and conditions that give good separation performance for AAV2, AAV5, and AAV8; the aim was to find a platform-like protocol that would work for most AAV serotypes and variants. The buffer system selected for this protocol for compatibility with all four serotypes tested was simple: pH 9.0 bis-tris propane (BTP) and 2 mM $MgCl_2$. In Buffer B, the elution salt is 250 mM Na-acetate.

Pre-screening with the Capto Q resin using 5% incremental buffer B steps to find the optimal elution of the empty capsids was performed to select the final two elution step conditions (Figure 2). These small incremental elution steps result in several peaks eluting, the first one or two containing empty capsids, followed by peaks in the middle containing a mix of full and empty capsids, and the last ones containing the full capsids. This can be

FIGURE 2

Capto Q resin pre-screening for elution step condition selection, for AAV2, AAV5, AAV8, and AAV9. The dotted line indicates the selected percent B for the elution of empty capsids in final step 1. Step 2 to elute the full capsids was using 100% B for all tested AAV serotypes except AAV9, which was 40% (100% B also an option).

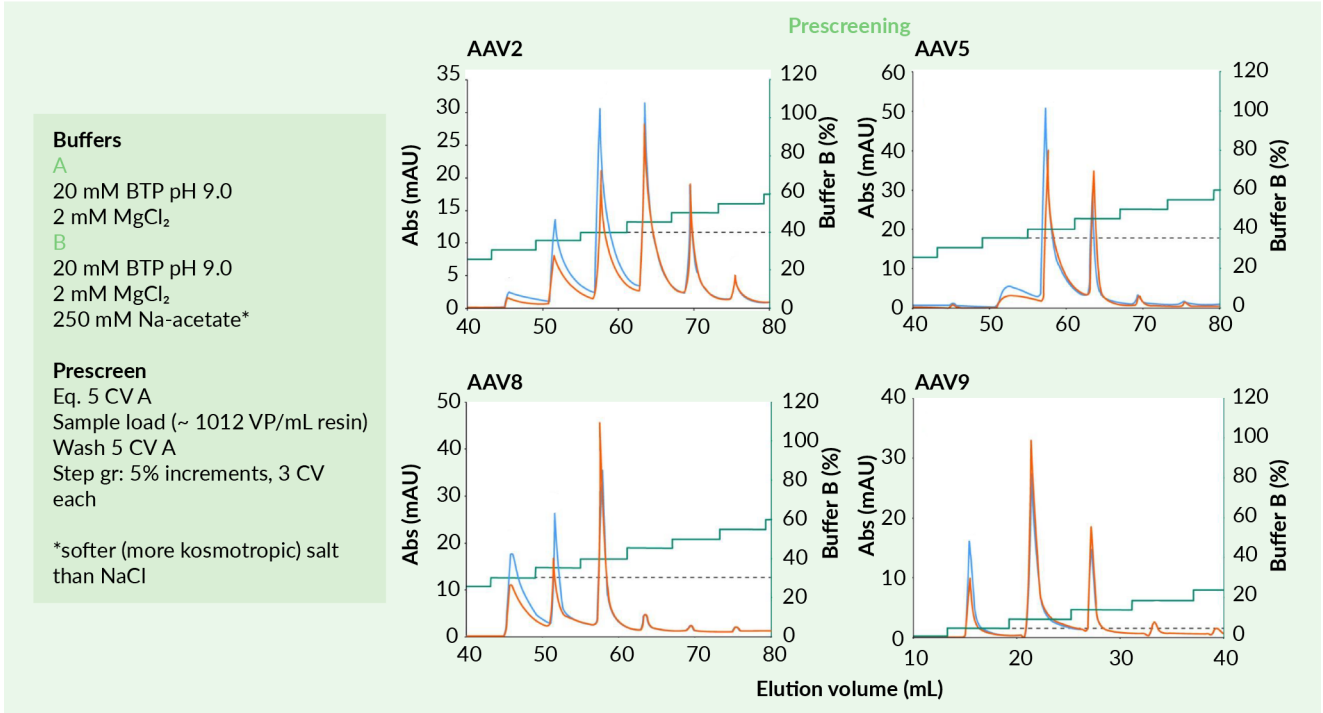


FIGURE 3

Selected conditions and results for the final two-step elution protocol.

Buffers
A
20 mM BTP pH 9.0
2 mM $MgCl_2$
B
20 mM BTP pH 9.0
2 mM $MgCl_2$
250 mM Na-acetate

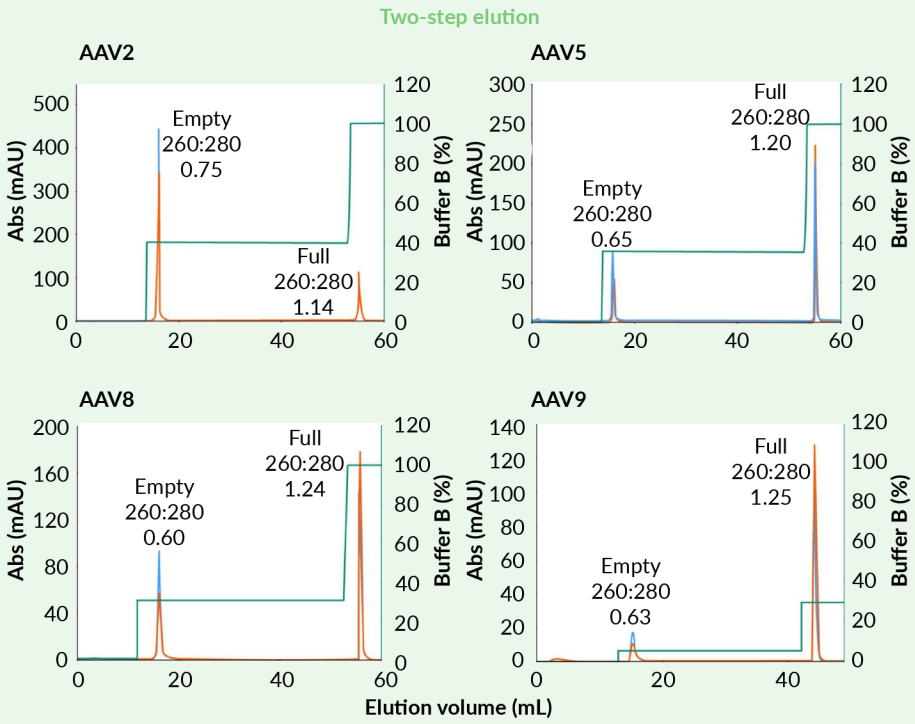
Two step elution
Eq 5CV A
Sample load (~ E12 VP/mL resin)
Wash 5 CV A

AAV2: Step 1 40% B 20 CV
Step 2 100% B 5 CV

AAV5: Step 1 35% B 20 CV
Step 2 100% B 5 CV

AAV8: Step 1 30% B 20 CV
Step 2 100% B 5 CV

AAV9: Step 1 5% B 20 CV
Step 2 30% B 5 CV



seen by the changes in the UV 260:280 ratios for the different peaks going from lower to higher. The selection of percent B buffer for step 1 is based only on UV ratios. The step before the step at which full capsids leak into the eluted empty capsid is selected for step 1 (Figure 2).

The two-step separation protocol that was selected based on the prescreening is shown in Figure 3.

The results for AAV2, AAV5, AAV8, and AAV9 are summarized in Table 1. The UV 260:280 ratios are typical for both full and empty. The VG recovery is very low in peak 1, as is the qPCR:ELISA ratio. Peak 2 shows a good VG recovery of > 80% for the AAV serotypes tested. The qPCR:ELISA shows nearly 100% full capsids, but assay variation should be taken into account. Results indicate high full capsid purity and yield

TABLE 1

Results summary for empty and full capsids for AAV2, AAV5, AAV8, and AAV9.

Serotype	Start sample	Peak 1 (empty capsids)			Peak 2 (full capsids)		
	qPCR:ELISA (% full capsids)	UV 260:280 (peak area)	VG recovery (%)	qPCR:ELISA (% full capsids)	UV 260:280 (peak area)	VG recovery (%)	qPCR:ELISA (% full capsids)
AAV2	7–10	0.75	NA	NA	1.14	NA	NA
AAV5	47	0.65	7	5	1.20	80	100
AAV8	11–35	0.60	3	1	1.24	80	95
AAV9	40	0.63	0.3	1	1.25	91	100

Mass balance based on total UV signal 70% to 100%. NA: Not analyzed.

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TABLE 2

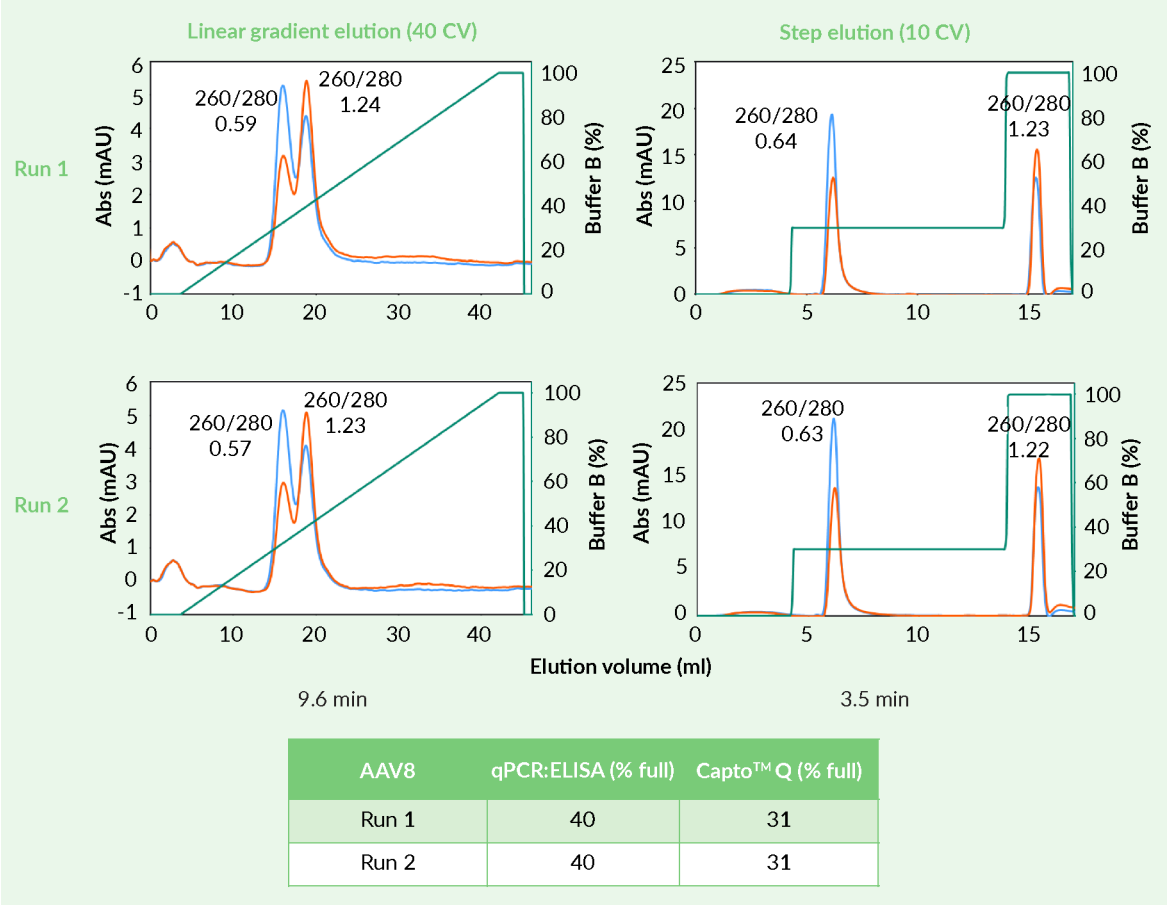
Analytical protocol with Capto Q column.

Prescreening protocol	Analytical two-step protocol	CIP protocol
Sample load: ~ 1×10^{12} VP in 2 mL Flow rate: 2 mL/min **Buffer A:** 20 mM BTP, pH 9.0, 2 mM $MgCl_2$ **Buffer B:** 20 mM BTP, pH 9.0, 2 mM $MgCl_2$, 250 mM Na acetate **Equilibration:** Buffer A, 5 CV **Wash:** Buffer A, 5 CV **Gradient:** Step elution, 5% increments, 3 CV each	**Sample load:** 1 × 10^{10} VP in 2 mL Flow rate: 5 mL/min **Buffer A:** 20 mM BTP pH 9.0, 2 mM $MgCl_2$ **Buffer B:** 20 mM BTP pH 9.0, 2 mM $MgCl_2$, 250 mM Na acetate **Equilibration:** Buffer A, 5 CV **Wash:** Buffer A, 5 CV **Gradient:** Two-step elution **rAAV8:** Step 1 30% buffer B, 10 CV Step 2 100% buffer B, 5 CV **rAAV9:** Step 1 5% buffer B, 10 CV Step 2 30% buffer B, 5 CV	**Flow rate:** 2 mL/min 5 CV ultrapure water 20 CV (1 mL/min or less) 1 M NaOH (up flow) 20 CV (or more until the pH is at least 8.5) 100 mM Tris HCl pH 7.5, 300 mM NaCl 5 CV ultrapure water 5 CV 20% EtOH

Column: Capto™ Q (HiTrap™ column, 1 mL)
System: ÄKTA pure™ 25 system, 10 mm path UV260:280 detector, bypassed mixer
Sample conductivity: 1–3 mS/cm

FIGURE 4

Analytical run results: AAV8.



AAV8	qPCR:ELISA (% full)	Capto™ Q (% full)
Run 1	40	31
Run 2	40	31

in peak 2. Ongoing orthogonal analysis with AUC and TEM analysis is being used to confirm this data.

AN ANALYTICAL PROTOCOL WITH CAPTO Q COLUMN IN LESS THAN 5 MINUTES

Separation for full and empty capsids using Capto Q resin with dextran surface extenders has been shown to be robust. This can be used as an analytical tool to achieve results in < 5 minutes. Table 2 shows the details of the analytical protocol used. Prescreening is still required, but only once per capsid. In this case, Capto Q HiTrap, 1 mL column, was run on ÄKTA pure 25 systems.

The results from the analytical runs with AAV8 are shown in Figure 4. The linear gradient elution gives overlapping peaks, which would not be highly accurate for determining the percent full capsids. This linear gradient protocol takes 9.6 minutes to run. The recommended two-step elution protocol shows separated peaks and takes only 3.5 minutes to run. This uses E10 particles per run. The percent full capsids was calculated to be 31% using Capto Q UV280 peak area, and 40% using qPCR:ELISA, which is known to be a method showing variation.

The results for the analytical runs with AAV9 are shown in Figure 5. The step elution protocol was used directly this time, as conductivity in the loaded sample is critical due to the weak binding of AAV9. A comparison of a simple 50-fold dilution to buffer exchange using a PD-10 column is shown. The two peaks show the expected UV 260:280 ratios and a low level of difference is seen between the results from dilution and buffer exchange (into buffer A). Results from the qPCR:ELISA showed a percent full of 46%, which is in line with the results found on the Capto Q column based on the UV 280 peak area.

The conductivity in the sample is critical, as ideally both empty and full will bind so both peaks can be eluted. The exact conductivity required to elute the empty can be affected by the buffer composition, capsid structure, and engineered variants. The conductivity values when using BTP pH 9, 2 mM $MgCl_2$, and 5% to 40% of 250 mM Na-acetate are shown in Table 3.

SUCCESS IN EMPTY & FULL CAPSID SEPARATION

Recommendations for empty and full capsid separation success are listed below:

- Use a UV detector with 10 mm pathlength and bypass the mixer on the ÄKTA system;
- Check sample conductivity before loading, and either dilute ≥10-fold or buffer exchange;
- Check sample purity as host cell proteins and DNA will increase UV background signals and affect separation performance negatively;
- Aggregation will affect the separation negatively, so consider additives, detergent, or salt as a remedy;
- Prescreening is important—for step 1 elution of the empty capsids, select the percent B buffer corresponding to the step before the inflection point (UV 260:280= 1);
- Make sure to set the pH of the buffer before adding $MgCl_2$ and for column CIP, wash with ultrapure water before NaOH to avoid precipitation;
- The load level is ≥1×10^{10} viral particles (VP).

To conclude, the ÄKTA pure 25 system can be used with a 10 mm path length UV detector for higher sensitivity, and the mixer can be bypassed for sharper elution steps by reducing dead volume. Capto Q (HiTrap

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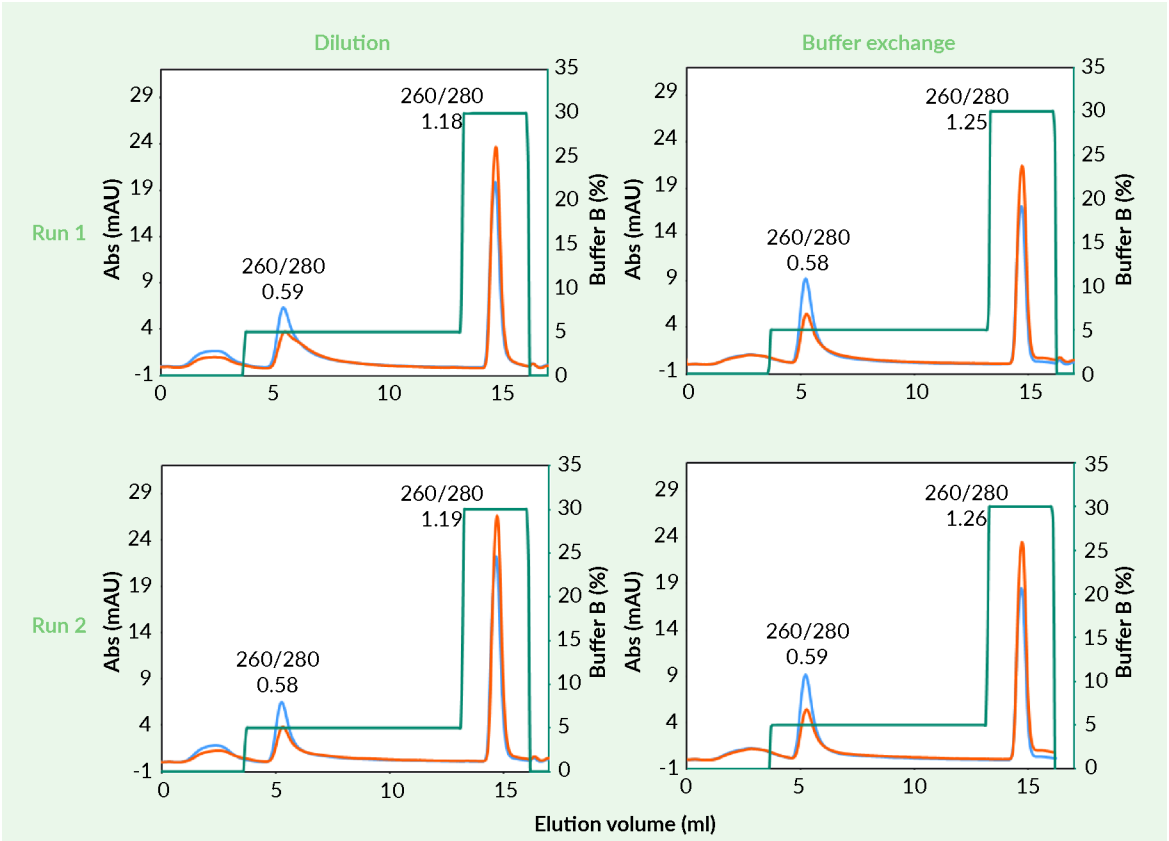
FIGURE 5

Analytical run results: AAV9.



AAV9	qPCR:ELISA (% full)	Capto™ Q (% full)
Dilution/run 1	46	53
Dilution/run 2	46	59
Buffer exchange/run 1	46	46
Buffer exchange/run 2	46	49

TABLE 3

Conductivity values for empty capsid elution of AAV serotypes.

AAV9	AAV8	AAV5	AAV2
5% B	30% B	35% B	40% B
2.7 mS/cm	6.75 mS/cm	7.56 mS/cm	8.41 mS/cm

column, 1 mL) can be used with a two-step elution protocol loading ≥ 1×10^{10} VP with a 5-minute runtime. The conductivity of the sample load is critical. Prescreening is needed once for each AAV capsid to determine the percent B buffer for step 1 elution of empty capsids. Results are shown for AAV8 and AAV9, but we expect the protocol will work for most AAV serotypes. A similar protocol is suitable for large-scale purification.

Q&A

Here, **Åsa Hagner-McWhirter,** Principal Scientist, Cytiva (pictured) answers your questions about using Capto Q anion exchange as an analytical tool for full and empty capsid assessment during process development.

Q How is the peak area defined? Does it start and stop before and after peaks 1 and 2?

AHM: Yes. We use UV 280 for the whole step 1 and 2 to get the total signal. To get the percentage of the empty capsids we divide the whole step 1 signal with the total signal. We also use UV 280 for the whole step 2 to get the percentage full capsids.

Q Have you used the peak height of the linear gradient to determine percentage full, and how does it compare to the area?

AHM: If we use the gradient elution to determine percentage full, we will have overlapping peaks. You can use the peak height to get a rough estimate of the amount of full versus empty. The more overlap you have, the less accurate it will be. We recommend using the optimized two-step protocol, and using the area because, in step 1, there is often some slight tailing. If you only look at peak height, then full may be overestimated. There can also be differences in the extinction coefficient between empty full capsids and AAV serotypes that affect the UV 280 signal. This can be included in the calculations to increase accuracy. It is also possible to explore other modes of detection, like fluorescence and light scattering.

We will produce more data to look into how the peak height versus the peak area compares, as we have some ongoing experiments in which we are mixing purified full capsids with purified empty capsids in different ratios.

Q How low of a percentage of full capsids can you analyze?

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AHM: We have been analyzing samples down to 10%. We will learn more about that when we do those mixes with purified full and empty in different ratios. This will allow us to see how small differences we can determine. We were not aiming to distinguish very small percentage differences here. We use this as a good process development tool, to quickly assess the percent full capsids in different samples and fractions during the optimization of this step. We also aim to look further at what we have in those different peaks using other analytic methods; we have ongoing analysis with AUC and are planning to perform cryoTEM to confirm the results.

Q Where do the partially filled capsids elute?

AHM: In the material used in the results shown here, we had below 10% of the partially filled capsids determined by cryoTEM. From the beginning, in the affinity eluate material, we have only a small amount so we do not know exactly where they would elute. We would work with more analytics to find out, possibly using known samples with partials. We have some preliminary data suggesting that the partially filled elute together with the empty capsids. In a purification process, the empty and the partials can therefore be reduced or removed.

BIOGRAPHY

ÅSA HAGNER-MCWHIRTER has been with Cytiva based in Uppsala, Sweden since 2003 and is a downstream and analytics SME, with a broad and deep understanding of viral vector processing. Due to her long experience and from customer interactions she has gained insights into common challenges and pitfalls in the area of viral vectors and vaccines as well as general protein purification and analysis. She has also worked with proteomics and fluorescent-based protein analysis technologies. Åsa holds a PhD in Medical Biochemistry from Uppsala University in 1999 based on research around biosynthesis of proteoglycans. The studies involved polysaccharide structure analysis, enzyme purification and cloning as well as characterizing an enzyme reaction.

AFFILIATION

Åsa Hagner-McWhirter PhD
Principal Scientist, Cytiva

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given she approval for this version to be published.

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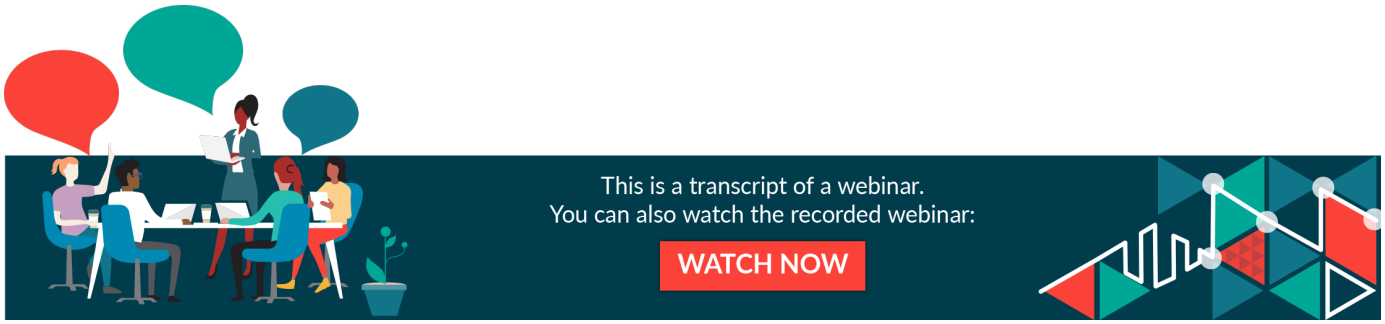

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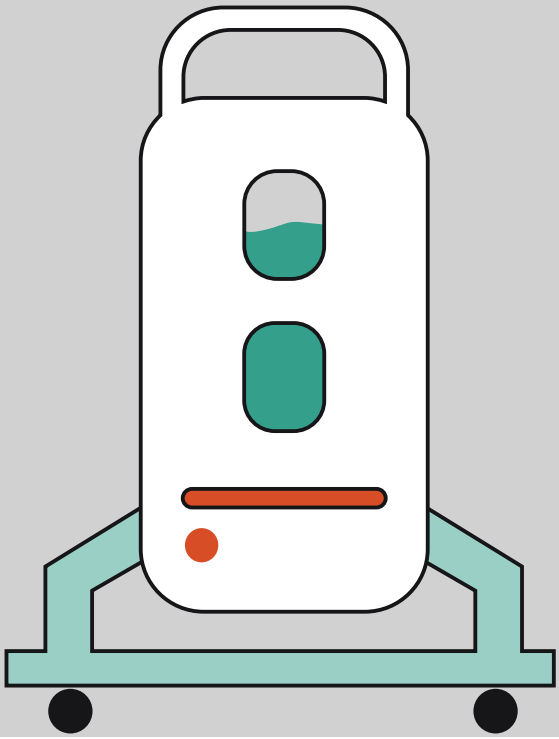


For local office contact information, visit cytiva.com/contact

CY32122-20AUG22-AD

FASTFACTS

An optimized & streamlined approach for upstream & downstream lentiviral production

Gregory Piscitello, Upstream Scientist, Viral Vector Technology and Innovation Team, MilliporeSigma
& **Paul Turiano,** Scientist II, Viral Vector Technology and Innovation Team, MilliporeSigma

To manufacture a sufficient quantity of final vector product, efficient and scalable production of lentivirus vectors is critical. To date, the VirusExpress® lentivirus production technology has been used to scale up several gene therapy programs through IND submission and into clinical trials. Here, several upstream and downstream process optimizations from the initial platform are described to maximize batch yields and quality for clinical and commercial production.

IDENTIFICATION OF IDEAL TRANSFECTION PARAMETERS USING A DOE APPROACH

In our upstream process, a Design of Experiments (DoE) approach was used to optimize transfection and improve the output titer of lentivirus without wasting expensive materials such as plasmids. As shown in **Figure 1**, in the first DoE, five ideal parameters were identified from the 33 examined conditions to get the most optimal output. In the second DoE, a mixture design examined the plasmid molar ratio to identify optimal plasmid levels for manufacturing. Through this DoE we achieved a 7.2 TU/mL titer at the shaker flask level.

DoE CONDITIONS CONFIRMATION & SCALE-UP

Following the small-scale DoE testing **(Figure 1)**, bench-scale testing was performed in 3 L Mobius® bioreactors. A titer range of 5×10^8–6×10^8 TU/mL titers (gravity feed, t=7 mins incubation) was obtained. Shaker flasks, used as controls, provided titers of 4×10^8–4.5×10^8 TU/mL.

Following the successful bench-scale runs, large-scale runs with the 50 L Mobius® bioreactor were carried out. **Figure 2** shows the lentivirus titers from the two 50 L bioreactor runs, along with the shaker flask controls.

ENRICHING LENTIVIRAL VECTOR RECOVERY THROUGH DOWNSTREAM OPTIMIZATION

The downstream process template was optimized at all steps from clarification to anion exchange (AEX) chromatography, tangential flow filtration (TFF), and lastly sterile filtration. The goal in creating this process template was to optimize process recovery and critical quality attributes (CQAs) such as aggregation to speed up development with robust operating ranges.

To highlight just one area of downstream optimization, in the AEX step, a range of concentrations of NaCl were tested from 0.5–2.0 M, resulting in an 88% total TU recovery, along with a >98% host cell protein clearance (with no detectable DNA in all fractions). There was no measurable TU/p24 above the 1.5 M salt concentration.

KEY BENEFITS OF THIS ROBUST MANUFACTURING LENTIVIRAL PLATFORM

- Risk reduction through products and processes
- Increased speed to clinic
- Decreased process costs
- Scalability with high harvest titers

Figure 1. Using DoE to create a defined platform for lentiviral vector manufacturing.

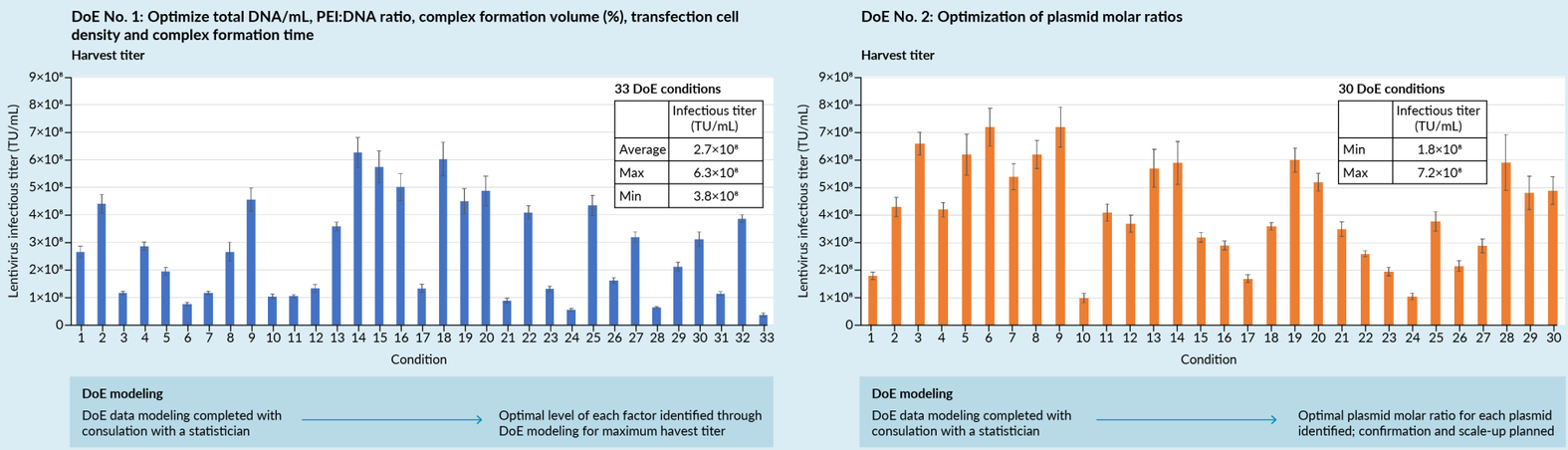
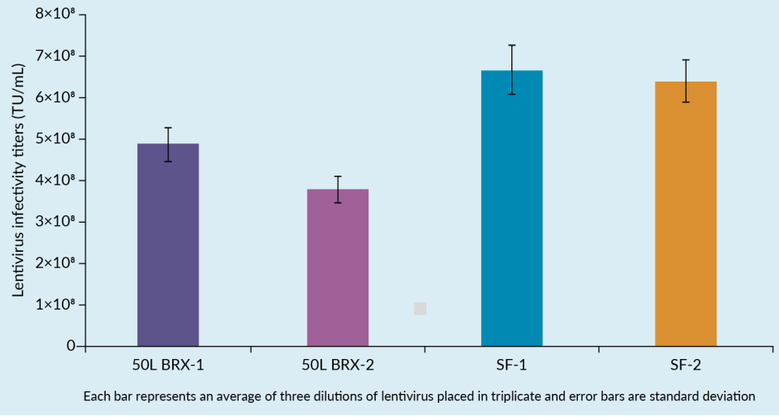


Figure 2. Lentivirus production in 50 L Mobius® bioreactor.



Each bar represents an average of three dilutions of lentivirus placed in triplicate and error bars are standard deviation

CELL & GENE THERAPY INSIGHTS

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In partnership with: MilliporeSigma

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RESEARCH ARTICLE

Validation of mRNA concentration determination by slope spectroscopy technology: a matrixed approach

Travis Alvine, Natalie Unsinn, John J Long & Joseph Ferraiolo

mRNA-based therapeutics are different from small molecules and other biologics that represent significant analytical challenges. mRNA characterization for pre-clinical/clinical testing and lot release are required to compete in the competitive marketplace and align with regulatory standards. Faster and more reliable results require innovative solutions to meet these analytical challenges. Nucleic acid concentration determination is measured by determining the ultraviolet (UV) absorbances at an analytical wavelength of 260 nm. These absorbance measurements allow scientists to measure nucleic acid concentration based on the known extinction coefficient for RNA. The spectral signature of their maximum absorbance peak at 260 nm is proportional to nucleic acid concentration. The advantages of this UV nucleic acid quantitation method are that it is simple, direct, and requires just a small volume of your sample for measurement. One challenge, however, that the analytical labs run into is its limitation for specificity, as matrix components that absorb similar wavelengths can lead to inaccuracies in the consequent nucleic acid concentration determination. We have observed that the standard fixed-pathlength UV in current traditional cuvette-based UV solutions using a 1 cm cuvette and/or smaller fixed pathlengths still does not resolve the quality of the given measurement and lead to hours of required investigation time. The use of dilution factors, which increase prep time and variability, and fixed-pathlength measurements in determining the concentration of a UV chromophore in solution does not provide an easily transferable and robust method that can be platformed within a company or process. Today, researchers can selectively quantify nucleic acid absorbance in the presence of chemical and nucleic acid impurities, notably DNA and dsRNA. Analytical software uses full-spectrum data and advanced algorithms to identify nucleic acid impurities and provide corrected nucleic acid concentrations.

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IMPROVING ANALYTICAL METHODS & TECHNIQUES

For cuvette-based spectrophotometry, sample handling and preparation (especially for in-process samples) creates challenges for bringing samples into the linear range of an instrument's detection capabilities. Additionally, performing serial dilutions for more complex sample matrices can have a significant impact on the calculated sample concentration due to pipetting accuracy and the risk of bubble introduction. As a result, the total assay variability may be larger than the acceptable range of concentration variance from the target value, calling into question the consistency of the method.

Unlike traditional UV-Vis methods that rely on a single absolute absorbance value, the slope spectroscopy method uses section data (absorbance versus pathlength data) to determine a slope value for quantitation of sample concentration using the slope spectroscopy equation ($m = \varepsilon c$) which is derived from the Beer–Lambert law (Figure 1). The R^2 value of the linear regression confirms that the absorbance values are changing proportionally when there is a change in absorbance over a change in pathlength following the Beer–Lambert law and therefore prove accuracy within every sample tested (Figure 2).

The CTech™ SoloVPE® variable pathlength spectrophotometer automatically adjusts the optical pathlength from 5µm–15mm in 5µm step increments. This provides the ability to determine the appropriate pathlength and linearity for significantly higher sample concentrations than those determined by fixed-pathlength spectrophotometers. The generated absorbance values are plotted into a linear slope regression with minimum R^2 of >0.999. Variable pathlength technology (VPT/slope spectroscopy) provides the speed, repeatability, and accuracy, to platform methods globally to eliminate the turnaround time and multiple personnel waiting for data results before continuing the next processing steps. As analytical testing capabilities continue

FIGURE 1

CTech™ SoloVPE® variable pathlength spectrophotometer.

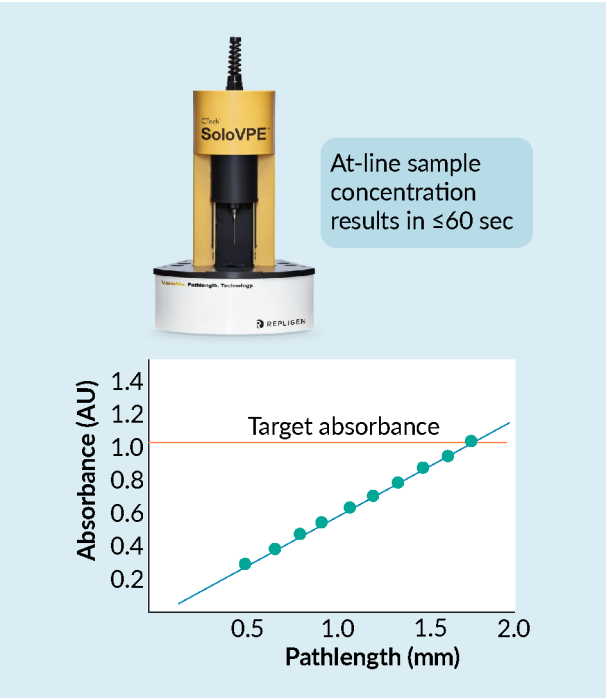
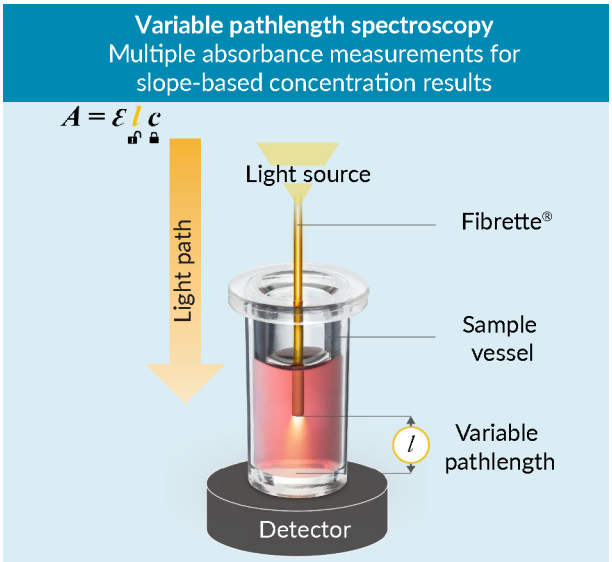


FIGURE 2

Variable pathlength spectroscopy.



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to improve, these new technologies must be qualified and validated in accordance with regulatory guidance to ensure the highest level of product quality and patient safety. This white paper summarizes the strategy and results generated from a platform mRNA content assay for mRNA concentration.

MATERIALS

- SoloVPE Instrument #1 [Part No. IN-SOLO5-VPE];
- SoloVPE Instrument #2 [Part No. IN-SOLO5-VPE];
- Cary 60 UV-Vis Spectrophotometer [IN-CARY60];
- Fibrette Optical Component [Part No. OF0002-P50];
- SoloVPE Sample Plastic Vessel [Part No. OC0009-P50];
- Solo Vessel Holder – Small [Part No. FA-CTIO1-PC26];
- Chem013 Measurement Standard [Part No. CHEM013-KIT];
- Tris-ethylenediaminetetraacetic acid (TE; 10 mM Tris, 1.0 mM EDTA, pH 8.0);
- Water for injection (WFI);
- 1 mM Sodium Citrate, pH 6.4;
- SpectraMax M5e Multimode Plate Reader;
- mRNA was produced in three representative sample matrices.

STUDY DESIGN

This method was validated as a content assay as described in ICH Q2 (R1) [1]. The following validation parameters were assessed as part of the validation study: accuracy, repeatability, linearity, intermediate precision, specificity, and range. In addition, method comparability (e.g., bridging) to the platform cuvette-based UV spectrophotometry method was included to provide sufficient data to demonstrate method comparability to the current method.

The samples used in this validation study consisted of a single mRNA molecule formulated in the following sample matrices: TE (10 mM Tris, 1.0 mM EDTA, pH 8.0), Water for injection (WFI), and 1mM sodium citrate, pH 6.4. Each mRNA test sample was serially diluted two-fold in the appropriate sample matrix to generate a total of five concentration levels. All prepared validation samples were aliquoted into single use samples and stored at -80°C prior to validation. The mRNA concentration of Level 1 for each matrix was determined by UV-Spectrophotometry per the platform cuvette-based UV spectrophotometry method. The mean concentration result (mg/mL) from a total of three vials of Level 1 for each mRNA construct was determined and served as the target (theoretical) concentration for Level 1 for each sample matrix. Based on the UV established value of Level 1, the remaining levels' mRNA concentration was determined as the theoretical concentration following each two-fold dilution.

A single experiment was performed for accuracy, repeatability, linearity, specificity, and range by testing mRNA levels 1–5 of mRNA concentration. mRNA samples in TE were prepared at 4.10 mg/mL (L1), 2.05 mg/mL (L2), 1.03 mg/mL (L3), 0.51 mg/mL (L4), and 0.26 mg/mL (L5). mRNA samples in WFI were prepared at 4.70 mg/mL (L1), 2.3 mg/mL (L2), 1.18 mg/mL (L3), 0.59 mg/mL (L4), and 0.29 mg/mL (L5). mRNA samples in 1 mM sodium citrate, pH 6.4 were prepared at 4.01 mg/mL (L1), 2.01 mg/mL (L2), 1.00 mg/mL (L3), 0.50 mg/mL (L4), and 0.25 mg/mL (L5). Each level was tested over three analytical runs to generate three reportable results at each level. Sample matrix without the active ingredient was used as the sample for specificity and tested over a single analytical run. A second experiment

was performed for intermediate precision completed by a second analyst varying day and instrument. Method comparability (e.g., bridging) was evaluated by demonstrating acceptable accuracy (% recovery).

Prior to method validation, the non-interfering characteristics of the matrices were evaluated. Based on those development results (data not shown), corrections (baseline and/or scatter) were unnecessary and were not included in sample measurements. Concentration of a mRNA sample is determined by a modified Beer-Lambert Law Equation as described in C=m/ε.

Concentration (C, mg/mL) can be found by dividing the slope of the sample (m, Slope Abs (260 nm)/mm) by the known extinction coefficient (25 mL/(mg*cm)). Each mRNA sample was measured at both 260 nm and 280 nm.

RESULTS

Validation results for accuracy are summarized in Tables 1–3. Relative accuracy (% recovery) was calculated for each mRNA level (1–5) for all mRNA constructs as described in the equation below using the measured mRNA concentration, and the theoretical mRNA concentration.

%Recovery= (measured mRNA Concentration)/(theoretical mRNA Concentation) × 100%

The % recoveries for all levels tested were well within the acceptance criterion of 85–115%.

Validation results for Repeatability are summarized in Tables 4–6. Repeatability was demonstrated by a single analyst over a total of three analytical runs by testing mRNA levels 1–5 for each sample matrix. %RSD was calculated for each level. The acceptance criterion of 10% RSD was met for all levels tested.

Validation results for Intermediate Precision are summarized in Tables 7–9. Intermediate precision of the method was demonstrated by calculating the % RSD of the combined results from two repeatability analytical runs completed by two analysts over 2 days and on separate instruments. The acceptance criterion of 10% RSD was met for all levels tested.

TABLE 1

Accuracy results for TE sample matrix.

Matrix	Level	Analyst	Instrument	Vial	Theoretical concentration (mg/mL)	Measured concentration (mg/mL)	Recovery (%)
TE	1	1	1	6	4.10	4.25	104
				11		4.31	105
				19		4.28	104
	2	1	1	6	2.05	2.19	107
				11		2.20	107
				19		2.18	106
	3	1	1	6	1.03	1.09	106
				11		1.09	106
				19		1.09	107
	4	1	1	6	0.51	0.54	106
				11		0.54	106
				19		0.54	106
	5	1	1	6	0.26	0.27	106
				11		0.27	106
				19		0.27	107

TE: Tris-ethylenediaminetetraacetic acid.

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TABLE 2

Accuracy results for WFI sample matrix.

Matrix	Level	Analyst	Instrument	Vial	Theoretical concentration (mg/mL)	Measured concentration (mg/mL)	Recovery (%)
WFI	1	1	2	6	4.70	4.58	97
				11		4.58	97
				18		4.62	98
	2	1	2	6	2.35	2.39	102
				11		2.40	102
				18		2.41	103
	3	1	2	6	1.18	1.22	104
				11		1.23	105
				18		1.22	104
	4	1	2	6	0.59	0.62	105
				11		0.62	106
				18		0.62	105
	5	1	2	6	0.29	0.31	107
				11		0.31	106
				18		0.31	106

WFI: Water for injection.

Validation results for Specificity are summarized in Table 10. Specificity was demonstrated by testing each mRNA matrix without analyte sample (Level 06) over a single analytical run completed by a single analyst. The acceptance criterion of 0.01 mg/mL or undetectable was met for all matrices.

Validation results for linearity are summarized in Tables 11–13 and are plotted in Figure 3. Linearity of the method was demonstrated by

TABLE 3

Accuracy results for citrate sample matrix.

Matrix	Level	Analyst	Instrument	Vial	Theoretical concentration (mg/mL)	Measured concentration (mg/mL)	Recovery (%)
Citrate	1	1	1	6	4.01	3.80	95
				11		3.78	94
				18		3.81	95
	2	1	1	6	2.01	1.92	96
				11		1.91	95
				18		1.93	96
	3	1	1	6	1.00	0.96	96
				11		0.96	96
				18		0.96	96
	4	1	1	6	0.50	0.49	98
				11		0.49	98
				18		0.49	98
	5	1	1	6	0.25	0.24	96
				11		0.25	100
				18		0.25	100

TABLE 4

Repeatability TE sample matrix.

Matrix	Level	Analyst	Vial	Measured concentration (mg/mL)	Average (mg/mL)	Standard deviation	RSD (%)
TE	1	1	6	4.25	4.28	0.030	1
		1	11	4.31			
		1	19	4.28			
	2	1	6	2.19	2.19	0.010	0
		1	11	2.20			
		1	19	2.18			
	3	1	6	1.09	1.09	0.000	0
		1	11	1.09			
		1	19	1.09			
	4	1	6	0.54	0.54	0.000	0
		1	11	0.54			
		1	19	0.54			
	5	1	6	0.27	0.27	0.000	0
		1	11	0.27			
		1	19	0.27			

TE: Tris-ethylenediaminetetraacetic acid.

regression analysis of measured mRNA concentration (mg/mL) against the theoretical concentration (mg/mL) for mRNA levels 1–5 for each matrix. Linearity was assessed over three analytical runs completed by a single analyst. The coefficient of determination was determined as 1.00 for all matrices, which met the acceptance criterion of 0.98. The slope, and Y-intercept were also determined and are shown in Tables 11–13.

The working range of the method for mRNA samples in TE was established from

TABLE 5

Repeatability WFI sample matrix.

Matrix	Level	Analyst	Vial	Measured concentration (mg/mL)	Average (mg/mL)	Standard deviation	RSD (%)
WFI	1	1	6	4.58	4.59	0.023	1
		1	11	4.58			
		1	18	4.62			
	2	1	6	2.39	2.40	0.010	0
		1	11	2.40			
		1	18	2.41			
	3	1	6	1.22	1.22	0.006	0
		1	11	1.23			
		1	18	1.22			
	4	1	6	0.62	0.62	0.000	0
		1	11	0.62			
		1	18	0.62			
	5	1	6	0.31	0.31	0.000	0
		1	11	0.31			
		1	18	0.31			

RSD: Relative standard deviation; WFI: Water for injection.

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TABLE 6

Repeatability citrate sample matrix.

Matrix	Level	Analyst	Vial	Measured concentration (mg/mL)	Average (mg/mL)	Standard deviation	RSD (%)
Citrate	1	1	6	3.80	3.80	0.015	0
		1	11	3.78			
		1	18	3.81			
	2	1	6	1.92	1.92	0.010	1
		1	11	1.91			
		1	18	1.93			
	3	1	6	0.96	0.96	0.000	0
		1	11	0.96			
		1	18	0.96			
	4	1	6	0.49	0.49	0.000	0
		1	11	0.49			
		1	18	0.49			
	5	1	6	0.24	0.25	0.006	2
		1	11	0.25			
		1	18	0.25			

RSD: Relative standard deviation

4.10 to 0.26 mg/mL as passing results were obtained for linearity, accuracy, and precision. The working range of the method for mRNA samples in WFI was established from 4.70 to 0.29 mg/mL as passing results were obtained for linearity, accuracy, and precision. The

TABLE 7

Intermediate precision results for TE sample matrix.

Matrix	Level	Analyst	Instrument	Vial	Measured concentration (mg/mL)	Average (mg/mL)	Standard deviation	RSD (%)
TE	1	1	1	6	4.25	4.29	0.025	1
		1	1	11	4.31			
		1	1	19	4.28			
		2	1	5	4.31			
		2	1	15	4.30			
		2	1	21	4.31			
	3	1	1	6	1.09	1.10	0.005	1
		1	1	11	1.09			
		1	1	19	1.09			
		2	1	5	1.10			
		2	1	15	1.10			
		2	1	21	1.10			
	5	1	1	6	0.27	0.27	0.000	0
		1	1	11	0.27			
		1	1	19	0.27			
		2	1	5	0.27			
		2	1	15	0.27			
		2	1	21	0.27			

RSD: Relative standard deviation; TE: Tris-ethylenediaminetetraacetic acid.

TABLE 8

Intermediate precision results for WFI sample matrix.

Matrix	Level	Analyst	Instrument	Vial	Measured concentration (mg/mL)	Average (mg/mL)	Standard deviation	RSD (%)
WFI	1	1	2	6	4.58	4.60	0.026	1
		1	2	11	4.58			
		1	2	18	4.62			
		2	2	5	4.64			
		2	1	15	4.58			
		2	1	21	4.59			
	3	1	2	6	1.22	1.22	0.004	0
		1	2	11	1.23			
		1	2	18	1.22			
		2	2	5	1.22			
		2	1	15	1.22			
		2	1	21	1.22			
	5	1	2	6	0.31	0.31	0.000	0
		1	2	11	0.31			
		1	2	18	0.31			
		2	2	5	0.31			
		2	1	15	0.31			
		2	1	21	0.31			

WFI: Water for injection.

TABLE 9

Intermediate precision results for citrate sample matrix.

Matrix	Level	Analyst	Instrument	Vial	Measured concentration (mg/mL)	Average (mg/mL)	Standard deviation	RSD (%)
Citrate	1	1	1	6	3.80	3.79	0.013	0
		1	1	11	3.78			
		1	1	18	3.81			
		2	1	5	3.78			
		2	2	15	3.78			
		2	2	21	3.79			
	3	1	1	6	0.96	0.96	0.000	0
		1	1	11	0.96			
		1	1	18	0.96			
		2	1	5	0.96			
		2	2	15	0.96			
		2	2	22	0.96			
	5	1	1	6	0.24	0.25	0.005	2
		1	1	11	0.25			
		1	1	18	0.25			
		2	1	5	0.24			
		2	2	15	0.24			
		2	2	21	0.25			

RSD: Relative standard deviation.

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TABLE 10

Validation specificity results.

Matrix	Level	Analyst	Vial	Theoretical concentration (mg/mL)	Measured concentration (mg/mL)
TE	06	1	6	0 (matrix only)	0.00
WFI			6		0.00
Citrate			6		0.00

TE: Tris-ethylenediaminetetraacetic acid; WFI: Water for injection.

TABLE 11

Linearity results for TE sample matrix.

Level	Average measured concentration (mg/mL)	Theoretical concentration (mg/mL)	Validation criterion
1	4.30	4.10	R^2 is ≥ 0.98
2	2.19	2.05	
3	1.09	1.03	
4	0.54	0.51	
5	0.27	0.26	
Identifier		Result	
Coefficient of determination (R2)		1.00	
Identifier		Results (report only)	
Slope		1.045	
Y-intercept		0.01	

TE: Tris-ethylenediaminetetraacetic acid.

TABLE 12

Linearity results for WFI sample matrix.

Level	Average measured concentration (mg/mL)	Theoretical concentration (mg/mL)	Validation criterion
1	4.59	4.70	R^2 is ≥ 0.98
2	2.40	2.35	
3	1.22	1.18	
4	0.62	0.59	
5	0.31	0.29	
Identifier		Result	
Coefficient of determination (R2)		1.00	
Identifier		Results (report only)	
Slope		0.97	
Y-intercept		0.06	

WFI: Water for injection.

working range of the method for mRNA samples in 1 mM sodium citrate, pH 6.4 was established from 4.01 to 0.25 mg/mL as passing results were obtained for linearity, accuracy, and precision.

Robustness of the method was evaluated as part of method development. Robustness – sample volume robustness (100 and 140 L) was demonstrated by a single analyst over a total of three analytical runs by testing

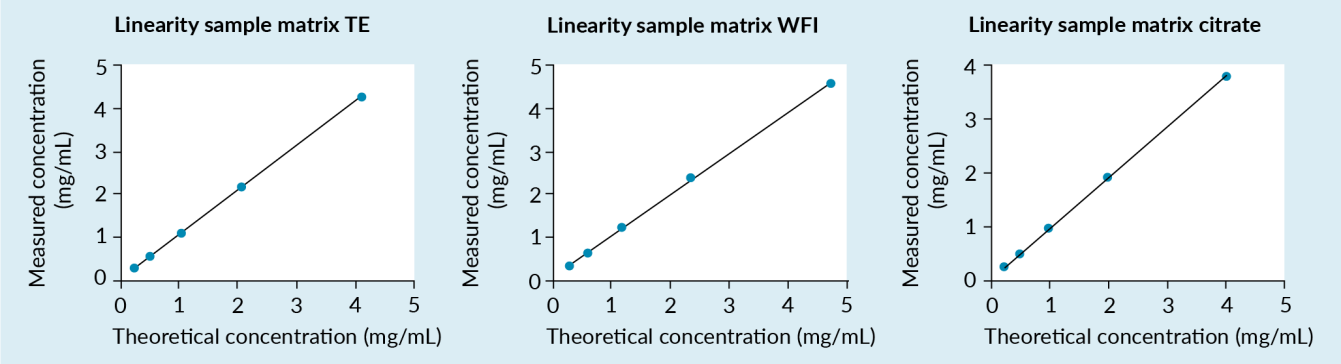
TABLE 13

Linearity results for citrate sample matrix.

Level	Average measured concentration (mg/mL)	Theoretical concentration (mg/mL)	Validation criterion
1	3.80	4.01	R^2 is ≥ 0.98
2	1.92	2.01	
3	0.96	1.00	
4	0.49	0.50	
5	0.25	0.25	
Identifier		Result	
Coefficient of determination (R2)		1.00	
Identifier		Results (report only)	
Slope		0.94	
Y-intercept		0.02	

FIGURE 3

Linear regression analysis (A–C) for each sample matrix.



Data are reported as mean ± SEM).

TABLE 14

Robustness–sample volume results.

Matrix	Level	Vial	Volume (L)	Measured concentration (mg/mL)	% difference
TE	1	4	100	4.32	2
		12	100	4.37	1
		22	100	4.34	1
		4	140	4.35	1
		12	140	4.33	2
		22	140	4.33	2

TE: Tris-ethylenediaminetetraacetic acid.
Note: The % difference is calculated from the measured concentration (mg/mL) of the 120-L sample volume.
Note: Theoretical concentration was 4.10 mg/mL.

mRNA levels 1, 3, and 5 for a single mRNA sample matrix. Percent difference from the measured mRNA result obtained in Accuracy (tested at 120 L) was calculated for each level. Results are summarized in Tables 14–16. These data indicate that the method is relatively unaffected by slight variations in sample volume.

Robustness–sample mixing of the method was demonstrated by a single analyst

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over a total of three analytical runs by testing mRNA levels 1, 3, and 5 for a single mRNA matrix. Percent difference from the measured mRNA result obtained in accuracy was calculated for each level. Results are summarized in Table 17. These data indicate that the method is relatively unaffected by these variations in sample mixing.

Bridging between the platform cuvette-based UV spectrophotometry method was confirmed as the acceptance criterion for accuracy was met.

TABLE 15

Robustness–sample volume results.

Matrix	Level	Vial	Volume (L)	Measured concentration (mg/mL)	% difference
TE	3	4	100	1.10	2
		12	100	1.11	1
		22	100	1.11	1
		4	140	1.09	2
		12	100	1.11	1
		22	140	1.10	2

TE: Tris-ethylenediaminetetraacetic acid.
Note: The % difference is calculated from the measured concentration (mg/mL) of the 120-L sample volume.
Note: Theoretical concentration was 1.03 mg/mL.

TABLE 16

Robustness–sample volume results.

Matrix	Level	Vial	Volume (L)	Measured concentration (mg/mL)	% difference
TE	5	4	100	0.27	3
		12	100	0.27	2
		22	100	0.28	1
		4	140	0.27	4
		12	140	0.27	3
		22	140	0.27	3

TE: Tris-ethylenediaminetetraacetic acid.
Note: The % difference is calculated from the measured concentration (mg/mL) of the 120-L sample volume.
Note: Theoretical concentration was 0.29 mg/mL.

TABLE 17

Robustness–sample volume results.

Matrix	Level	Mixing condition	Vial	Measured concentration (mg/mL)	% difference
WFI	3	Hand inversion	4	1.21	0
			14	1.23	2
			22	1.21	1
		Aggressive vortex	4	1.20	0
			14	1.19	1
			22	1.23	2

WFI: Water for injection.
Note: The % difference is calculated from the measured concentration (mg/mL) of the 120-L sample volume.

CONCLUSION

These data demonstrate that the SoloVPE variable pathlength spectrophotometer is a preferable alternative to traditional UV spectrophotometry. The versatility of variable pathlength technology (VPT/slope spectroscopy) is impactful for a Contract Development and Manufacturing Organization (CDMO), as it allows for the development and validation of platform analytical methods, resulting in many advantages over traditional UV spectrophotometry.

The challenge for CDMOs is the complexity in developing and validating analytical methods that can support several clients all with unique constructs. The SoloVPE technology, combined with a comprehensive platform validation covering multiple concentrations and formulation buffers, provides great benefits to CDMO clients and ensures the quality and the consistency of the CDMO's products for the reasons highlighted below:

- The SoloVPE technology is platformable. A single test method can be leveraged to support multiple mRNA products with no need to redevelop and validate multiple platform analytical methods to test mRNA concentration. The versatility of the SoloVPE reduces the cycle time for product-specific work, helping to support streamlined product release;
- The SoloVPE is accurate and repeatable. In contrast to traditional cuvette-based UV spectroscopy which relies on a single data point measured from the sample to determine the concentration, SoloVPE measures multiple data points from the sample at several pathlengths to determine the concentration. In addition, sample handling is effectively eliminated as no dilution of the test sample is required;
- The SoloVPE is easy to use. Complex methodologies can introduce error leading to unnecessary investigations and delays in product release. SoloVPE reduces method complexity while delivering a technology that is extremely robust and well suited for a release laboratory setting.

Onboarding new technologies like the SoloVPE allows CDMOs to continue to provide comprehensive analytical testing capabilities to support their clients' needs.

REFERENCE

1. ICH Q2 (R1) Validation of Analytical Procedures.

BIOGRAPHIES

TRAVIS ALVINE leads the analytical method validation group and manages analytical method validation, method transfer, and method implementation into the Quality Control release laboratory at Aldevron's Fargo location. He has been with Aldevron for over 6 years, with experience in analytical method development and validation over that time.

NATALIE UNSINN operates within the analytical method validation team through validation of methods, collaboration across sites, and implementation of methods to the quality control release team. She has been with Aldevron for over 3 years, with experience in quality control and analytical method development and validation.

JOHN J LONG has over 20 years of experience working in analytical methods for large molecule therapeutics including Vaccines, Biologics, and Gene Therapy products. He has experience across the analytical life cycle including method development, validations, and transfers worldwide. John has

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supported products from early development through licensure as well as support for marketed products. He has broad experience in compliance, investigations, inspections, and regulatory submissions.

JOE FERRAIOLO leads the bioanalytics applications team and is in charge of the SoloVPE variable pathlength spectroscopy system for at-line applications. He has been with the company for more than 25 years, with over 15 years of development and validation experience in analytical applications. He specializes in UV analysis and leads the development and commercialization of high-value products and flexible solutions that address critical steps in the production of biologics.

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AUTHORSHIP & CONFLICT OF INTEREST

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