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# CELL & GENE THERAPY INSIGHTS

## SPOTLIGHT ON:
## Gene therapy CMC and quality control

**Guest Editor:** Christine Le Bec, Head, CMC, Gene Therapy at Sensorion

CELL & GENE THERAPY INSIGHTS

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SPOTLIGHT

FOREWORD

# Gene therapy CMC and quality control

**CHRISTINE LE BEC,** PhD, joined Sensorion Pharma, a small biotech company, in early 2020 as Head of CMC Gene Therapy. She is responsible for CMC development (process and analytical development, product characterization) including non-clinical and clinical manufacturing, CMC transfer to CDMO/CRO and CMC regulatory issues. Prior to joining Sensorion Pharma, she worked for more than 20 years at Genethon, a French non-profit organization, in the field of Gene Therapy vectors (AAV, lentivirus, baculovirus) for rare diseases. She has a strong expertise in the development, qualification, validation of analytical methods for product characterization, release testing of gene therapy products and in stability studies. She also has a solid knowledge of international regulations and reviewing CMC documents for clinical applications.

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Over the last 20 years, gene therapies and especially adeno-associated virus (AAV) have been evaluated in clinical trials for the treatment of a wide variety of monogenic and non-genetic diseases. Three AAV-based products have been licensed to date in the US and EU for treatment of rare genetic diseases: Glybera® (alipogene tiparvovec) for lipoprotein lipase deficiency, Luxturna® (voretigene neparvovec-rzyl) for a rare inherited retinal dystrophy (*RPE65*$^{-/-}$) and Zolgensma® (onasemnogene abeparvovec) for spinal muscular atrophy.

Many regulatory documents are available and in particular, guidance documents for Chemistry, Manufacturing and Controls

(CMC) for human gene therapy investigational new drug applications. Current Good Manufacturing Practices (cGMP) must be followed for the methods, facilities, and controls used in manufacturing of gene therapy products. As clinical gene therapy products are developed and move forward to market authorization, the manufacturing should be robust, reproducible, more automated for GMP compliance, and scalable for large-scale production. In terms of downstream process, optimizing the purification steps is very important to reduce process residual impurities, to enrich for the full AAV particles, and to increase product recovery. Specific analytical assays are achieved to assess vector productivity, vector purity, biological activity, and to assure lot-to-lot consistency in product safety and performance. To sustain this, reliable, fast, robust, GMP-compliant analytical methods are needed. In recent times, analytical technologies have developed considerably to support on-line process development and product characterization, with increased automation helping to reduce method variability, timeframes, and sampling sizes needed for Quality Control.

This *Cell & Gene Therapy Insights* Spotlight brings you an excellent selection of articles that address different aspects of gene therapy CMC and quality control.

Gabriella Denning, from Expression Therapeutics gives an overview of CMC considerations and the new FDA guidance for Gene Therapy products. She comments on product identity, purity, and potency assays as well as sterility, safety, and stability studies. The article concludes by pointing out the CMC evolution during the product development stage.

Julie Yu Wei *et al.* from Ultragenyx Pharmaceuticals present an updated panel of analytical tools for vector genome quantification, potency evaluation, aggregation measurement, subvisible impurity, viral protein content, and protein impurity. They compare the conventional methods to the novel technologies and discuss their relative weaknesses and strengths, and their applicability on accelerating process and formulation development.

There are different AAV production platforms available today: adherent or suspension cells; human cells, insect cells or producer cell lines; transient transfection or infection systems. David Chu *et al.* from Capsida Biotherapeutics expand on upstream production and purification processes and the impact on product quality and performance associated with these modalities. They also highlight the importance of establishing critical quality attributes for vector performance and early drug product formulation assessment for long-term stability.

Finally, we have two interviews with experts from the gene therapy manufacturing and quality control field. Eduard Ayuso from Dinaqor shares his views on the development of AAV vector strategies from research to clinical manufacturing, and his thoughts on Dinaqor having its own capabilities for AAV manufacturing. He also focuses on in-line analytical tools for upstream process characterization and the bioanalytical technologies for product characterization.

Lauren Drouin from LogicBio talks about novel AAV capsids and their characterization, high-throughput analytical tools to accelerate process development, and Quality Control automation to obtain greater accuracy and better repeatability. The interview continues with a discussion of potency assays, which ensure product efficacy – one of the most critical release tests to develop and validate.

This Spotlight can only provide a limited insight of manufacturing of gene therapy products, current analytical tools, and regulators' perspectives on requirements - the focus is largely on AAV vectors and the challenges to accelerate development. I hope you will enjoy reading these articles as much as I did.

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## AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY CMC & QUALITY CONTROL

SPOTLIGHT

EXPERT INSIGHT

# Analytical toolkit for rapid formulation development of rAAV gene therapies

Julie Yu Wei, Ying Cai & James Warren

Recombinant adeno-associated virus (rAAV) gene therapy products have been studied in hundreds of clinical trials for treating multiple rare and monogenic disorders. Commercial success of rAAV gene therapy is also coming to fruition, as two products have recently achieved FDA approval for the treatment of Leber Congenital Amaurosis and Spinal Muscular Atrophy. Driven by clinical and commercial demand, significant advances have been made to analytical methods to characterize and release these products. rAAV vectors have demonstrated sustained stability across diverse bioprocessing conditions and are assumed to be stable in the Phosphate Buffered Saline (PBS) buffer with low levels of surfactant. Still, relatively few accounts of rAAV product formulation and stability data have been published to date, likely caused by substantial sample requirement, time urgency, and limited analytical tools. The traditional formulation development methods are few and many of which have low efficiency, poor precision, or accuracy. Here we present an updated panel of analytical methods compared to conventional methods with a focus on measuring stability-indicating attributes. This analytical toolkit has the capability of examining rAAV at the molecular level under various stress conditions to provide a holistic view of the physical and biochemical attributes of the product. The increased throughput and fast turn-around using small amount of material, while providing multi-attribute monitoring, enables rapid formulation development for fast decision-making and accelerated speed to clinic.

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## INTRODUCTION

Adeno-associated virus (AAV) was discovered in 1964 as a contaminating virus in adenovirus preparation. AAV is a small virus of approximately 24 nm in diameter, where the capsid is made of 60 subunit proteins consisting of three viral proteins (VP1, VP2, and VP3) assembled in icosahedral configuration with an approximate ratio of 1:1:10 [1]. 12 major AAV serotypes (AAV1-AAV12) and over 100 variants have been identified that naturally infect human tissues [1]. Recombinant AAV (rAAV) has been constructed to replace viral DNA with therapeutic transgenes with the size of 4–5 kb. The ability of rAAV to maintain persistent episomal transgene expression in dividing and non-dividing human cells in a broad spectrum of tissues at specific sites makes rAAV widely applicable for gene expression for precision treatment of genetic defective diseases.

Serotype-specific tissue tropism makes rAAV highly valuable in precision-medicine. Tissues targeted by rAAV include the retina, liver, pancreas, kidney, lung, heart, central nervous system (CNS), and muscle. The tropism is related to the variable regions (VRs) of virus capsid [2], cell surface receptor [2,3], cellular uptake, intracellular processing, nuclear delivery of the vector genome, uncoating, and second-strand DNA conversion [3]. The rAAV capsid protein can be engineered to improve tropism to expand into rAAV-resistant tissues and improve transduction. The expanding list of rAAV variant constructs and multi-specificity of tissue will need better molecular characterization and a platform to rapidly screen a viable formulation to protect rAAV from degradation during delivery, as well as to ensure compatibility with the target tissue environment.

The current formulation of rAAV for frozen storage has been extensively studied [4–9]. Frozen storage can be successfully achieved by supplementing phosphate formulations with various sugar excipients to avoid freeze–thaw stress [4], a surfactant to prevent loss of viral titer [4], and appropriate amount of NaCl to minimize dilution-induced aggregation [9] occurring in thawing-induced partitioning of rAAV. Alternatively, Tris-based buffer formulations have been successfully used in place of phosphate to further mitigate pH changes across freezing [10]. Most clinical rAAV products are stored under this frozen condition using one of the formulations described above. As products are developed to treat indications with higher patient populations and wide geographic distribution, it will also be beneficial to establish conditions that promote long-term stability under non-frozen conditions.

## CONVENTIONAL ANALYTICAL METHODS FOR FORMULATION DEVELOPMENT

The expanding number of rAAV clinical trials has increased the demand for formulation development to address concerns with multiple serotypes, multiple tissues, expanding delivery routes, and stability under non-frozen conditions. To meet concurrent time constraint, a formulation development platform toolkit is needed that incorporates a set of analytical methods with low sample requirements, fast turn-around by high throughput testing, and reliable measurement of key relevant parameters.

An optimal formulation should protect the rAAV product from degradation and thus retains the infectivity and potency, which is essential for the product to transduce target tissue. Typical degradation products include broken or incomplete capsids, viral protein fragments, soluble aggregation, and insoluble aggregates such as subvisible particles (SVP). The rAAV product function also depends on the genomic load delivered to target tissue, capsid protein structure, viral protein ratio, and post-translational modifications (PTMs). Events during transduction, such as receptor binding, cell trafficking, endosome escape, nuclear localization, and uncoating, are intimately connected with the viral protein amino acid sequence and their PTMs. Evaluation


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of rAAV product quality attributes required for transduction is important to include in formulation development, particularly for direct injection into target tissue where formulation has a direct influence on the infectivity cycle. Analytical tools that study those attributes are mRNA expression for transduction, ddPCR for genome titer, DLS and SEC for soluble aggregation and monomer, automated microscopy for SVP, mass spectrometry for PTM, and nanoDSF for predicting thermal stability.

## POTENCY-FUNCTIONAL EVALUATION

The purpose of formulation development is to ensure the formulated rAAV product maintains its functional activity so that the vector is capable of host-cell receptor binding, cell entry, intracellular trafficking, and expression of the therapeutic gene, leading to a biologically active gene product. Potency evaluation involves multiple cellular events that are commonly assessed through infectivity assay (medium tissue culture infectious dose $TCID_{50}$), transgene mRNA expression, protein expression, and functional assay (protein activity) for measuring the transgene product in cell-based or animal-based models. Each type of assay offers complementary information to help resolve the multiple factors that contribute to the vector performance. *In vivo* studies are typically established early in the discovery phase of rAAV products and are sometimes utilized in the characterization of potency for rAAV clinical products.

Potency of rAAV products can also be typically assessed in cell-based assays where the vector is applied to a platform *in vitro* cellular system and the resulting transduction is assessed by infectivity measures and/or expression of mRNA following the transcription of the vector genome within transduced cells. A common practice is to rely on *in vitro* $TCID_{50}$ assay, to measure the infectivity of rAAV, which is also a surrogate measure of the stability of the capsid and genome. The $TCID_{50}$ has been reported to have a variability of ± 0.5log10 [11] (although this variability can be improved with development), which lacks the necessary precision to differentiate a minor to moderate infectivity drop in stability studies. Cell-based mRNA expression assays measure part of the infectivity pathway by quantitating amount of mRNA produced following transduction of the vector product to an *in vitro* cell culture system. This cell-based assay has a faster turn-around time than $TCID_{50}$ and can be setup in multiple 96-well plates for parallel testing to achieve higher throughput. The cell lines used for potency evaluation could be sensitive to formulation excipients and need to be assessed for their interference. For example, some cancer cell lines, including hepatic carcinoma cells, have shown sorbitol-induced inhibition [12].

In short, cell-based potency assays are effective and sensitive to use for formulation development and accelerated stability studies to assess the impact of various biochemical environments and storage conditions on the biological function of the product. However, these methods can be time and resource-intensive, requiring significant training, expertise, time, and effort to analyze a multi-factorial experimental design.

## VECTOR GENOME EVALUATION

rAAV products are typically composed of a mixture of empty, intermediate, and fully filled capsids. Only full capsids can be expected to deliver the true therapeutic potential of the product, while empty capsids and intermediate species are considered process-related impurities. Thus, measuring the viral titer from the full capsid is critical for assessing function and stability. The distribution of full and empty rAAV particles can be measured by classic methods such as cryogenic transmission electron microscopy (cryo-TEM) and analytical ultracentrifugation (AUC), and the copy number can be evaluated through quantitative PCR (qPCR). Cryo-TEM offers a visualization of the genetic material and a

visual count of the full capsid, while AUC gives a detailed account of the species (empty, intermediates variants, fully filled, overfilled, aggregation, fragment, particulates, contamination from small molecules) with identification and reliable quantitation, as well as an estimation of the size of DNA contained in each of the species. AUC/TEM, when combined with particle concentration could yield accurate and precise estimation of viral load. However, both methods have notable limitations. AUC consumes large amount of material per run, TEM has low throughput and long turn-around time, while qPCR has a high assay variability and buffer matrix may interfere or inhibit the PCR amplification reaction.

The traditional method, qPCR, has a high assay variability and buffer matrix may interfere or inhibit the PCR amplification reaction [13]. Digital droplet PCR (ddPCR) has recently become the industrial standard for rAAV product genome quantification due to the superior accuracy, precision, and reduced sample matrix interference as compared to qPCR. In ddPCR, the accuracy is achieved by sample partition into droplets for direct quantitation, hence removing the dependence of the result on PCR amplification efficiency. The accuracy of ddPCR is further enhanced by the application of Poisson distribution toward 95% confidence interval calculation of the measurements. Comprehensive comparisons of qPCR and ddPCR have been published [14–17]. The current consensus is ddPCR has significantly reduced variability compared to qPCR for samples containing PCR inhibitors, especially for concentration near the quantitation limit, and more sensitivity toward appearance of low amount of genetic material [14,15,17].

Genome titer assays are a fundamental component of a stability panel because conditions which promote loss of capsid integrity can be detected by these methods with a high degree of accuracy and precision, and qPCR is easily multiplexed to enable high-throughput testing. However, genome titer has limited ability to detect degradations that develop under certain conditions, which may lead to a decline in biological function. For example, minor capsid changes or capsid aggregation may compromise infectivity but otherwise appear unchanged as measured by genome titer alone.

## AGGREGATION & MONOMER

One of the critical quality attributes is aggregation [18]. Concentrated AAV or low ionic strength formulation may promote aggregate formation and result in decreased transduction efficiency. SEC is often used to characterize the HMW and LMW species of rAAV product. However, this method has two limitations. The size of soluble aggregates that can be detected by SEC is limited by the frit pore size and protein-column interaction, and aggregation in concentrated rAAV in its formulation could be changed upon dilution into SEC mobile phase [19]. DLS could detect aggregation in its native formulation without dilution, up to 2 μm, to enable meaningful formulation comparison. It measures the hydrodynamic size of a particle based on the Brownian motion. From Rayleigh approximation, dimers have ~64 times more light intensity compared to a monomer because the intensity of scattering is proportional to the 6th power of the particle diameter. Thus, the technique is more sensitive to aggregation than smaller size degradants. The size accuracy is medium as particle diffusion rates vary with effective particle size (particle shape), temperature, viscosity, water hydration, dilution error. In addition, aggregate quantitation is poor. Despite the limitations, DLS has been used for studying ionic strength associated aggregation formation [9] and other formulation studies [20].

New generation light scattering instruments have demonstrated improved precision and accuracy compared to the traditional DLS. The Prometheus® Penta (NanoTemper, Cambridge, MA) and DynaPro® (Wyatt, Santa Barbara CA) are among them. In addition to measuring AAV size and aggregate content


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in its formulation without dilution, they offer a new function to evaluate AAV particle size change from heating. We evaluated AAV with both instruments and a similar precision of ~2% CV in size was achieved. Penta reported the particle size of ~26 nm for AAV monomers, consistent with the cryo-TEM reported value of 24–26 nm [21]. DynaPro also reported low CVs for the size and concentration of AAV monomer and aggregates [20]. Overall, the new generation light scattering instruments are ideal for early formulation screening and stability studies. The method is rapid, non-destructive, low sample volume, uses native condition, and not requiring method development.

A group of methods orthogonal to the SEC is field-flow fractionation (FFF), notably the asymmetric flow field-flow fractionation (AF4), where particles are separated by size based on diffusion in the native buffer caused by laminar and cross flow. The advantage of high resolution and a large dynamic range (1 nm to 1 µm) complements SEC. In conjunction with multi-angle light scattering (MALS), it has been demonstrated as a valuable tool in characterizing the stability of rAAV in various solution environments [22]. However, the FFF method is not a routinely available tool and requires technical expertise to customize test protocols for different samples [23]. Therefore, it is less desirable for early-stage rapid formulation development.

## SUBVISIBLE PARTICLES IMPURITY

One area of aggregation that has not been widely published by the gene therapy area is subvisible particles (SVP) in the drug product. Although systematic delivery by IV infusion of the drug product involves extensive dilution and filtration that minimize the risk of delivering SVP into patients, the risk may be higher for direct delivery into a localized tissue. SVP are regulated for therapy targeting retinal tissue according to USP<789>Particulate Matter in Ophthalmics, and delivery to other tissue follows defined limits listed in USP <788> for particle counts ≥25 µm and ≥10 µm. Moreover, regulatory agencies expect drug developers to monitor and control particles from 2 to 10 µm, and monitor particles ~1 µm or less.

As previously reviewed, DLS can detect aggregation up to 1–2 µm. For particles ≥2 µm two standard methods, light obscuration and microscopy, are given in guidance <787>, <788>, and <789>. The implementation of those two SVP testing methods for rAAV gene product analysis is hindered by the large sample requirement (1–25 mL) (<788>), disqualifying them as a routine method for rAAV formulation studies. Recent technology advancement in this area has minimized the sample requirement down to 3 µL to 150 µL, including automated microscopy (HaloLabs), single particle optical sizing by light absorption and scattering (AccuSizer® from Entegris), and coulter counting (SpectraDyne™). While automated microscopy and AccuSizer are automated and fast, coulter counting from SpectraDyne is manual and low throughput. Automated spectroscopy (HaloLabs) is in 96-well plate format and accuracy is improved with automated imaging that removes subjective errors, making it appropriate for application in the gene therapy area [24].

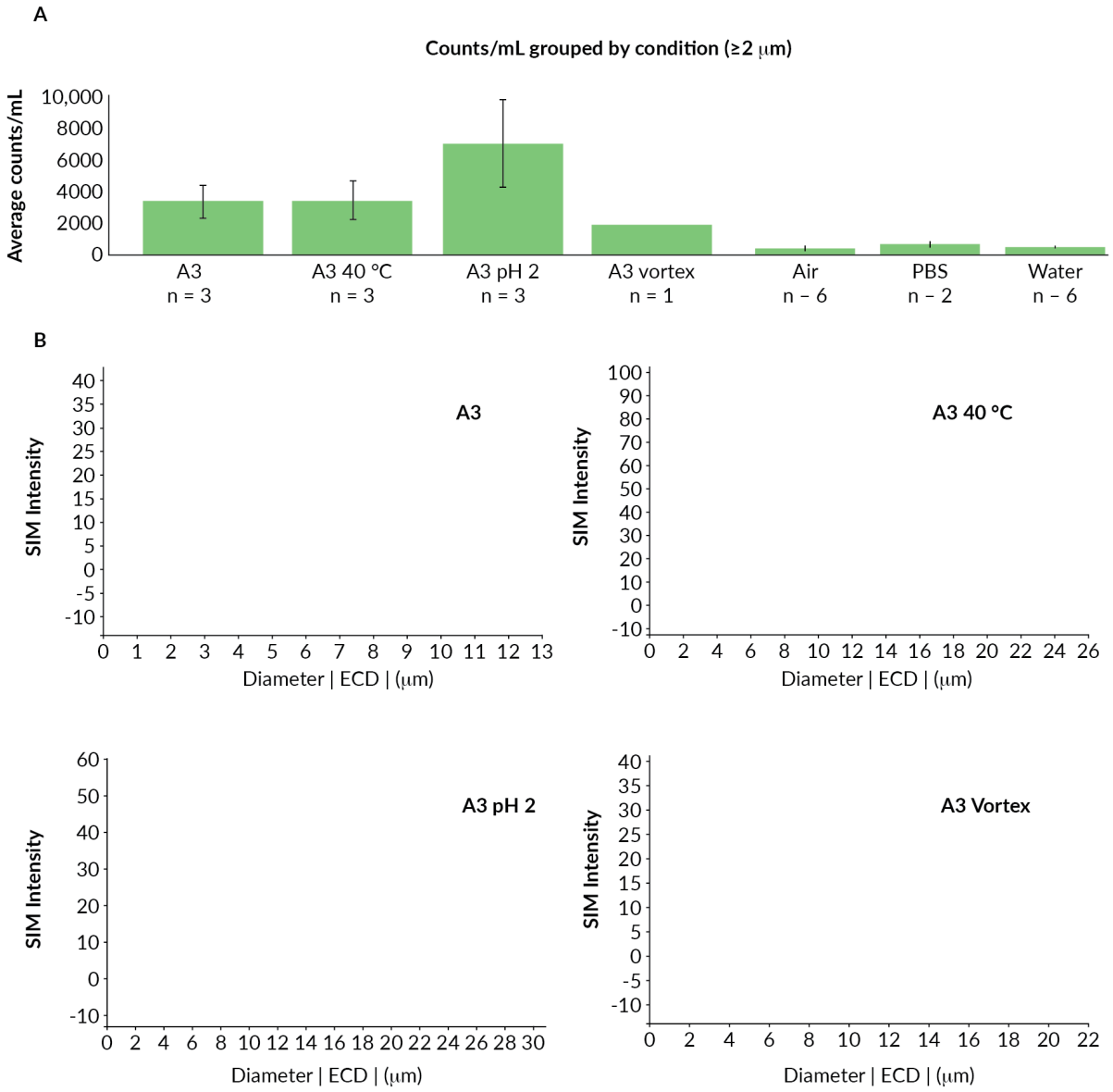
Our preliminary evaluation of automated microscopy shows that SVP ≥2 µm in rAAV preparation may increase by different stress factors such as high temperature, low pH and high shear (Figure 1). The particle number has a precision indicated in the error bar, similar to current standard SVP methods. The particle behavior is measured three dimensionally by its width (diameter) and height (SIM intensity). The specific pattern in response to stress shows this technology will be important for drug product formulation evaluation.

## VIRAL PROTEIN RATIO & PROTEIN IMPURITY

The balance of the three VPs (VP ratio) is required for proper structure/shape and

## FIGURE 1

**rAAV subvisible particle measured by automated microscopy.**




**(A)** AAV8 unstressed compared to stressed. The baseline counts are from air, water, and PBS. **(B)** Size distribution of SVP ≥2 μm in A3 material before and after stress. The x-axis is the diameter of the rAAV subvisible particle from 0–30 μm, and y-axis is the light intensity of the particle. The diameter indicates the size of the aggregate, while the intensity indicates the layer of particles. The experiments were conducted with Horizon instrument (HaloLabs) where 50 μL was loaded in triplicates onto 96-well plate and dried prior to measurements.

function [25,26], and the product-related VP impurity is a clinical safety concern. Each of the VP has a biological function (VP1 directs cell trafficking and endosome escape, VP3 forms the correct shape), and inappropriate proportion results in decreased potency [25]. Because the VP ratio is an important potency-indicator, its consistency should be maintained during storage. VP impurity, often a product of degradation of the viral protein, signifies instability and could be a safety concern. Both the VP ratio and impurity can be disturbed by a number of factors during storage, including physical and thermal stress induced denaturation and chemically induced PTM change.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SDS-PAGE is the classic electrophoresis method. It uses several µL materials and can be set up in multiple gel format so that parallel testing can be performed. The main issue for SDS-PAGE is its low precision in quantitation and narrow concentration dynamic range. CE-SDS, also known as SDS capillary gel electrophoresis (CE-CGE), has improved advantages over SDS-PAGE in separation resolution and precision due to plug-type movement which minimizes diffusion. This results in a very narrow peak width allowing reliable quantitation and lower limit of detection. Sample stacking also increases sensitivity over SDS-PAGE [27]. The run time of the CE-SDS is only a matter of minutes. With automation setup it greatly increases turn-around time. One caveat of CE-SDS is its sensitivity is affected by higher salt concentrations due to competing injection with the analyte and band broadening. In addition, high level of certain buffers and excipients could be interfering.

## DEAMIDATION & CHARGE VARIANTS

Deamidation is an important stability indicating attribute, as it is linked to decreased transduction efficiency and loss of potency [28]. Deamidation occurs when an asparagine or glutamine loses the amide group and converted to carboxylic acid via hydrolysis. Therefore, it can be monitored indirectly from change of charge variants or decrease in protein pI during the stability study.

Conventional measurement of charge variants was carried out by ion-exchange chromatography (IEC) or isoelectric-focusing gel electrophoresis (IEF). But these methods have limitations of high variability, low resolution and poor precision. The new generation tools such as capillary isoelectric focusing (CIEF) [29] and imaged CIEF (iCIEF) have addressed these issues [30]. iCIEF further eliminates the mobilization step, which enables the highest possible resolution within a short run time of 15–20 min. We have used iCIEF to measure the shift of rAAV charge variants in the same formulation buffer during an extended hold study (Figure 2). In Figure 2A, an increase of acidic species was shown for the sample exposed to higher temperatures. In Figure 2B, the control sample started with a pI range of 6.2 to 6.9. The sample pI shifted to a range of 6.0 to 6.5 after 4 days (Figure 2C); and 5.9 to 6.3 after 7 days for the same sample (Figure 2D). There are limited reports of iCIEF for AAV characterization and stability studies. However, this method has great potential for early formulation development by its high resolution, minimal development time, low sample volume, and fast run times. A reference standard is recommended for using iCIEF to compare charge variants in different formulations as formulation ionic strength may affect the charge profiles.

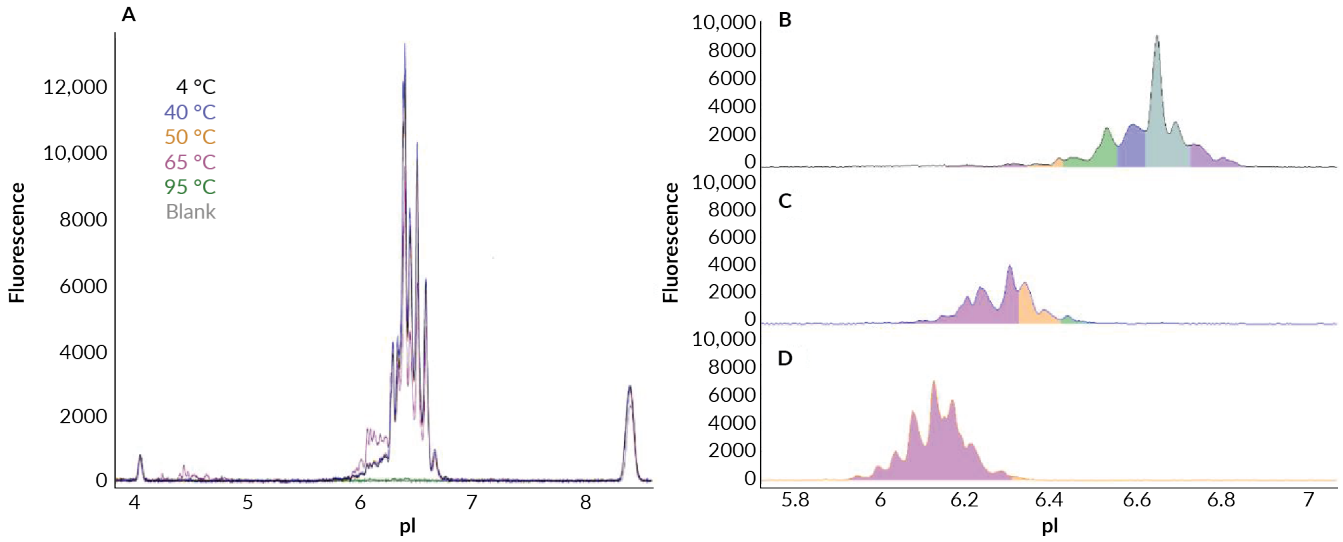
## POST TRANSLATIONAL MODIFICATIONS (PTMS)

The VP ratio, protein impurity, and surface charge in potency and stability can be related to the structure by mass spectrometry. There is an increasing interest in characterizing the primary structure and PTMs by mass spectrometry to obtain key information regarding the structure. Jin *et al.* developed intact and peptide map method for rAAV and identified the key PTMs [31]. VP1 N-terminal acetylation and VP2 phosphorylation play a direct role in its infectivity. N-glycosylation of rAAV is implicated as a possible factor for tropism and VP ratio. Wide spread of deamidation was observed in the serotype AAV8 and correlated to activity loss [28]. The connection between PTM and structure/function makes mass spectrometry one of the critical tools to develop for next generation formulation development.

Two main directions on mass spectrometry are developing in parallel to support characterization of rAAV product quality. PTM characterization by peptide mapping using reverse-phase high performance liquid chromatography hyphenated with mass spectrometry (RP-HPLC-MS) [31], or variant of viral

## FIGURE 2

**AAV8 charge profiles for (A) samples being held at different temperature for 5 mins; (B) a control sample at the beginning of hold, (C) the same sample in (B) being held for 4 days at 37°C, and (D) the same sample in (B) being held for 7 days at 37°C.**




Samples were processed with Bio-Techne protocols and detected by native fluorescence with a 10-second exposure time [30].

protein intact mass analysis including RP-HPLC-MS [31] and CE-MS [32]. Peptide map generates nearly complete sequence coverage and produces confident PTM assignment on VP1u and quantitation based on comprehensive fragmentation coupled with sequence variant searching. With proper optimization, peptide map can use <10 µg of material per run and achieves moderate throughput experimentation. However, peptide map assignment generally requires replicates including a comix to ensure repeatability. Manual verification and long data processing time also determines the applicability of this method toward formulation development. The CE-MS is an important technology to develop, due to its functional cross-over with CE-SDS and iCIEF. Viral protein impurity and VP variants from CE-SDS and charge profile from iCIEF can be identified by mass. CE-MS has been shown to successfully separate VP1, 2, and 3 with adequate signal [32]. One of the advantages of CE-MS is that it uses intact mass on the separate VP, thus greatly reducing experimental and analysis time. The sample requirement of chip-based CE-MS is extremely low, on the order of nL, and the separation and mass acquisition only take minutes. The high throughput nature and rich information output makes chip-based CE-MS an ideal tool for formulation development.

Certain post-translational modification of rAAV capsid protein residues may develop as a result of biochemical or thermal stresses and these have been correlated to subsequent decline in infectivity or potency of a vector product [25]. The full range of impacted PTMs that result in potency loss have only started to be explored. Here we will give two examples: deamidation and oxidation. Asparagine or glutamine deamidation may occur at AAV capsid proteins at sites which contribute to the infectivity of the vector, resulting in product quality changes. Characterization of rAAV capsid PTMs may establish 'leading indicators' of change in biological function, and as such are important to include in formulation and stability studies. Oxidation is a critical factor for consideration in formulation development. Studies have demonstrated inhibitors of free-radical oxidation such as EDTA, triethanolamine, and citrate can enhance the stability of viral vectors [33]. Oxidation of rAAV can be measured by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HILIC-FLR-MS [34]. The use of viral protein intact mass from chip-based CE hyphenated with high mass resolution/accuracy mass spectrometer can be used to estimate both oxidation and deamidation.

Our preliminary assessment of chip-based CE-MS shows that upon thermal stress, AAV9 undergoes significant increase in deamidation to ~100% (Table 1; indicated by increase in mass of VP3 under stressed condition) resulting in an average mass that is 1 mass unit higher than the unstressed. Higher order of deamidated products can be seen ~20%. This result encourages the implementation of chip-based CE-MS as a routine tool for formulation development to monitor PTM changes. Early detection of a formulation which fails to suppress undesirable PTM generation is important to maintain product quality at a given temperature over time.

## THERMAL STABILITY

AAV capsid melting temperature ($T_m$) is a stability indicating quality attribute and often associated with the serotype. Capsid deamidation or degradation can be reflected in the reduction of transition temperature, and $T_m$ is altered by one single acidic or basic amino acid difference in the capsid [35]. It was also reported that $T_m$ is affected by formulation pH and decreased more rapidly near pH 4.0, caused by VP1u externalization and AAV uncoating during endosome/lysosomal escape [36]. Therefore, studying AAV thermal transition profiles can enable selection of heat stabilizing excipients or optimization of buffer pH conditions.

Differential scanning calorimetry (DSC) is a technique commonly used to characterize thermal transitions of proteins. It measures the dependence of protein structure on the temperature expressed as heat capacity function (Cp) over temperature. An alternative method is differential scanning fluorimetry (DSF). A protein-binding dye (typically SYBR green) is used for binding to the hydrophobic region of the protein. The fluorescence is significantly increased during protein denaturation phase when core hydrophobic patches are exposed. A DNA-binding dye such as SYBR gold is also used to monitor the release of genome during heating. However, DSC requires large sample volumes, and DSF could have buffer matrix interference, therefore both are less favorable for early formulation development.

The newer generation of DSF instrument has addressed the previous limitations. NanoDSF features small amount of sample (10 µL) and in high throughput capacity (48 samples per 2 h), greatly improving the capability of calorimetry experiment. It also eliminated the need of external dyes by using the intrinsic native fluorescence (NF)

**TABLE 1**

**Comparison of VP3 mass from two lots of AAV8 and one lot of AAV9 with and without stress.**

| Sample | PTM | Theoretical mass (Da) | Observed mass (Da) | Mass difference (Da) | Mass error (ppm) |
|---|---|---|---|---|---|
| AAV8 lot 1 VP3 | N-Term Acetylation (1) | 59804.0726 | 59803.65 | 0.4226 | -7.2 |
| AAV8 lot 2 VP3 | N-Term Acetylation (1) | 59804.0726 | 59803.18 | 0.8926 | -15.1 |
| AAV9 lot 1 VP3 | N-Term Acetylation (1) | 59732.0126 | 59732.16 | -0.1474 | 2.3 |
| AAV9 lot 1 stressed VP3 | N-Term Acetylation (1), deamidation (1) | 59732.9973 | 59733.31 | -0.3147 | 3.9 |
| | N-Term Acetylation (1), deamidation (2) | 59733.9820 | 59734.00 | -0.0180 | 0.8 |

The samples were treated with the vendor's peptides BGE, 2 nL sample volume was loaded onto HR-chip, and mass data were acquired using Thermo Scientific QExactive HF instrument.

from the protein tryptophan. We have used nanoDSF to compare heat stressed samples and observed elevated Cp baseline and loss of early transitions before reaching $T_m$. The NanoDSF, however, does not replace DNA detection by DSF, as DNA does not contain intrinsic fluorescence.

In summary, DSF offers a rapid, cost effective, and robust method for AAV formulation development. However, it may be difficult to compare thermal transitions with different AAV products. Heat is only one of the stress factors leading to AAV degradation. It was reported that individual viral protein thermal stability in different buffer formulations did not exhibit significant influence on AAV transduction efficiency [35]. The $T_m$ could relate to storage stability [37] and therefore, may be used to select formulation buffers of improved AAV heat stability, but other potency methods may still be needed at an early stage to establish correlation.

## COLLOIDAL STABILITY

Stable high concentration rAAV formulation is of great interest for some delivery routes such as CNS administration. However, high concentration presents a challenge of material limitation for formulation development. The behavior at high concentration can be studied by self-interaction of rAAV as a function of concentration. The self-interaction, or protein-protein interaction (PPI), is notable at a high concentration, and contributes to colloidal instability which is described by several undesirable biophysical properties including phase separation, suspension, turbidity, and increased viscosity, all of which associated with aggregation. The group of tools for PPI studies include spectroscopy method, osmotic second virial coefficient B22 calculation and kD analysis by DLS, zeta potential, and viscosity. This combination was shown to predict long-term stability [38]. Popular methods in spectroscopy include bilayer interferometry (BLI) from Octate family of instruments [39] and turbidity assay by UV-spectroscopy in conjunction with various sample preparation methods [40,41]. Both of which feature high-throughput and fast turn-around time. Those are relative methods with reasonable precision. High throughput and accurate DLS is indispensable for kD/B22 [42] and the instruments are discussed above. The viscosity must be measured directly at high concentration [43]. Viscometers that use low amount of sample include micro-VISC family of instruments and Viscosizer, where the latter has automation capability in high throughput manner. Recent advancement in zeta potential measurement now allows for accurate and high throughput measurement in the native formulation buffer (TRPS from Izon). The combination of self-interaction tools listed here equips for development of high concentration formulation in rAAV field.

## OVERVIEW OF ANALYTICAL METHODS

This review discussed a panel of conventional and new generation of analytical tools applicable for rAAV formulation development and stability studies. Table 2 below summarized the strengths and weaknesses of these methods. DLS, nanoDSF and iCIEF offer great potential to enable rapid formulation development and accelerate stability studies.

The tools evaluating PPI have not been fully explored for rAAV modality and are therefore not included in Table 2. Other analytical tools not discussed in this paper include direct structural measurements and binding assays may offer new potential for formulation development.

## TRANSLATIONAL INSIGHT

Definition of the optimal formulation for a rAAV gene therapy product is a critical milestone in the late discovery and early CMC-development phases, as the formulation buffer has implications on safety and tolerability of the product, the short- and


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**TABLE 2**

**Overview of analytical methods.**

| Attribute | Methods | Strength | Weakness |
|---|---|---|---|
| Viral titer | qPCR | Fast, high throughput, low material requirement. Release method | Low accuracy and precision |
| | ddPCR | Low material requirement, accuracy and precision high. Release method | Low throughput |
| Potency | $TCID_{50}$ | Overall assessment of infectivity in a platform format. Release method | Slow, low throughput, low accuracy and precision |
| | mRNA expression | Assessment of transduction in a platform format. Release method | Mid-range precision |
| | *In vitro* protein expression | Assessment of biological activity *in vitro* | Specific method development needed |
| | *In vivo* expression | Assessment of biological activity in animals | Significant amount of work and time resources required |
| Aggregation | AAV size by DLS | Fast, high throughput, low material requirement, accurate and precise sizing | Lower sensitivity toward smaller size species |
| | Aggregation by DLS | Fast, high throughput, low material requirement, aggregation in native formulation | High assay variability for aggregation quantitation |
| | Aggregation by SEC | Fast, high throughput, low material requirement. Release method | Limitation of aggregate size, aggregation maybe changed by diluting into SEC mobile phase |
| Capsid | CE-SDS | Fast, higher throughput than SDS-PAGE, medium material consumption. Release method | Sensitivity is limited by buffer matrix such as salt content |
| Deamidation | CE-MS | Fast, high throughput, low material requirement | Currently characterization tool |
| Thermal transition, $T_m$ | DSF | Fast, high throughput, low material requirement. Can detect changes to both capsid and genome | Nonspecific interaction of dye with buffer components |
| | DSC | Standard for thermal stability, high precision and accuracy | Low throughput, high material consumption |
| | nanoDSF | Fast, high throughput, low material requirement | Cannot detect change of genome content |
| Charge heterogeneity | iCIEF | Fast, high throughput, low material requirement. High precision. Can be developed as a release assay | High salt content interferes with assay |
| Subvisible particle | Automated microscopy | Fast, high throughput, low material requirement, USP release method | More effort for qualification |

long-term stability of the product, and may also have an impact on the biodistribution and transduction of targeted tissues. Formulation screening during the discovery and early-CMC development stages can be challenging due to lack of sufficient amount of representative material or due to lack of time or resources required to run complex cell-based methods to assess impact to vector product potency. Many sponsors choose to implement platform formulations which have previously demonstrated satisfactory stability/performance on historical programs rather than undertake formulation screening studies at early stages of development. Most importantly, because formulation is such a significant component of the drug product, formulation changes implemented during or after Phase 1 may result in delays to clinical development in order to bridge or confirm safety of the new formulation. Many of the methods summarized in this document permit rapid screening of multiple buffer chemistries using minimal vector product sample volumes across accelerated or stressed stability studies in order to de-risk novel formulation buffers at early stages of development.

As programs progress to later CMC development, some of the methods used in pre-formulation analysis may be carried through as formal assessment of product quality in QC characterization, stability, or release testing. Alternatively, some of the methods described herein may be maintained as development tools to periodically assess the product quality over the course of the product lifecycle. Methods such as ddPCR, SEC and DLS, can be established as platform methods as they may be applied across multiple products with minimal re-development (for PCR assays this is dependent upon target sequence). Methods such as CE-SDS, iCIEF, mass spectrometry, and nanoDSF are sensitive to differences in product serotype and as such will likely require serotype-specific or product-specific development. Once established they could also be platform methods. Many of these methods have already been qualified and/or validated for clinical or commercial product release, while others such as DLS and iCIEF demonstrate sufficient levels of precision and accuracy and may be used as routine qualified characterization or release methods in the future.

## CONCLUDING REMARKS

With two rAAV gene therapy products having recently gained marketing authorization in the US and hundreds of similar products currently in clinical development, the future of rAAV gene therapy is promising. As new products are developed targeting diverse tissue biodistribution such as liver, CNS, cardiac, and skeletal muscle, and the concentration of these drug products are pushed higher to deliver more product in a smaller volume, and as current long-term storage duration and temperatures are modified to support supply chain for larger patient populations, formulation development for rAAV vector products will take a more prominent stage. Traditional molecular biological and virological methods such as qPCR, $TCID_{50}$, and mRNA expression will need to be supplemented with newer chemistry methods, some of which have been used in the characterization of biologics historically, in order to establish fit-for-purpose and phase-appropriate panels for formulation and stability screening. In particular, methods such as SEC for aggregation, DLS for particle/aggregate size, CE-SDS and iCIEF for purity and capsid protein characterization, chip-based CE-MS for PTMs, and capsid characterization by nanoDSF have demonstrated strong applicability and promising performance as the industry moves forward toward establishing a more comprehensive understanding of the structure-function relationships for viral vector products.

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY CMC & QUALITY CONTROL

SPOTLIGHT

INNOVATOR INSIGHT

# Expression systems for viral vector production: advantages of the Sf9 baculovirus system and simple solutions to address its specific analytical challenges

Yi Fang Lee & Srinath Kashi Ranganath

The gene therapy landscape has exploded in the last few years, bringing a multitude of viral vectors to the clinic. At the forefront of this evolution is the application of lentivirus, adeno and adeno-associated virus (AAV), and plasmid-based therapies to genetic diseases of all types. With each program that progresses through the clinic, the body of manufacturing and clinical knowledge grows – and so does the availability of regulatory guidance. Adeno-associated virus (AAV) is increasingly popular, and the baculovirus-Sf9 platform has been established as a promising alternative to mammalian cell-based methods. However, the Sf9 baculovirus production system poses some unique analytical challenges. Here, we will discuss two of these issues – quantifying residual DNA and detecting the adventitious agent Sf-rhabdovirus – and solutions developed by Thermo Fisher Scientific designed to meet regulatory guidelines and ensure product quality and safety when utilizing the baculovirus-Sf9 platform for AAV production.

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## AN ESTABLISHED ALTERNATIVE TO RAAV PRODUCTION IN MAMMALIAN CELLS

As an alternative to recombinant AAV (rAAV) production in mammalian cells, the baculovirus Sf9 platform has been notably established as a GMP-compatible and scalable system generating as many vector genomes per cell as mammalian cell-based methods – i.e., up to 2 x $10^5$ vector genomes per cell in crude harvests.

The baculovirus Sf9 production system offers many advantages over other production platforms when considering various safety issues. The system uses serum-free media, and despite the discovery of adventitious virus transcripts in Sf cell lines, most of the viruses infecting insects do not actively replicate in mammalian cell lines. There is also no need for a helper virus or plasmid to produce recombinant AAV in insect cells, besides the baculovirus.

Although this system offers many advantages, the Sf9 baculovirus production system poses some analytical challenges relating to process impurities from the system itself. The manufacturing process for Glybera™ drug product (uniQure), the first gene therapy product produced using the Sf9 baculovirus system, is an illustrative example. A comparability assessment of the product from different process stages was performed; of critical importance was the comparability between the process used for the clinical studies, and the commercial process. The results indicated that the product quality has improved throughout the development of this manufacturing process. In most analyses, the commercial process quality was comparable if not better than the clinically used process, except for a significantly higher carry-over of baculovirus DNA.

In this relevant case, the residual baculovirus genetic sequences unintentionally encapsulated in AAV1 capsids, and co-purified with the product, which was one of six major product quality objections raised during the licensure assessment of the Glybera drug product by the European Medicines Agency.

Another important analytical challenge is detection of Sf9 rhabdovirus, which is an adventitious virus infecting insect cells, and is present in most Sf9 and Sf21 cell lines used in bioproduction.

Solutions to these two important challenges are discussed below.

## QUANTITATION OF RESIDUAL SF9 HOST CELL & BACULOVIRUS DNA

To address the issue of residual DNA in the Sf9 baculovirus system, there is a need for an analytical method to accurately quantitate residual Sf9 and baculovirus DNA. The FDA describes the residual DNA limits for non-tumorigenic cells to be less than 10 nanograms per dose, and the DNA size to be below approximately 200 base pairs in the recent CMC guidance for industry document revised in 2020.

Similar guidance was provided by the FDA in a briefing document from 2012 for vaccines produced in cell lines derived from human tumors. The most recent WHO recommendation also sets the upper limit for residual cellular DNA at 10 nanograms per dose.

In a typical AAV production process, process impurities such as residual DNA and host cell protein are expected to be present. Therefore, they should be characterized and substantially reduced and controlled to an acceptable range by appropriate purification steps. Residual host cell DNA is present in two forms – as a nuclease-sensitive process-related impurity that is non-specifically co-purified with the desired AAV vector product, and as a nuclease-resistant product-related impurity that is encapsulated within AAV particles. Minimizing these distinct forms of residual host cell DNA requires different manufacturing process optimization strategies, and a robust residual DNA testing method is therefore necessary to determine the residual DNA levels in the process.

The design and control of an in-house residual DNA assay poses a number of


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challenges. Designing a custom residual DNA assay requires extensive development and validation to ensure that the primers are specific to the target sequences, and are not amplifying non-specific targets. For a multi-target system such as the Sf9 baculovirus platform, multiple assays are needed for both Sf9 and baculovirus.

Establishment and maintenance of a robust standard or DNA control requires significant expertise and is highly time consuming. These assays require multiple components, and strong inventory management is required for the individual components to ensure lot-to-lot reproducibility. Additional infrastructure may also be required for each component around incoming quality control.

## The resDNASEQ™ Quantitative Sf9 Baculovirus DNA Kit

The resDNASEQ Quantitative Sf9 Baculovirus DNA Kit is a duplex quantitative PCR-based system for the simultaneous detection of residual DNA from the Sf9 baculovirus platform used in the development of gene therapy, cell-based vaccines, and similar biotherapeutics. The assay is reliable, rapid, and enables sensitive and specific quantitation of Sf9 host cell DNA and baculovirus DNA.

All resDNASEQ kits are provided in a comprehensive product solution format, with all-inclusive reagents and genomic DNA standards. The rapid testing and streamlined workflow allow for a fast time to result of under 5 hours with optimized sample preparation.

The reliable performance of the kit also allows its use in multiple stages of the gene therapy manufacturing process. The kit is designed to meet the specifications listed in the USP 509, the residual DNA testing guidance document published by the United States Pharmacopeia.

Validation was conducted by eight operators spanning three continents, using multiple lots, and over four days. Two sample preparation methods were used; manual sample preparation and automated sample preparation with KingFisher Flex using the PrepSEQ™ Sample Preparation kit. Real-time PCR was evaluated on the Applied Biosystems™ 7500 Fast™ and the QuantStudio™ 5 instruments.

The key performance criteria evaluated were:

- PCR efficiency
- Linearity
- Range of the standard curve
- Precision
- Reproducibility
- LOQ/LOD
- Specificity
- Sample preparation spike recovery
- Singleplex versus duplex performance

These criteria were evaluated based on the ICH Q2 R1 guidelines, and the criteria for precision and accuracy were taken from the USP 509 document.

The assay is able to achieve an R2 of greater than 0.99 and a PCR efficiency of 100 +/- 10%, (Figure 1). Regarding the precision, as outlined in our USP, the requirement is to meet less than 30% CV for the quantity values. The kit is able to achieve this comfortably, with less than 30% of the back-calculated quantity percent CV. The LOD is 30 femtograms, the LOQ is 300 femtograms, and the assay range is 300 femtograms to 3 nanograms. The assay range was evaluated in multiple gene therapy matrices derived from bioreactor at harvest, samples from after chromatography, and samples after final purification.

## FIGURE 1

**Specifications of the quantitative Sf9 and baculovirus DNA kit.**

**01 Comprehensive product solution**
All-inclusive kits with genomic DNA standards and all reagents

**02 Rapid testing and streamlined workflow**
Time-to-results < 5 hours with optimized sample prep

**03 Ultrahigh sensitivity**
0.3 pg LOQ for Sf9 and Baculovirus DNA

**04 Reliable performance**
Reliable results across multiple stages in the gene therapy manufacturing process

| Specification | |
|---|---|
| Linearity | $R^2$>0.99 |
| PCR efficiency | 100% +/-10% |
| Precision | ⊠30% back-calculated quantity CV |
| Limit of Detection (LOD) | 30fg |
| Limit of Quantitation (LOQ) | 300fg |
| Assay range | 300fg to 3ng |

| Gene Therapy Matrices tested | |
|---|---|
| Sample derived from a bioreactor at harvest | ✓ |
| Sample after chromatography | ✓ |
| Sample after final purification | ✓ |

## High sensitivity & broad dynamic range

The broad linear range provided by the assay allows for testing of a wide range of Sf9 and baculovirus DNA samples (Figure 2). This is particularly important for rigorous process characterization studies used to assess the impact of the small process changes on the residual DNA content.

## Workflow integration

All Thermo Fisher resDNASEQ assays use the same workflow as illustrated in Figure 3, which involves extraction of the nucleic acid material from the test articles using the PrepSEQ sample preparation kit, followed by the detection and quantitation using the redDNASEQ kits on a QPCR instrument. The sample preparation can be performed manually or in a semi-automated manner using the Pharma Kingfisher™ Flex instrument, allowing flexible throughput of ~10–500 samples per week.

The qPCR instruments we recommend for this type of testing are the 7500 Fast, or the QuantStudio™ 5 PCR instruments, because they are compatible with the multiplex nature of the assays, and provide the ability to integrate the AccuSEQ™ software. This allows for fully automated data analysis on a single software in a 21 CFR part 11 compliant environment, and prevents the need to migrate the data into excel or perform other manual calculations.

The PrepSEQ Sample Preparation Kit is a universal kit for all our QPCR-based applications and can be used to extract residual host cell DNA, plasmid DNA, and mycoplasma DNA. The kit was also evaluated for extraction of over 60+ enveloped or non-enveloped viruses that contain single or double-stranded DNA or RNA. The kit has demonstrated robust extraction efficiency from samples containing simple to complex matrices, including ones with low pH, high salt, and high protein concentration.

It is important to note that while this assay may be able to run on other real-time PCR instruments, we have performed the validation using AccuSEQ software that is only compatible with the 7500 Fast and QuantStudio 5 PCR instrument. The AccuSEQ software is designed specifically for these PCR instruments and assays to enable compliance with


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21 CFR part 11 features. These features are security, audit, and e-signature capabilities. Any adjustments tools or changes to the data are recorded and audited so that everything is fully traceable.

## DETECTION & QUANTITATION OF SF-RHABDOVIRUS

Rhabdovirus was first identified in Sf9 in 2014 by the FDA Center for Biologics Research and Evaluation [1]. It was then independently reported by other institutes, suggesting that it is a universal contaminant of Sf9 cells.

The virus is a 13 kb negative sense strand RNA virus, belonging to the rhabdoviridae family. Variants have been reported within Sf9 cell lines, and the Sf21 parental cell line, but they are largely conserved.

Studies have suggested that the virus is non-infectious to mammalian cells, and thus does not replicate in human cell lines. However, the safety data is quite recent, and the long-term effects of the virus are still unknown. Therefore, there is a compelling reason for this virus to be monitored for its absence in bioproduction using Sf cells. In the publication on discovery of the rhabdovirus by the FDA, the authors emphasize the need to demonstrate the absence of Sf-rhabdovirus in manufactured biological products at different stages of manufacturing.

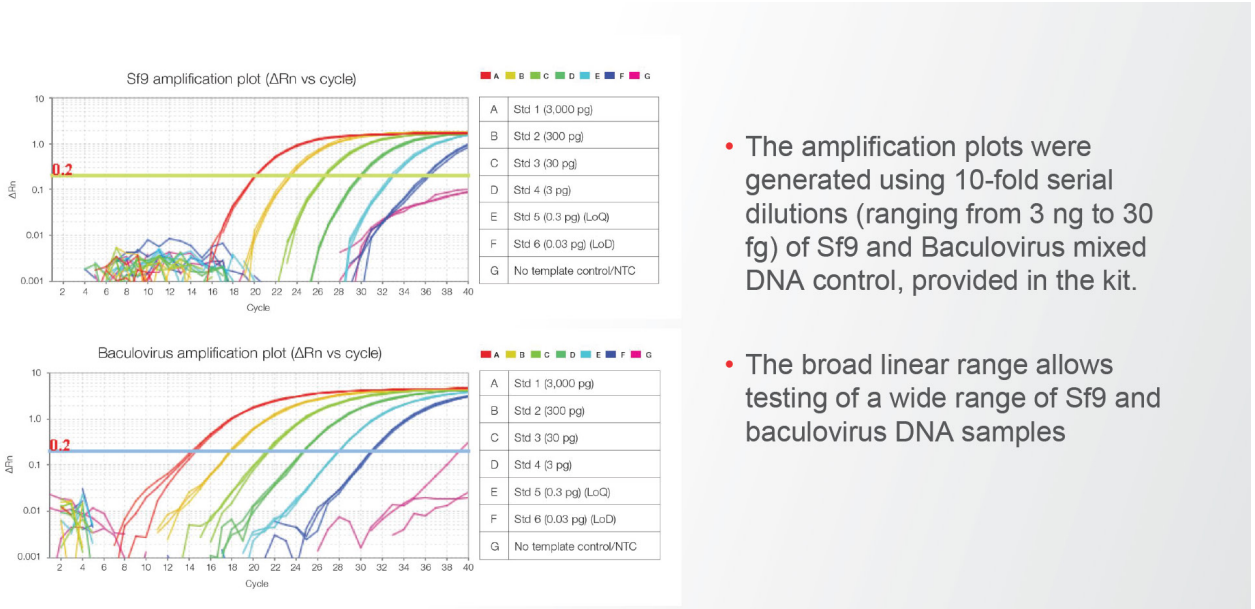
Based on FDA Q5A viral safety guidance, an appropriate virus testing program in assessment of virus removal and inactivation is required for biological products. Similarly, adventitious viral agent testing is required by the FDA under human genome therapy INDS guidelines.

Virus safety is a critical aspect of biopharmaceutical production, and relies on the well-established principles of prevent, detect, and remove to assure drug safety for patients (Figure 4). These principles are the foundation of every viral safety strategy.

The testing strategy for Sf-rhabdovirus also follows these principles. Given the wide variety of samples that may require testing, it is important to create a rapid and robust assay that is rapid, and most importantly suitable

**FIGURE 2**

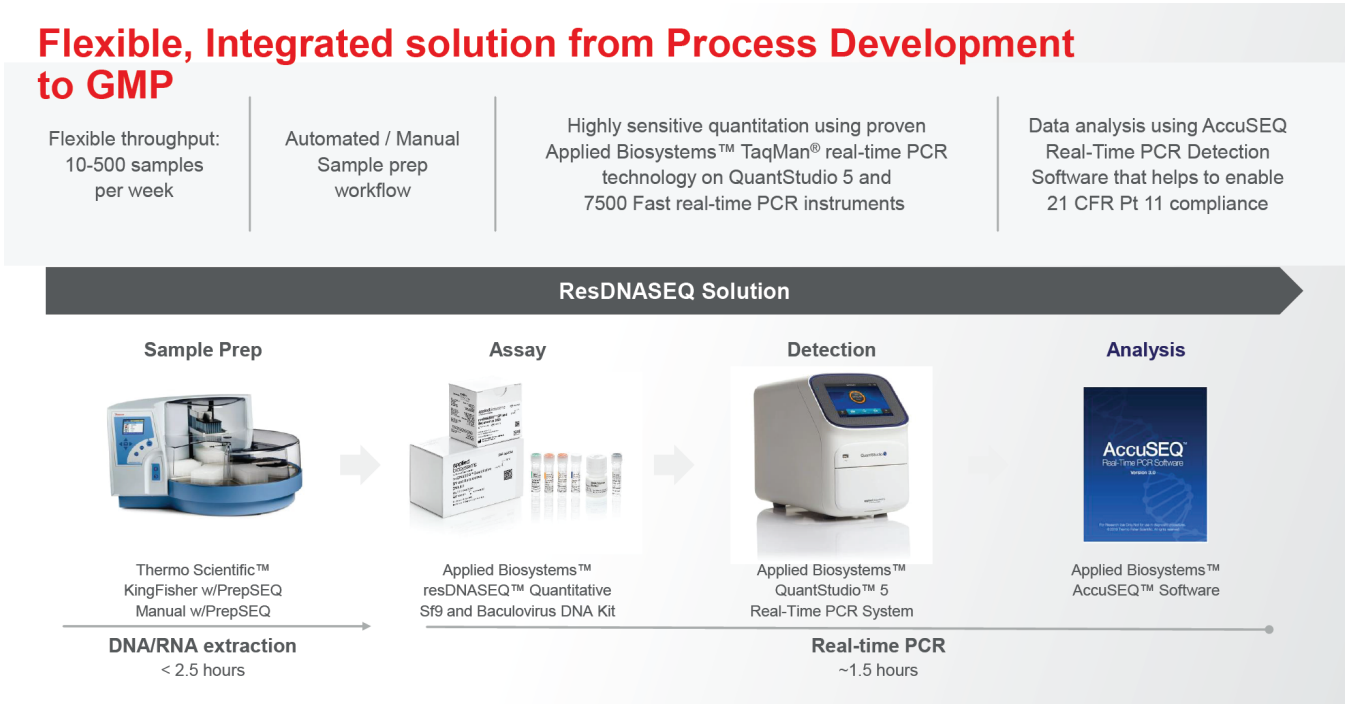
**High sensitivity and broad dynamic range.**




Figures A and B show amplification plots for both Sf9 and baculovirus using ten-fold serial dilutions ranging from 3 nanograms to 30 femtograms of the mixed DNA control provided in the kit.

**FIGURE 3**

Representative workflow of a Thermo Fisher resDNASEQ assay.




for various stages of the biopharmaceutical production process.

The ViralSEQ™ Quantitative Sf-Rhabdovirus Kit intends to address the requirement for fast and sensitive quantitation of Sf-rhabdovirus genome RNA. Coupled with the PrepSEQ nucleic acid sample preparation kit, the ViralSEQ Quantitative Sf-Rhabdovirus Kit is compatible with samples from various stages in the manufacturing process.

Similarly to the quantitative Sf-baculovirus resDNASEQ kit, the Sf-rhabdovirus kit follows a workflow from sample extraction to real-time PCR readout, with report generation and regulatory compliance compatibility. The main difference is that a reverse transcription step is required prior to real-time PCR (RT-PCR) detection. We recommend the ABI Veriti™ 96-well Fast Thermal Cycler in our validated workflow.

The RT-qPCR workflow is a two-step process. In the first step of reverse transcription, genome strand-specific primer will reverse transcribe the RNA. The primer incorporates a non-viral tag sequence to the cDNA. In the qPCR step, the primer specifically amplifies the products from the tagged cDNA test line for specific detection of the genome RNA (Figure 5).

## Validation summary

Validation was conducted by six operators over two locations in Singapore and the USA, using three validation lots over 5 days. Two separate preparation methods were used; manual sample preparation and automated sample preparation with KingFisher Flex using the PrepSEQ kit. The real-time PCR was evaluated on the ABI 7500 Fast Real-Time PCR, and the QuantStudio 5 Real-Time PCR Instrument.

Together, 79 runs were analyzed for the validation study. The main performance criteria assessed were:

- PCR efficiency
- Linearity
- Range of the standards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- Precision
- Reproducibility
- Limitation of quantitation (LOQ) / limit of detection (LOD)
- Specificity
- PrepSeq spike-recovery

We observed that the standard curve has a good PCR efficiency of close to 100%, and a good linearity of $R^2 > 0.99$ More importantly, we found that the result is reproducible at both the US and Singapore test sites. In terms of sensitivity, we found that the assay is robust, from 10 copies to 107 copies, with an LOQ of 30 copies per reaction, and an LOD of 10 copies per reaction. The LOD is cut off at above 95%.

We evaluated for inter-variable precision across plates, including inter-operators, inter-lot, inter-instrument, and inter-day analyses. In general, the average back-calculated percentage coefficient of variation was less than 30% across all standard dilutions down to 30 copies.

The detection of viral mRNA may undermine the accuracy of the tests in detection of truly infectious viral copies. Therefore, we designed the assay to only detect the genome RNA specifically. To test for genome RNA specificity we tested 107 copies of positive-strand RNA, which are representative of the mRNA strands. We found that despite the high copy numbers, the cycle threshold (CT) is either undetected, or above the LOD, suggesting that the assay is highly specific towards the genome RNA strand, which has a much lower CT value.

We tested for exclusivity of the assay with a panel of genomes from different species or cell lines, and observed non-detection of amplification with the exclusion panel, suggesting that the assay is specific to rhabdovirus.

The recovery of spike controls with the PrepSEQ nucleic acid kit, manually and on

**FIGURE 4**

**Viral safety assurance strategy for assuring the absence of Sf-rhabdovirus in manufactured biological products.**

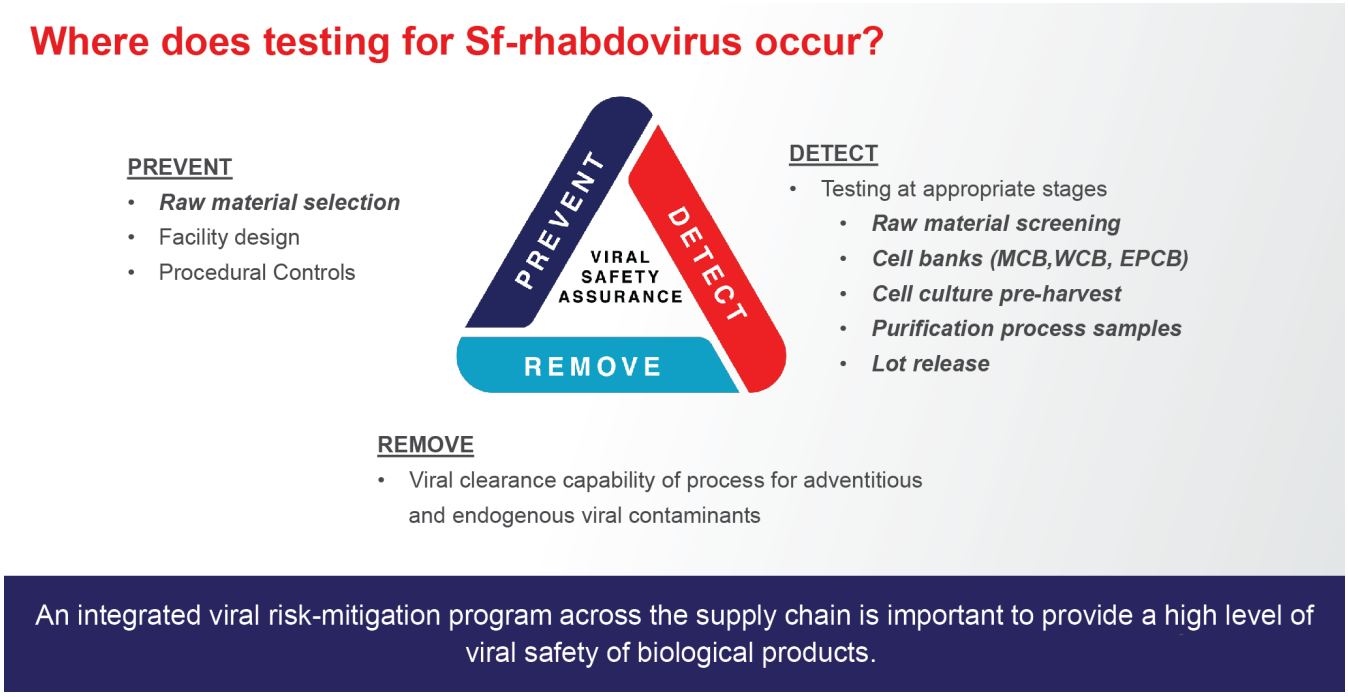
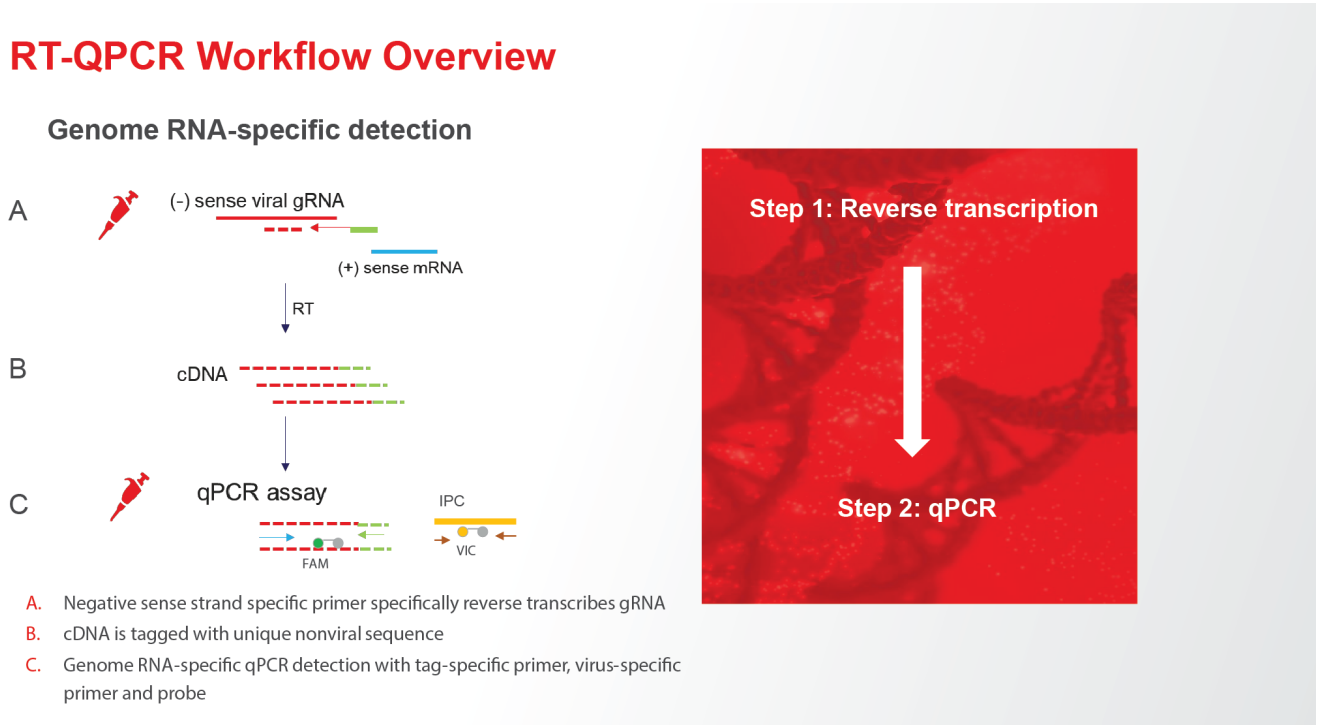



Figure sourced from [2].

## FIGURE 5

RT-qPCR workflow overview.




A. Negative sense strand specific primer specifically reverse transcribes gRNA
B. cDNA is tagged with unique nonviral sequence
C. Genome RNA-specific qPCR detection with tag-specific primer, virus-specific primer and probe

automated platform KingFisher Flex, was examined. RNA controls were spiked at different varying concentrations from 500,000 copies to 500 copies in the presence of a matrix emulating the final bioprocessing purification. A good recovery of between 70 and 130% for all concentrations was observed in both manual and automated sample preparations. Different intermediate bioprocessing matrices were also tested during the RNA verification study. These matrices include constituents from the bioreactor at harvest, and after chromatography. Recovery was between 70 and 130% for all matrices through the KingFisher platform.

PCR inhibitors are often a concern in assay accuracy, so sample preparation with inhibitors that are commonly found in bioprocessing was studied: benzonase, often used in DNA degradation, tween-20, a detergent, and LV-Max, a cell culture medium. The results showed no significant difference in CT value or recovery between assay detection in PBS versus the inhibitors after our sample preparation. This shows that the sample extraction by the PrepSEQ kit is sufficient to minimize the inhibitors, and thus not affect the accuracy of assay detection.

## INSIGHT

The Sf9 baculovirus expression system is an effective alternative to mammalian substrates for commercial production of AAV and other biologics. It has unique advantages for AAV production, including easy scale-up, growth in serum-free media, and no need for helper virus or plasmid raw materials for AAV production. It also comes with its own analytical challenges, including the need to demonstrate clearance and quantitate residual Sf9 host cell and baculovirus DNA, and Sf-rhabdovirus contaminant.

Thermo Fisher Scientific has simplified these challenges by staying attuned to emerging regulatory guidance and developing solutions, from sample preparation to analysis, designed to meet regulatory guidelines. We have developed solutions for residual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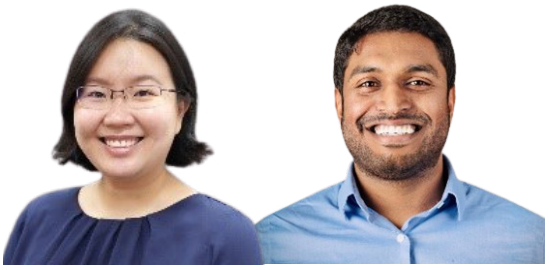
DNA testing, with a highly sensitive and specific duplex qPCR assay for the simultaneous quantitation of residual Sf9 host cell DNA and baculovirus DNA. The ViralSEQ Quantitative Sf-Rhabdovirus Kit provides a highly sensitive and specific quantitation of Sf-Rhabdovirus via a strand-specific qPCR assay with a high dynamic range.

## REFERENCE

1. Ma H, Galvin TA, Glasner DR, Shaheduzzaman S, Khan AS. Identification of a novel rhabdovirus in Spodoptera frugiperda cell lines. *J. Virol.* 2014; 88(12): 6576–85.

2. American Pharmaceutical Review.

# ASK THE AUTHORS



**Yi Fang Lee**
R&D Scientist, Thermo Fisher Scientific

**Srinath Kashi Ranganath**
Staff Scientist – Field Applications, Thermo Fisher Scientific

**Q** Does the validation data you provided in the presentation eliminate or shortcut the validation process required for regulatory acceptance of use of this assay in the workflow?

**SKS:** This is a very commonly asked question in this field. What we have covered here proves that the assay is validatable in simulated sample matrices. However, it is important that customers validate the assay in-house, using the actual sample matrices – particularly the ones that they set the item specifications on.

To summarize, you could leverage some information from our validation. For example, the specificity of the primers remains the same, regardless of whether you test that in PBS or

whether you test that in your sample matrix. Some of the components may be leveraged, but things like LOQ, looking at spike recovery, and those kinds of parameters must be evaluated in your sample matrices that are representative of the manufacturing process at your site.

**Q** What did you use as the standards for the residual qPCR assays?

**YF:** For the rhabdovirus, we are using in vitro transcribed RNA covering the assay region. As for baculovirus, Srinath would you like to speak to that?

**SKS:** For Sf9, it is the Sf9 genomic DNA extracted from Sf9 cells. The baculovirus cannot live by itself, so that was cultured in Sf9 cells, followed by subsequent extraction of the baculovirus from that mix – so both genomic DNA.

**Q** A question from the audience: we used the Sf9 rhabdo-free cell lines and media. How would implementing this assay be useful for our process?

**YF:** Potential Sf rhabdovirus contamination has been expressly noted by the US FDA as a potential concern. So even though its safety and priority is still unknown, we know that reinfection of Sf-rhabdovirus cell lines has been shown to be possible.

Therefore, we encourage testing for Sf-rhabdovirus in at least the master cell bank, the working cell bank, and bioreactor points of the workflow, in a Sf-rhabdo-free Sf9 expression system. This will act as a risk mitigation strategy to monitor and ensure that these cells remain Sf-rhabdovirus free.

This will also strengthen the regulatory requirement or quality of a robust virus testing program for the Sf expression system.

**Q** Can the duplex Sf9 baculovirus ResDNASEQ kit be used to measure just baculovirus res DNA?

**SKR:** This is a problem that I encountered when I was an analyst executing the assay in the lab myself.

The assay is designed as a duplex assay. But if you get results that pass for the Sf9 target but do not meet criteria for the baculovirus target for any reason, then you are able to just run baculovirus by itself. So you can run the assay singleplex or duplex, and our validation data shows that you're able to generate similar results using either approach.

**Q** You mentioned that both assays can be run in a manual or semi-automated method. Could you please outline or quantify the advantages of the semi-automated method over the manual?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**SKR:** I have personal experience running both methods. One of the main advantages is having a shorter hands-on time, allows you to do other analyses. It also gives you the ability to spend more time on data analysis.

The other advantage is the higher throughput. You are able to extract up to 96 wells on the KingFisher Flex instrument. When you're processing the samples manually, you are working with the 16 position magnetic racks, so you can only work with 16 tubes at a time. Any more than that can get really tedious and might affect the accuracy, precision, and consistency of the data.

Another advantage of KingFisher is that it requires very little training. So when there is employer redundancy, new hires, or you're trying to scale up and you want to bring on more people to run the methods, KingFisher makes it very easy.

With a manual method you have to train each analyst in a very skillful way so that you see very little variation amongst different analysts.

I have also found that the KingFisher flex results in better and more consistent recovery in general when compared to manual methods.

**Q** Could you please go over the validation data for the rhabdovirus kit? Did you evaluate the specificity of the kit for other species of RNA and DNA?

**YF:** We have validated an exclusion panel of different species, including *E. coli* and mammalian cells. We did not detect any amplification of the rhabdovirus in the panel of different species. For the validation data, if you would like to have more details feel free to reach out to our team who can provide more details

**Q** What is the ratio of full versus AAV empty particles produced in insect cells?

**SKR:** This isn't directly related to the content we have presented, but I can touch on it. From my understanding of the process, and some of the articles and literature, it seems like the packaging efficiency is similar or better than some of the other mammalian cell-based viral vector production systems. It also depends on the particular rAAV serotype, as it's not the same for every serotype.

I have seen packaging efficiencies of greater than 90% using the Sf9 baculovirus system.

**Q** Are insect cells and baculovirus DNA immunogenic in humans?

**SKR:** Again, the literature shows that, for example, vaccines produced in using the baculovirus system have greater immune response. But is the DNA itself

immunogenic to humans? There is very little in the literature that talks about the immunogenicity of the insect cell and baculovirus DNA.

One thing we have shown is that the viruses that are commonly found in the Sf9 cells do not replicate in humans, or do not infect humans by themselves. So that is one of the advantages of using this system.

## Q How much of a concern is rhabdovirus contamination in insect cell production platforms?

**YF:** The rhabdovirus has been shown to be reported in different Sf cell lines by various different independent laboratories, so I think that it is a universal concern.

It's non-infectious to human cell lines, but because it is quite a recent discovery, the long-term immunogenic effects are still fairly unknown, so we are still advocating for testing of rhabdovirus.

## Q Could you share the protocols for residual Sf9 bac-DNA for harvest, process intermediates, and final drug substance?

**SKR:** In general, most of our ResDNASEQ methods are geared towards using a single protocol for all sample types. That is how I recommend you design your protocols in order to have a fail-proof method when you transfer it over to your quality group.

The way we do that is somewhat of a unique approach. I think it might best help if you work with your FAS, in order to optimize your samples to get the optimized protocols that are as close to final stage as possible, and then evaluate that method.

In general, the protocol that we start out with is what is listed in the user guide. But I understand some specific sample types, especially things that start in the harvest stage, might require some optimization. The process intermediates and the final drug substance usually share the same end protocol.

## Q What is the minimal sample volume required for each of the tests discussed?

**SKR:** For the Sf9 baculovirus, if you are testing a sample neat, and testing it by the USP method: triplicates unspiked and triplicates spiked, you need exactly 600 microliters. Our protocols start with 100 microliters per vial in the KingFisher, or 100 microliters per tube in the manual method.

So regardless of the method you use, you need exactly 600 microliters if you are testing neat. But it is always good to have retains in case you need to repeat the assay.

**YF:** It would be the same for the rhabdovirus. In terms of reaction and volumes, we recommend 8 microliters for the extracted samples.


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## Q Is there any cross-reactivity between Sf DNA and baculovirus DNA when using the Sf9 Baculo ResDNASEQ kit?

**SKR:** To address one of the other questions I mentioned that the baculovirus DNA is produced in Sf9 DNA, and the fact that we are multiplexing the assay to be able to detect Sf9 and baculovirus targets in the same vial.

It is important that we do not have any specificity issues as they relate to the primers themselves, so we have evaluated that. When we spiked baculovirus DNA and amplified using the Sf9 primers, we saw a signal below the lowest standard, which told us there is no non-specificity towards each other within the assay. That was one of the most important parameters we looked at when we first designed the assay. There is no cross-reactivity.

### BIOGRAPHIES

**Yi Fang Lee**
**R&D Scientist, Thermo Fisher Scientific**

Yi Fang joined ThermoFisher Scientific as an RND Scientist since Jan 2020. She has led the development of the ViralSEQ Quantitative Sf-rhabdovirus assay. Her previous work experience was in cancer biology and assay development for circulating tumor cells in a microfluidics-based system. Yi Fang received her PhD education at the Genome Institute of Singapore, majoring in oncogene discovery from transcriptome- wide studies. She has published journals on cancer biology, sequencing and microfluidics systems.

**Srinath Kashi Ranganath**
**Staff Scientist – Field Applications, Thermo Fisher Scientific**

Srinath is a Staff Scientist – Field Applications with the Pharma Analytics group at Thermo Fisher Scientific, supporting customers in implementing, optimizing and validating the Pharma Analytics workflows for biomanufacturing processes across various therapeutic modalities. Prior, Srinath served as a Bioassay Scientist and an SME for the development and optimization of assays for residual DNA and other process impurities for 6 years. Srinath has an MS in Pharmaceutical Sciences from Campbell University, NC. His thesis is focused on understanding the expression profile of certain intracellular signaling molecules and how altering their function will affect the downstream cell signaling.

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## AUTHORSHIP & CONFLICT OF INTEREST

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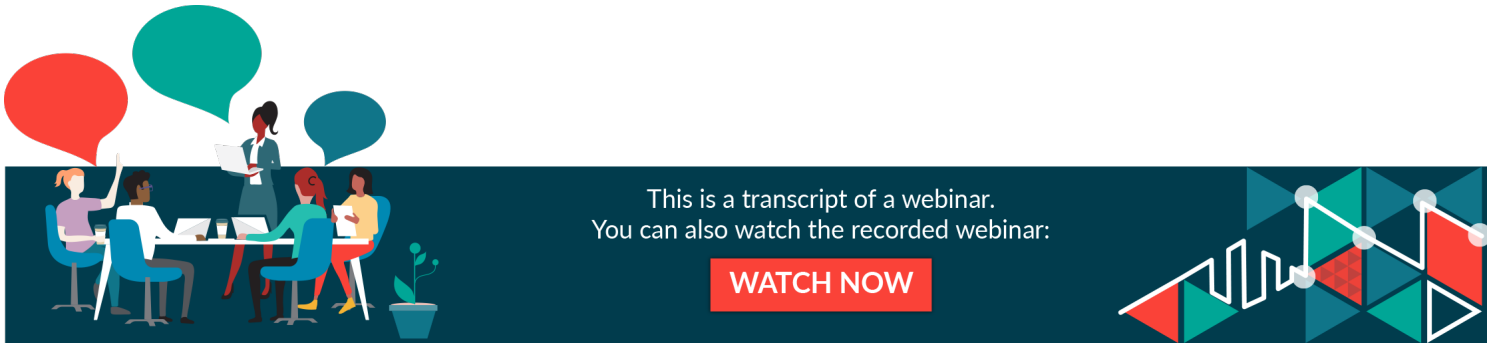



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SPOTLIGHT

PODCAST INTERVIEW with:

**Rachel Legmann,** Director of Technology, Gene Therapy, and
**Joe Ferraiolo,** Associate Director Bioanalytics, Repligen

# Advancing real-time monitoring of AAV vector processes

*Cell & Gene Therapy Insights* 2021; 7(9), 1183–1194

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**Q** How can we make vector bioprocessing faster and more cost-effective – and improve the identification and measurement of critical quality attributes – by harnessing cutting edge analytical tools?

**RL:** In order for us to understand how we can make vector bioprocessing faster and more cost effective, it is important to firstly touch upon what are the main technical challenges that currently exist in developing a robust viral vector on time and on budget.

One major challenge is that the process development cycle time is long. When the process development team requires many development cycles for achieving a good process, consistency, good compatibility between the development lots, and optimal product recovery yield, then there is a major delay in proceeding to reliable and low-risk scale-up engineering runs followed up with manufacturing runs.

The current analytical methods for evaluating the AAV titer – for example, an empty-full capsid ratio, during the final steps of purification and during lot variability assessment, a stability shelf-life study, and a lot pooling strategy – are time consuming and do not accurately inform about product critical quality attributes. When you have limited process control, your process is at high risk, and you cannot make the appropriate decisions because you are walking slowly in the dark with minimal visibility to identify what is the true optimal operational design space.

To expedite your development time and improve your purification recovery yield and process consistency, there is an urgent need for robust titer and empty-full ratio data generated in real time, and with faster turnaround time. It should ideally be possible to process a large number of samples with no sample manipulation required.

Currently, more and more gene therapy and vaccine companies want to maintain independence with their supply chain of raw materials for viral vector and mRNA-based products, such as plasmid. Having rapid and reliable titer and purity assays for the starting material is very important for good critical quality attributes of the product.

"The current analytical methods for evaluating the AAV titer ... are time consuming and do not accurately inform about product critical quality attributes."

- Rachel Legmann

**Q** Where are the key throughput-related issues for vector manufacturing assays/analytical tools currently? And how to address them moving forward?

**JF:** Currently, much of the work being done with in-process testing requires


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wait times and hold up times while the material is being processed, which unfortunately doesn't expedite the flowthrough of the actual product itself. What we are looking to do is to give scientists a tool to be able to analyze their samples in real time, without any sample manipulation, so they can understand exactly where they are in relation to the process. We are more focused here on giving the scientists the tools to make real-time decisions in the process, without requiring additional time to post measurements, without hold up time, or time to strengthen the analytics technology that exists currently.

**RL:** The main bottlenecks are in the development of the viral vector during the manufacturing process, and having large amounts of samples, which really presents a big challenge to moving forward with accuracy and making the right decision.

The first point of concern is the last two purification steps – the final run, when you have to remove the non-product-related impurities and the empty capsid from your final product – and during the final tangential flow filtration (TFF) step. In these steps you have so many samples with different formulations and this is where you have a bottleneck, because you need real-time data to make a very rapid decision.

Another key point during development is implementing this into a pooling strategy. When you are working on a pooling strategy and you are not processing the whole production harvest at one time, but instead splitting the harvest into multiple lots, then you have multiple samples that you have to analyze simultaneously. However, you must make a decision rapidly in order to avoid losing the batch and you must check the consistency of each lot prior to the pooling – that is critical.

There are further bottlenecks in development relating to measuring consistency of multiple development lots. For example, a customer running multiple development lots will need to know the optimal time to move forward with the engineering run in manufacturing. At that point you have multiple samples that must be analyzed really quickly and in the manner Joe described. And finally, when you come to define the many conditions affecting product shelf-life for the best possible formulation, storage, and shipment, you once again have multiple samples and another real bottleneck to deal with.

**JF:** Just to give a little context to Rachel's comments, here's how that would work in the real world.

One of the ways in which we are able to monitor the process is by using a UV-Vis method called Slope Spectroscopy. It is not an absolute absorbance-based technology but is more a technology that looks at the change of absorbance over multiple path lengths of interest, generating a linear slope progression in order to calculate just how accurate that slope is to replace absolute absorbance measurements with a slope regression and R2 value.

The SoloVPE® is capable of undiluted sample analysis in less than one minute. This enables the Slope Spectroscopy method to provide a process feedback control that allows for real-time decision making.

The ability to test samples without the need for any manipulation or sample dilution allows for a highly accurate reading of that sample made at the time of measurement. These slope values can then be used to assess where particular batches are within a process, and allow the scientist to make real-time decisions based on which samples continue through the process, which are

pooled with other processes, and which are potentially held at time, allowing further processes occurring in the future to be added to the particular lots going out for continued analysis.

**Q** What tools are available for measuring the impact of various AAV vector engineering methods on the capsid and its transfection profile – and what do these data mean for vector manufacturing at large?

**RL:** As we all know, the adeno-associated virus (AAV) is the most popular viral vector used in gene therapy today. Currently, there are four main workhorse techniques to measure AAV titer and empty-full capsid ratio: digital droplet PCR (ddPCR), ELISA, transmission electron microscopy, and analytical ultracentrifugation. ddPCR is precise but has a smaller dynamic range, therefore requiring exact sample dilution. Other methods that are currently being evaluated in the gene therapy market include high-performance liquid chromatography (HPLC), capillary electrophoresis, dynamic light scattering, and traditional UV-Vis spectroscopy.

Traditional UV-Vis spectroscopy utilizes a standard UV-visible spectrophotometer that uses a 1 cm fixed path length. When analyzing samples that are outside of the concentration range of the spectrophotometer, it is necessary to dilute or perform other manipulations on the sample in order to obtain a reliable reading. Careful manipulation of a sample can take between 30 minutes and 3 hours, depending on the operator and their expertise. And more importantly, it increases the risk of error in the final measurement data.

Essentially, data that are processed immediately allow more rapid process understanding and a design of experiments (DoE) approach that can potentially reduce process development time for titer impurity measurement for multiple drug modalities, including proteins, plasmid, and AAV. However, none of the aforementioned methods deliver results quickly or easily, so that they can be implemented in real process time. This impacts process control ability and creates process risk, which can cause complete shutdown, and most unfortunately of all, impact the patient who is looking to receive a safe drug.

The problem in the gene therapy market remains the fact that analytical tools are insufficient due to high variability (of between 20–40%), low throughput, and a lack of capability to execute in real process time. Therefore, there is a great need for a better in-line process control, offering quick and direct total viral vector analysis with high dynamic range during development to enhance throughput and improve decision making. Real-time monitoring will reduce the risk of batch loss by eliminating the dependency on offline testing and indeed, there is a tremendous ongoing effort in the field to decrease labor costs through in-process tools that allow real-time measurements. The SoloVPE system is one such solution that addresses the disadvantages of current analytical methods.

**JF:** When we started exploring our methodology for Slope Spectroscopy to see if it would be applicable for AAV, one of the things that really startled us was the average 20–40% acceptance range that is currently tolerated by the industry. This was a world that was not well known to us: when we talk about either high concentration

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monoclonal antibody, plasmid purity, or oligonucleotide concentration, we are typically used to working with groups that are looking at under 5% variability.

We wanted to understand how and where a UV-Vis technique could potentially offer a solution to the industry – to leverage our experience gained and the progress we've made in reinventing how UV should be done to see if we could potentially influence lowering that range, increasing the throughput due to no sample manipulations or dilution, and getting rid of the variability within the measurement itself.

"...the technology has now advanced to the point where Slope Spectroscopy represents a better analytical tool for providing real-time feedback, and eliminating the wait times..."

- Joe Ferraiolo

It is important to emphasize that we are not looking to remove or replace those current analytical methods we described earlier – that is not a viable approach. However, through collaborations with our customers, the technology has now advanced to the point where Slope Spectroscopy represents a better analytical tool for providing real-time feedback, and eliminating the wait times associated with the current process to influence how quickly or slowly the process goes through step-to-step changes.

**RL:** Indeed, the great correlation between the SoloVPE and ddPCR/ELISA (the two separate methods that between them form the gold standard in the market right now) demonstrates that this system enables real-time process decision making, thereby mitigating risk for pooling strategy and improving the overall decision. So again, it's something that can be used not instead of the standard analytical tools approved by the regulators, but specifically to deal with multiple samples.

**Q** Can you provide an overview of current Repligen analytical solutions for therapeutic product quality attributes – what are the current limitations, what constitutes acceptable variability, and how we can get better in this department?

**JF:** When Slope Spectroscopy was invented, it was born out of frustration with the current methodology available at the time. Any UV-based technology developed in the last 30 or 40 years has been based on a 1cm path length. If the sample happens to fit within the linear range of that spectrophotometer, it is possible to make the measurement; if not, it is a case of having to introduce sample prep and dilution error to the potential assay. So, the goal was to provide a platform solution regardless of concentration of sample. When compared against other fixed path length technologies, even though the path length might be smaller, it still relies on that sample fitting within the linear range of the given path length to make the measurement. So unfortunately, it still has the same problematic issues as traditional fixed path length UV at 1cm.

> "...the SoloVPE system can transfer more easily into GMP environments where a trained scientist may not be operating the system."
>
> - Joe Ferraiolo

Fundamentally, the SoloVPE or FlowVPX® technology establishes the best linear regression fit within Beer Lambert's law to provide a slope value which is paired with an R2 value of three 9s or higher, assuring accuracy in the measurement. What we have essentially done is taken spectroscopy and made it into a pass-fail method based on the R2 criteria of three 9s or higher.

With proteins and plasmids, and any DNA/RNA, we are looking at what is essentially a two-wavelength measurement, at 260 and 280 nanometers, and the system calculates concentration by either taking these measurements by themselves or taking a ratio of the two. Most plasmids and oligonucleotides are highly concentrated and very viscous. The only action the scientist has to take is pipetting their sample (undiluted and with no sample prep) into one of the SoloVPE vessel and pressing the 'Start collect' button. The software is completely automated and will generate that linear curve based on multiple path length reads within the measurement. This is obviously very quick – each measurement takes about a minute to run – and you get a real-time picture of exactly where that process is.

More importantly, the SoloVPE system can transfer more easily into GMP environments where a trained scientist may not be operating the system. It may be a lab technician instead, who has no fundamental idea of what Beer Lambert's law is, but who is more than capable of pipetting sample into a cuvette and running the measurement on the SoloVPE, because there is no sample preparation or dilution required.

We typically leverage plasmid purity, and anything related to high concentration nucleotides, because of the ease of transfer. Once the method is validated, it's easily transferred to sister sites or contract manufacturers using the same slope method.

We are not only interested in helping one part of the process. We are keen to find out where Slope Spectroscopy becomes the platform from company to company and from site to site, and achieves a repeatability of <+/-2%. This to our minds is what the platform approach means for the industry: to not only expedite the way that data goes from one lab to another, but to reduce the amount of time and error associated with each method of transfer.

Regarding AAV process monitoring and screening, it is about getting away from some of the theoretical values that have been published (or not published, in some cases) and addressing the fact that there is no real, fundamental, defined method for this analysis. All we know for sure is the two wavelengths of interest when we are talking about UV, where the measurement should be made. In our case, we take a dual wavelength slope ratio, which essentially becomes our R value, our ratio. That OD value can be used as a tool for process monitoring, pooling applications, or final concentration.

There are some published extinction coefficients available for the AAV material, so we are now talking about leveraging a one-minute method that can be locked down in GMP environments to calculate concentration, as well as monitoring any steps post-chromatography.


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**RL:** From the process development perspective, when I am producing a plasmid using *E. coli* fermenter, I like to use the system on the upstream side, when you are actually using the OD600 in order to rapidly measure the density of the microorganism with no dilution. It is a major benefit not only for the evaluation of the quality of the produced plasmid, but also for the option of very rapidly monitoring the growth during the fermentation process.

**JF:** That is one of my favorite applications to work on, not because of the type of sample but more for the benefits that using this technology gives to the process.

One of the challenges that currently exists with OD600 is the fact that you always have continued cell growth throughout the entire process. One can imagine that if a scientist or analyst is trying to leverage traditional UV-Vis method, they would have to pull samples at the time of measurement, and they would then have to potentially store those samples, dilute them, and then report back the measurement. And while all of this is happening, the cells are growing, so it's a bit like chasing a moving target. With the at-line version of the SoloVPE System, it's as simple as pipetting a sample from the process into the system, undiluted and with no sample prep, and then you have your measurement in less than a minute.

With the FlowVPX technology, which is our in-line solution for Slope Spectroscopy, this process becomes even more robust and is also simplified. It also allows us to leverage the kinetic software to assign the time points when we would like the analysis to be made. Everything is completely automated, so there is zero sample manipulation. Essentially, you are watching concentration over time within your process.

For AAV empty-full correlations, we have done a lot of work with companies that have certified standards for AAV. We have leveraged these standards samples to test and compare comparability studies conducted using SoloVPE System and qPCR, which is the current technique being used. There is excellent agreement between both techniques, meaning the technology can not only be used to leverage process monitoring – if it appears within the given range of about 1 e+11 and higher, we can assess concentration as well.

## Q How to drive further improvements in AAV vector process analytics?

**RL:** Coming from the monoclonal antibody world and moving into a far more complex product such as AAV, I would love to improve the process in several places. It is really exciting to see if I can use this platform (or any other platform) in order to get better production and performance of the process, which means having a better yield and a better purity of my product.

One of these points is in the purification steps with chromatography. Obviously, people are using the regular UV spectroscopy, but it's very important to have a more reliable and rapid tool with lower standard deviation – a quick result from which to make the right decision. I'm talking about the final chromatography run, which is the critical point where you are separating the empty and full capsids. And we all know that it's not only important to have the population with a certain amount of full capsids. It's also important that it is consistent – that

every time we are getting the same thing. Otherwise, we cannot move forward. This tool will help us to be able to develop and rely on the best final chromatography process.

As mentioned earlier, I would also use the system for the final process step before fill-finish (TFF), when it's also very important to know the titer and the impurities of the empty-full capsid ratio. And product stability is a further point of application, in order to measure and monitor the product under different extreme conditions to identify the best shelf-life. I would definitely use it for lot-to-lot variability, too – both in development and in the manufacturing process.

As Joe mentioned, the system is not going to replace ddPCR and ELISA, or maybe future accurate tools. But it will certainly help us to understand the consistency and the reproducibility of the process, in addition to the other assays that we will be doing at the end on the final product. It will also establish robustness through conducting a real-time risk assessment for many engineering runs, and that will significantly shorten the validation time. That means better performance for the process itself as well as the monitoring of it. And our pooling strategy, as we mentioned, is mitigating the risk of batch failure.

Lastly, I would like to see the improvement delivered through using a rapid analysis tool in the quick testing of samples before the drug substance formulation. Off-line measurements are of course required, but you do need to have a degree of consistency between all the drug substances you are analyzing.

**JF:** The current methodologies that are used all come with their own sets of issues. But the common thread that runs through all of this – and especially in relation to what our customers need – is that it's not just about being quick, it's also about being accurate.

Unfortunately, a lot of the current technologies out there don't cover both. That is really the need we are trying to address. We know the limitations of UV-Vis and what it can and cannot do, but in the places where it can be leveraged, the technology now exists to provide the marketplace with a better tool for the job than those that are to hand. Whether it's monitoring the process steps or implementing the system in a GMP environment where the final release test has been done with the technology, we can provide the industry with a better tool that does go after both speed and accuracy.

What we try to achieve with this is not just to claim the technology is good for literally anything you can think of that has a UV chromophore, but it's understanding where that tool is best deployed within a process, and giving our customers a better way to leverage the information from the analytics to make better decisions in a much faster timeframe.

## Q How is Repligen driving innovation of in-line process analytics?

**JF:** Slope Spectroscopy has been a game changer within the industry. I mentioned earlier that there hadn't been significant progress in the evolution of UV-Vis in decades, frankly.

What we have tried to do over the years is not only to understand where the best fit for the technology might be, but to provide our customers with a true solution. As I mentioned earlier, we are not just interested in one area – we like to see where the technology can be implemented, and then give our customers the best support moving forward in how to implement


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the technology at that particular process point. That's the case whether it is in development, where we are just monitoring the process (particularly in the non-regulated environment), all the way through on-the-floor manufacturing and QC, to where the technology is being used for final release – and ultimately, transferred to other companies, sister-sites or contract manufacturers.

Looking forward, we are looking to drive the innovation of the technology in-line – that's really where the technology is headed. Both Rachel and I feel the best use of the technology within the process flow is being able to give scientists real-time information by literally just watching a screen and understanding where they are within a process, related to either concentration or slope optical density readings, in order to make real decisions. And not just making those decisions quickly, but having the backing of each linear regression providing the proof and evidence that the scientist needs in order to have confidence in the values that are being reported by the technology.

I think 'quicker' and 'more accurate' – the buzzwords that are typically thrown around AAV analytics – hold true for this technology. What we're really trying to do is place an emphasis on showing everybody examples of how this technology has been implemented with real evidence, and getting our customers to share that experience with us – whether it's related to turnaround time, cost savings within the lab related to not having to serial dilute samples, or ultimately moving each example into a fully-fledged, in-line process monitoring tool.

**RL:** What is critical for me is that after we have done a long, extensive, and tedious process development, we want to know that this will help us in moving from lab bench scale (where we're doing all the developments that are critical for reducing and mitigating risk) to drug substance, and continuing to progress to a pilot with a FlowVPX and then maybe at GMP

Another key point is the importance of having in-line measurements, especially for product titer impurities. In the world of gene therapy, with AAV vector as a product, it is important for the future when we are going to move into continuous processing, following the footsteps of the monoclonal antibodies. The trend is the same: we want to reduce the cost, so we need to move to continuous processing. And in-line analytical tools will enable this transition.

Even if someone is currently working with a semi-continuous or batch process, inline Slope Spectrscopy is an excellent way to have better feedback control. And it paves the way for the future use of continuous processing for gene therapy utilizing single-use, closed systems, where you can actually do all that analysis inline. This is the dream for everyone who is developing AAV vector-based products currently.

"Slope Spectrscopy ... paves the way for the future use of continuous processing for gene therapy utilizing single-use, closed systems, where you can actually do all that analysis inline."

- Rachel Legmann

**JF:** One final note. We have talked about the fact that the system is quicker, there are no sample dilutions, and it's very accurate

– but the amount of time savings cannot be overstated. Especially, as Rachel mentioned, in relation to high concentrations of product.

We have discussed multiple applications for the technology and in several cases, once the method has been properly developed and transferred into GMP, there are multiple scenarios where you will not potentially need staff on hand 24 hours a day, 7 days a week. A lot of that time is currently spent sending samples out for analysis and waiting for the results, during which time the technicians are literally doing nothing. It is just holding up the process until QC has analyzed those samples and sent them back, determining whether the process can continue or if it needs any type of modification.

We have multiple publications, posters, presentations, and examples from companies that have eliminated some of their shifts from the production area, and that are simply providing a better quality of life for the individuals who are working there.

So again, the faster turnaround time is great, making the product more robust is great. But I think one of the things that tends to be overlooked is the fact that we are not looking to save you a couple of minutes off your day; this technology is designed to take weeks or months off your year, by saving you having to wait around for those processes that are no longer applicable because you are using the SoloVPE or FlowVPX technology to make those decisions in real time.

**RL:** Furthermore, every engineering run in the 200–500-liter scale range can cost almost a million dollars just on the plasmid and other materials. Reducing the cycle time and doing fewer development processes means really significant cost savings.

## BIOGRAPHIES


### Rachel Legmann

**Director of Technology, Gene Therapy, Repligen**


Rachel has more than 25 years of experience in the field of scalable biologics and gene therapy manufacturing of therapeutic products, viral vectors and proteins for gene therapy and biologics. She completed her Ph.D. in Food Engineering and Biotechnology at the Technion-Israel Institute of Technology, Israel. Rachel joined Repligen in 2021 as a subject matter expert leading the global gene therapy organization helping customers achieve their technical and operational objectives in their manufacturing of vector-based therapeutics and vaccines with a focus on gene therapy processes including upstream, downstream, analytics and scalability. In addition to supporting global customers and building high level networks, Rachel is supporting various internal cross-functional activities and external collaborations. Prior to joining Repligen, Rachel held several scientific and leadership roles at Microbiology and Molecular Genetics department at Harvard Medical School, CRO SBH Sciences, Seahorse Biosciences part of Agilent, CDMO Goodwin Biotechnology and Pall Corp part of Danaher.


### Joe Ferraiolo

**Associate Director Bioanalytics, Repligen**


Joe is the Associate Director Bioanalytics for Repligen. He is in charge of the Bioanalytics Applications department related to the SoloVPE Variable Pathlength UV solution. He has been with the company for more than 20 years, with over 10 years of development and validation


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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 biologic drugs, gene therapy solutions, and monoclonal antibodies.


## AUTHORSHIP & CONFLICT OF INTEREST

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# Are you finishing strong in cell therapy manufacturing? Tackling your final fill and finish challenges with automation

Dalip Sethi and Annie Cunningham

Fill and finish is a critical, high-value step in cell therapy manufacturing; failure is both costly for the manufacturer and potentially catastrophic for the patient waiting to receive therapy. This key step is frequently done manually, which poses a number of significant risks. In contrast, the Finia® Fill and Finish System, designed to automate this process, may reduce contamination and labor costs while improving reproducibility and supporting GMP compliance.

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## MANUAL FILL AND FINISH: THE RISKS

In autologous cell therapy manufacturing, production of the therapy begins with collection of cells at the bedside, normally done at a clinical site or hospital. Once the cells are collected, they are cryopreserved and sent for manufacturing, where isolation, washing, modification, and expansion take place. At the end of this process there is a final formulation, fill and finish step prior to cryopreserving the cells for transport back to the hospital for infusion into the patient.

There are two crucial reasons that this formulation, fill and finish is a high-value step in manufacturing. The most important consideration is that these cells come from a patient needing the therapy. The time used for manufacturing is time that a patient spends

## DATA INSIGHTS

- On average, the Finia process more accurately dispensed a 50 mL target volume compared to the manual process.
- There were no statistically significant differences between cell densities and viabilities from the Finia and manual processes.
- Finia required considerably less hands-on and full-time equivalent labor time compared to the manual process.
- The manual process resulted in 15 open events compared to zero open events with the Finia process. Automation considerably reduces contamination risk.
- Post-thaw viability was maintained at greater than 90% for cell products from all three donors for both Finia and manual processes.
- Mean post-thaw viability for Finia samples remained above 90% for up to 48 hours in culture.

waiting for their therapy, and any failure at this final step may be catastrophic for the patient. Secondly, there is the economic impact. Modifying host cells is expensive. As this is the last step before the cells are passed back to the patient, a lot of time, money and effort has already gone into manufacturing them.

A typical manual final formulation, fill and finish step is performed in a high-grade GMP clean room and requires extensive GMP and hands-on training to reduce the risk of failure. There are very stringent standard operating procedures (SOPs) associated with these manual processes, and a second operator is required to ensure that everything written in the SOP is carried out, documented, and signed off for product release. To prepare cells for cryopreservation, a cryoprotectant, such as dimethyl sulfoxide (DMSO), is added which protects the cells during cryopreservation and thawing. Addition of cryoprotectant creates an exothermic reaction. To reduce the impact of heat generation, a system of cold packs and syringes is generally used to ensure that the cells are kept at the correct temperature.

Risks and considerations associated with this process include [1]:

- Operator-to-operator variability
- Homogeneity of the cellular product when producing several bags from one batch of cell product
- DMSO exposure time
- Sealing and air removal when using cryopreservation bags, to avoid breakage
- Temperature variability
- Contamination
- Paper-based or electronic documentation errors

In contrast, proper automation of the fill and finish process can maintain viability and sterility, reduce error, and ensure reproducibility and consistency.

## THE FINIA® FILL AND FINISH SYSTEM

Finia is an automated solution for the final formulation, fill, and finish step. The system can perform automated mixing, cooling, air removal and aliquoting, and sealing of the cryopreservation bag. The system is functionally closed and utilizes sterile, single-use disposable sets (Figure 1). Finia manages data through a server-based application called the Cell Processing Application (CPA), which facilitates cGMP compliance. The CPA manages access through user credentialing and permissions. Protocol and material configurations are downloaded from CPA to Finia, which enforces the workflow stream and prevents materials from being added in the wrong order through a barcoding system. The CPA also performs electronic data capture, including run reports and errors, and can manage multiple Finia systems from a central server.

## DATA SNAPSHOT: THREE FINIA STUDIES UTILIZING THE FINIA SYSTEM

Three different studies were performed by Terumo Blood and Cell Technologies to answer specific research questions about Finia.


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## FIGURE 1

**Features of the Finia Fill and Finish System.**

Designed for an automated fill and finish process

- An automated, functionally closed system for:


- Uses sterile, single-use disposable sets
  - Up to three product bags and one QC bag
- Operates with Cell Processing Application (CPA) to facilitate cGMP compliance:
  - Data recording and reporting including process alarms/flags
  - Device security and workflow management
- Reproducibility/consistency of formulation
- Accurate volumes of materials into mixing bag and product bags
- Minimization of air in the final product container for cryopreservation

cGMP: current Good Manufacturing Practices; QC: quality control.

## STUDY #1: FINIA VOLUME ACCURACY VERIFICATION

To begin, data was gathered by the Terumo Blood and Cell Technologies engineers to characterize Finia volume accuracy across multiple volumes. This study was water-based and involved 20 runs across four different product bag target volumes, resulting in 55 different individual product bag data points (Figure 2). The results highlight Finia volume accuracy across a wide volume range.

## STUDY #2: COMPARATIVE STUDY – MANUAL VERSUS FINIA

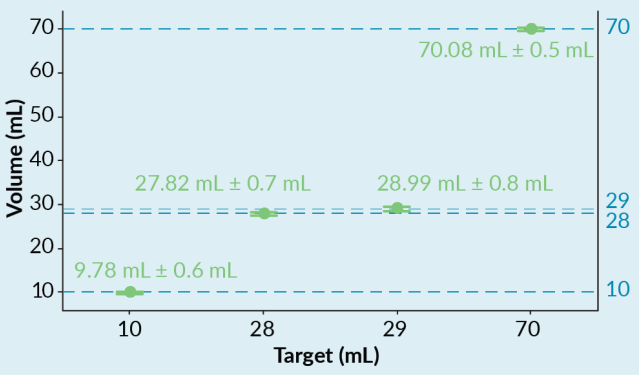
Next, a paired study design was devised to compare a robust manual process to the Finia automated process. Jurkat cells were expanded using the Quantum® Cell Expansion System. Each day for three days cells were harvested from a Quantum system and split into separate bags for three runs of the Finia process and three runs of the manual process. For each run, the fill and finish processes were performed simultaneously. Each Finia or manual process resulted in three different product bags with 50 mL target product volume, and the cellular products were analyzed for cell count and cell viability using trypan exclusion.

### Product bag volumes

The first parameter studied was product bag volume (Figure 3). Each product bag was weighed post-process. The volume was calculated by

## FIGURE 2

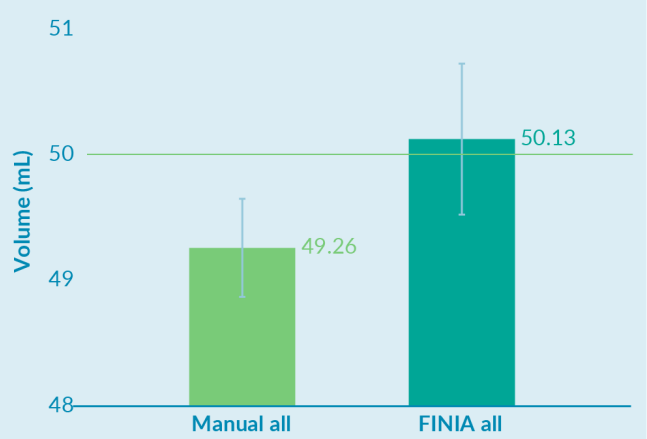
**FINIA volume accuracy against four different volumes.**




Four different target volumes are shown on the X axis: 10 mL, 28 mL, 29 mL, and 70 mL. Each point shown in the graph is an average of a sample size of 15 for the 10 mL, 28 mL, and 29 mL targets and a sample size of 10 for the 70 mL target. Individual standard deviations are used to calculate the intervals.

## FIGURE 3

**Average product bag volume from each process.**




An average volume was calculated from 27 total product bags from each process across all runs. The target volume was 50 mL per product bag. The average from each process is listed above its respective bar, with standard deviation bars. Statistically significant differences were observed between the manual process (green) and Finia process (turquoise).

subtracting the weight of the empty bag and dividing by the specific gravity of the cell product. Based on these data, the Finia process was more accurate; on average, Finia product volumes were closer than manual process volumes to the target volume of 50 mL.

## Homogeneity of Finia products

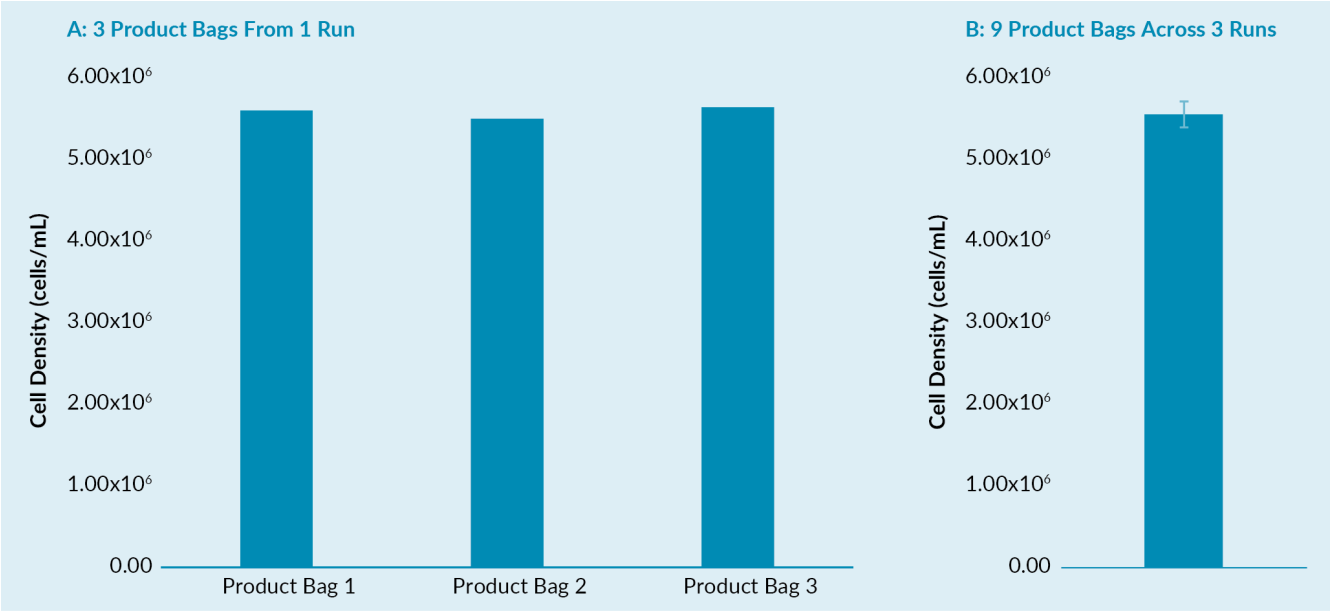
Next, cell density of the Finia products was measured to assess homogeneity. During a fill and finish run on Finia, the cells were mixed with cryoprotectant in a master mixing bag before being aliquoted into each product bag. As shown in Figure 4, cell density is very similar across Finia product bags within one run (Figure 4, Graph A) and across multiple runs (Figure 4, Graph B). This data demonstrates the homogeneity of the mixture in the master bag and how accurately Finia can aliquot into individual bags.

## Absolute and relative viability post-process

Cell health was also assessed using absolute and relative viability (Figure 5). Viability was assessed from each product bag post-process using trypan blue exclusion. Relative viability is calculated using the measure of post-process viability as a percentage of pre-process viability. Numbers were comparable between the manual and Finia processes with no statistically significant differences

## FIGURE 4

**Cell density representative of A: an autologous product and B: an allogeneic product.**




**A:** three product bags from one run chosen at random; **B:** nine product bags across three runs, with standard deviation of ± $1.59 \times 10^5$.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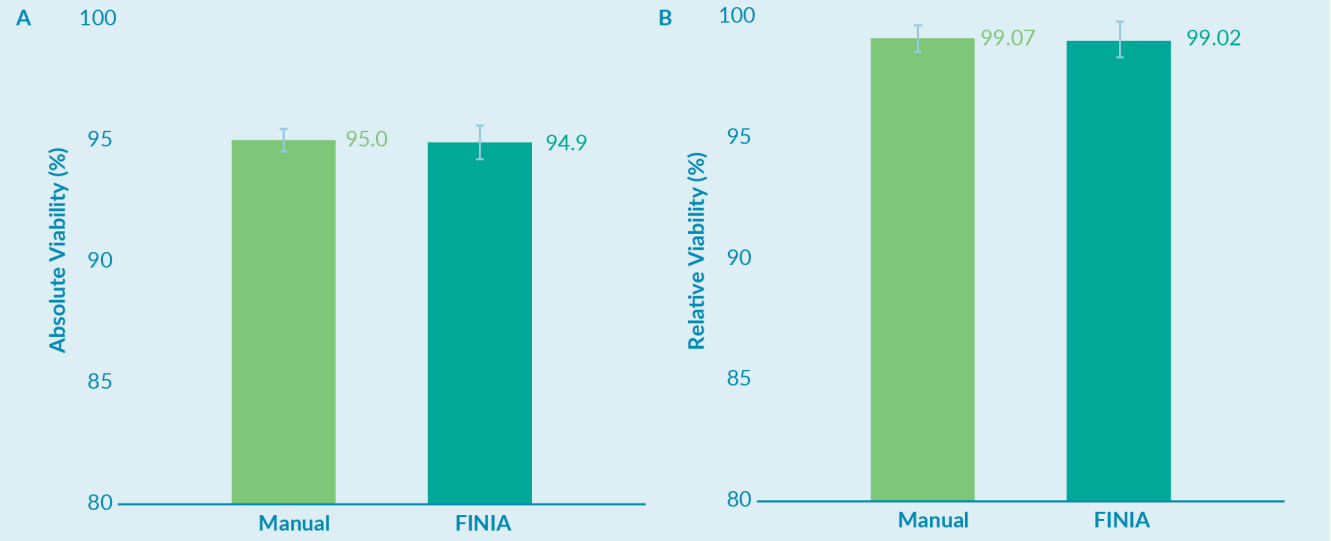
## FIGURE 5

**A: absolute cell viability and B: relative cell viability post-process.**




Both manual and Finia processes maintained very healthy cells relative to their starting material. These data are an average of 27 product bags from each process. Manual process is shown in green and Finia process in turquoise.

observed, illustrating that both approaches can result in very healthy cells post-process.

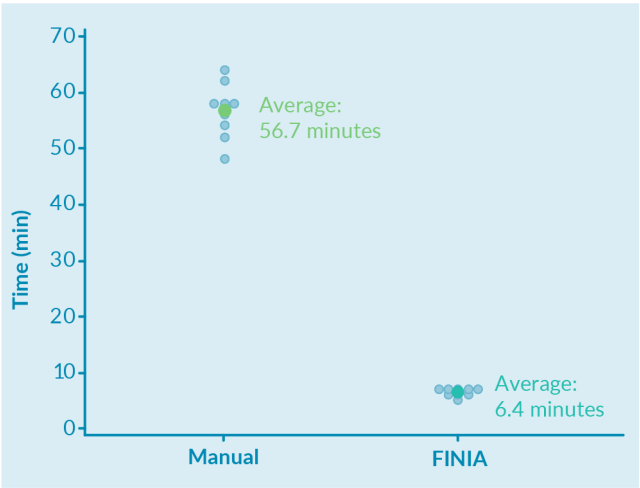
## Hands-on and employee time

Hands-on time is defined as the length of time for which the operator is interacting with each of the processes. For the manual process, hands-on time is the length of time an operator spent handling cells, adding cryoprotectant, aliquoting into bags, removing air, and sealing the product bags. For Finia, this is the length of time for which the operator is required to interact with the device by adding the single use disposable set, choosing the protocol, and adding materials to the product line in a sterile manner. For both processes, hands-on time does not include any pre- or post-process effort or cooling time.

Going a step further than hands-on time is the measurement of employee time, which is intended to represent the total amount of labor that went into these processes (Figure 6). For the manual process, this is the entire length of time for which operators were needed for the process and includes the ten-minute cell hold. It also includes a second operator acting as a quality control (QC) person in the manufacturing area to ensure process documentation. For Finia, this is the length of time for which the operator is required to interact with the device. It is important to note that an operator can multitask or run multiple Finia systems at the same time.

## FIGURE 6

**Measured employee time from nine runs of each process.**




Each datapoint shown in blue is employee time used during that run. The average for the manual process is shown in green and Finia in turquoise. Statistically significant differences were observed. Statistical test was a paired *t*-test.

## Open events

Fifteen open events were recorded for the representative manual process. Every time a syringe interacted with a bag of material or a bag spike was placed into a bag was recorded as an open event. In contrast, zero open events occurred with the functionally closed Finia system. This process used a TSCD®-II Sterile Tubing Welder, which allows the system to remain functionally closed. Each material – cells, cryomedia or buffer – was attached to the Finia disposable set in a sterile manner.

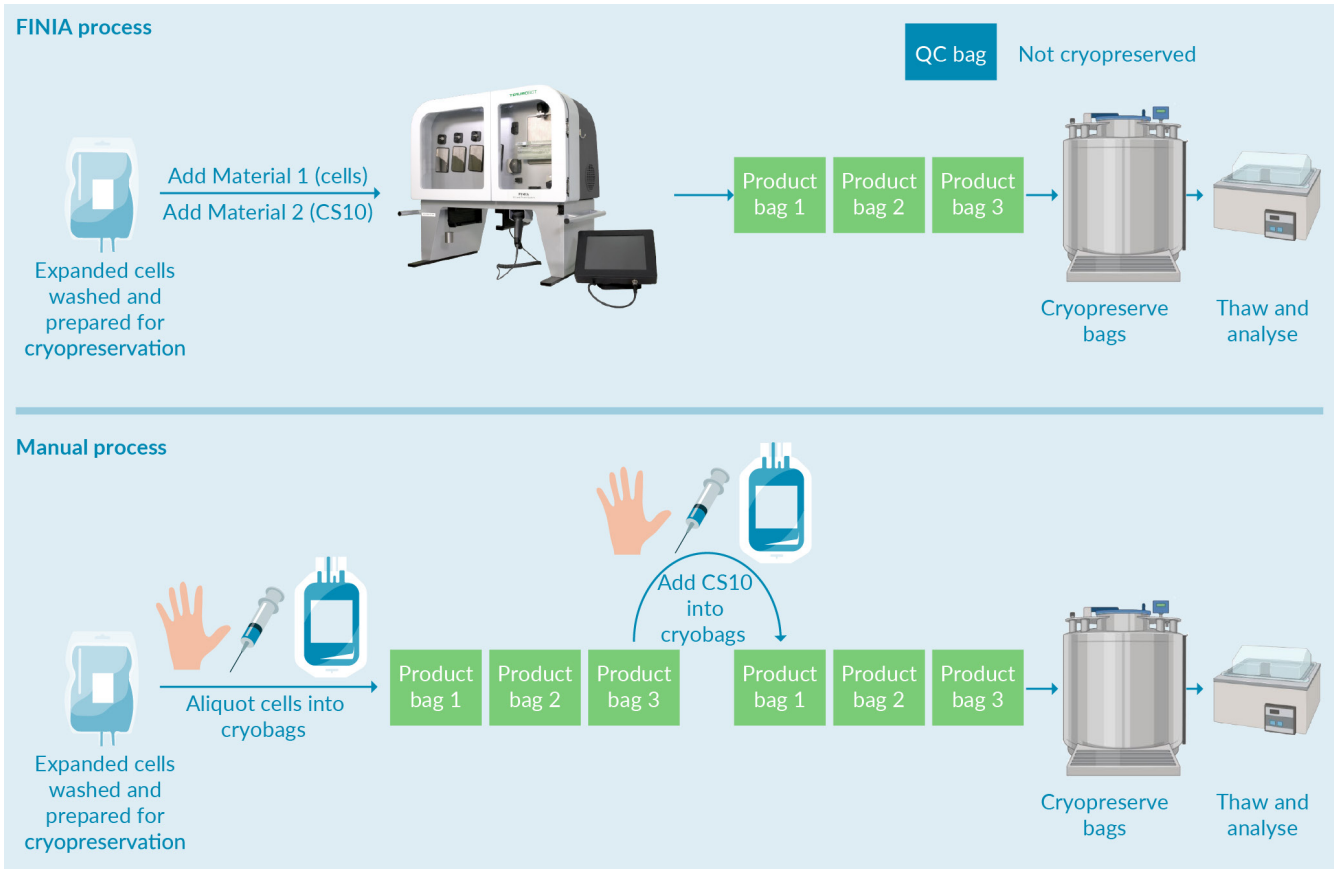
## STUDY #3: CRYOPRESERVATION – MANUAL VERSUS FINIA

The third study compared manual and Finia processes from both a cell health and a full cell therapy workflow perspective. This study used expanded primary T cells from three healthy donors to assess quality metrics before and after Finia as well as postcryopreservation recovery.

A leukopak was acquired using the Spectra Optia® Apheresis System. Cells were isolated using Human CD3 positive selection kit (Stemcell). CD3+ T cells were activated using CD2/CD3/CD28 soluble activator (Stemcell) and expanded on the Quantum system. After 7 to 8 days of expansion, the cells were harvested, washed, and prepared for the fill and finish process. The Finia and manual processes can be seen in Figure 7. As in the earlier studies, this was a comparative study where the cells were separated and used by different operators to run the fill and finish processes simultaneously. Upon completion of the fill and finish process, the product bags from both manual and Finia

**FIGURE 7**

**Manual and Finia formulation, fill and finish processes performed for this study.**





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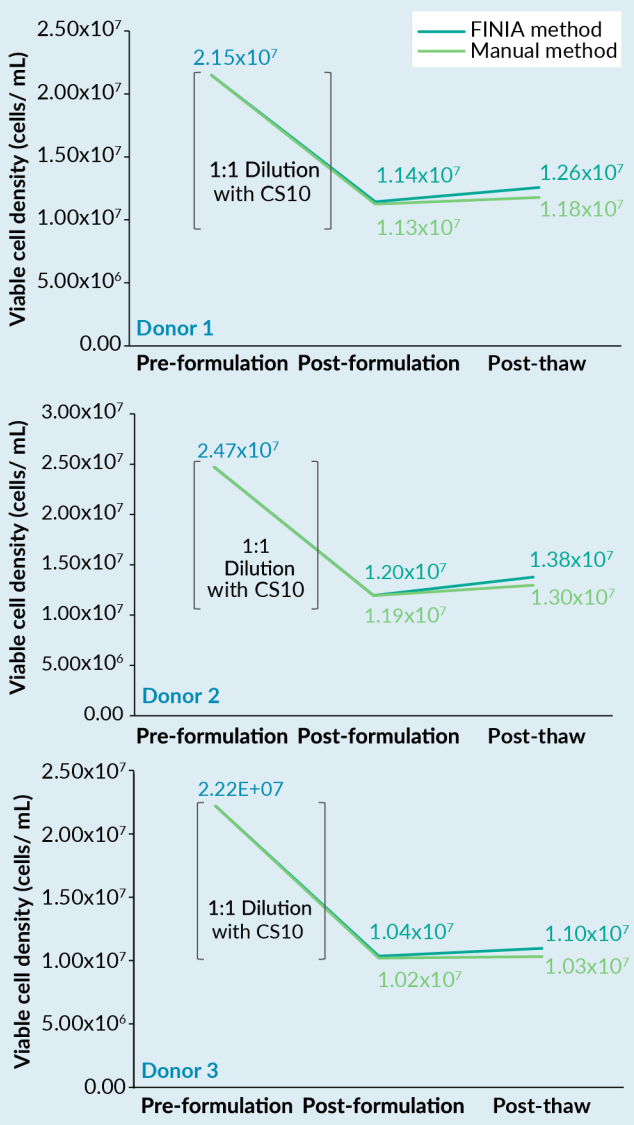
processes were added to the controlled rate freezer at the same time and then placed into a liquid nitrogen vapor phase freezer for at least 72 hours. Finia also produces one QC bag for post-process testing. After 72 hours in the liquid nitrogen freezer, the product bags were individually thawed in a water bath, diluted using cell thaw media, washed, activated, and expanded in flasks for up to 48 hours in complete media.

## Viable cell densities

The first parameter studied was viable cell density across multiple time points, as seen in Figure 8. The pre-formulation timepoint is the cell density of the starting material prior to being separated into the Finia or manual process streams (n = 1). During the fill and finish process, the cells were diluted with cryoprotectant, resulting in a one-to-one dilution. The post-formulation timepoint was

## FIGURE 8

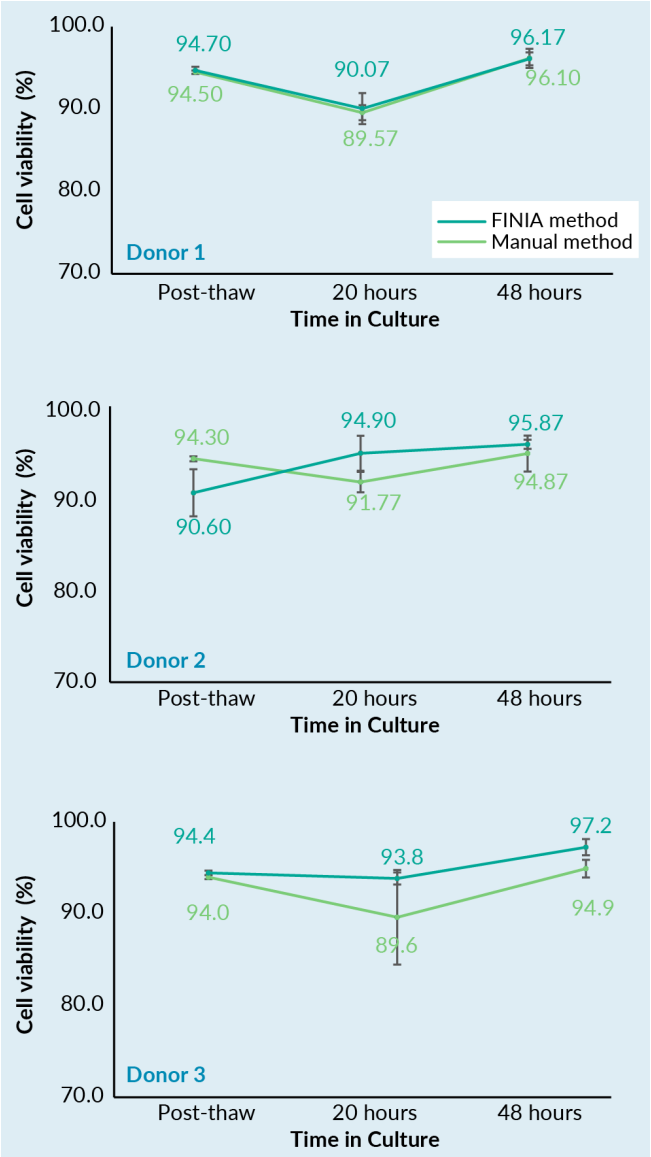
**Viable cell densities across multiple timepoints.**




Samples were compared between the Finia (turquoise) and manual (green) processes for three donors. No statistically significant differences between processes were observed.

## FIGURE 9

**Post-thaw samples for each donor.**




Samples were compared between the Finia (turquoise) and manual (green) processes. All three donors were averaged to observe statistical differences at the 20-hour timepoint.

taken from the QC bag from Finia (n = 1) and from a small aliquot from each product bag from manual process (n = 3). The post-thaw timepoint is from the thawed product bags from each process (n = 3).

## Absolute viability and cell health

The next parameter assessed was absolute viability as measure of cell health. Viabilities above 90% were maintained throughout the processes including pre-formulation, post-formulation, and post-thaw for all three donors.

After each product bag was thawed, the cells were placed into a flask, activated, and grown in culture for up to 48 hours. An average of all three donors was taken at the post thaw, 20-hour, and 48-hour timepoints. Post-thaw culture viability was observed to be greater than 90% for both Finia and manual processes (Figure 9).

In summary, the Finia system can offer accurate and reproducible results along with time, labor, and cost savings, and a reduced risk of contamination and error – highlighting the value automation can provide in cell manufacturing.

## REFERENCE

1. Sethi D, Cunningham A. De-risking the final formulation, fill and finish step in cell therapy manufacturing: considerations for an automated solution. *Cell Gene Ther. Insights* 2020; 6(10): 1513–1519.

TERUMO BLOOD AND CELL TECHNOLOGIES

### AUTHORSHIP & CONFLICT OF INTEREST

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## ✓ Maintain cell viability and uniformity.[2]


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- Precisely remove air from bags automatically to reduce the risk of product failure due to bag breakage.
- Reduce the need for labor-intensive manual documentation and the risk of error with the unique Cell Processing Application software that facilitates GMP compliance and workflow configuration management.

**Learn more at TERUMOBCT.COM/Finia.**

cGMP: current Good Manufacturing Practices; DMSO: dimethyl sulfoxide.

*Product available in select markets.

Customers are responsible for validating the use of the Finia system for their intended therapy.

References 1-3: Data on file.

 CT-FFAF-00161

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SPOTLIGHT

EXPERT INSIGHT

# Challenges in AAV manufacturing: the interplay of process variations, in-process characterization, and drug product quality

David Chu, Hari Acharya, Emily Springfield & Laura Adamson

A large focus for cell and gene therapy in recent years has been Chemistry Manufacturing and Controls (CMC) regulations related to production processes and analytical testing. Regulatory agencies have issued multiple guidelines for these novel biological products focusing on improving production, reproducibility, and testing expectations. With the increase in the number of clinical trials, there has also come a suite of clinical holds related to product quality or process reproducibility concerns. In remarks released from the FDA in 2018, they discussed how in contrast to traditional drug development, nearly 80% of the review process in cell and gene therapy applications is focused on the manufacturing process and product characterization methods. The focus of this article is discussing the current modalities for manufacturing, the available methods for evaluating in-process and final product characteristics, and the impact both the complex matrices and final formulation have on understanding changes to product quality and performance.

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## PROCESS VARIATIONS & IMPACT ON PRODUCT QUALITY

### Production & purification process variations

The upstream process for producing adeno-associated vectors (AAV) involves three main parts:

1. Expansion of the production cell line;
2. Introduction of the genetic components to make the viral vectors; and
3. Harvesting and separation of the vectors from the producer cell components.

Although the field is moving towards suspension platforms, there are still many production processes performed in adherent cultures. Both platforms have controlled and monitored parameters to ensure consistency during expansion.

Once the cells are expanded, the genetic components are introduced, and an intricate dance begins. This is a complex cascade between the helper sequences provided by either the plasmid or the helper viruses, the AAV replication genes, and recruitment of cellular proteins to allow for rescue and replication of AAV inverted terminal repeats and packaged sequences from the carrier DNA construct and expression of capsid proteins to form a gene therapy vector packaging the therapeutic gene [1]. Table 1 summarizes the variety of components and methods currently utilized. Besides the large variation in mechanisms to induce production in the target cell line, vectors are also harvested in a broad time range from 2 to 7 days after initiating production. Vectors may be harvested from only the cells, supernatant, or both parts of the culture. Given the large number of variations in how the vectors are produced and complexity of the production cascade, the difficulty in understanding the impact of process changes and differences in product characterization across companies and product types is not surprising.

Some of the key considerations for selecting a production platform is product quality and scalability. Initial studies comparing production methods showed decreased infectivity in baculovirus-Sf9 produced material and increased infectivity in herpes simplex virus (HSV)-produced material compared to a transient transfection process [2,3]. Refinement of the AAV packaging genes in the baculovirus-Sf9 system improved infectivity of the AAV vectors, demonstrating modifications of the helper and AAV genes themselves can significantly impact quality of the resulting product [4,5]. This has been further demonstrated in transfection systems where modifications of the helper or *Rep* genes, including use of Rep sequences from other AAV serotypes, has increased overall productivity, number of full capsids, and vector infectivity compared to wild-type AAV2 sequences [6,7]. In a recent paper from Rumachik *et al.*, AAV vectors produced from plasmid transfection of HEK293 cells were characterized and compared to those from baculovirus transduced Sf9 cells [2]. Distinct differences were reported in productivity, genome methylation, and post-translation modification, with HEK293 produced material demonstrating increased *in vitro* and *in vivo* potency. Additionally, there were small but significant differences observed in the *in vitro* performance of vectors purified from cells compared to supernatant. This trend was not consistent across cell models. Together, this shows there is a significant impact from all three parts of the production process on the performance of the vector.

The upstream production process may have only three main steps, but each step has multiple process parameters which may impact product quality. As a company develops a program, they must define these process parameters and select a production method early in their development cycle that is sufficient for the predicted clinical demand. Different production methods may be used depending on the stage of the program e.g., pre-clinical versus early stage versus commercial. Changes to the production methods at these stages may introduce variability in product quality making data translation from study to study


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**TABLE 1**

**Summary of AAV upstream methods.**

| Cell lines (source) | Gene delivery method | Variations on the process |
|---|---|---|
| HeLa (mammalian-human) | Viral/integrated | Ad5 + stable integration of Rep and/or Cap sequences |
| HEK293(with or without T antigen) (mammalian-human) | Plasmid | Dual or triple transfection<br>Adherent or suspension |
| | Closed, linear DNA | Dual or triple transfection<br>Adherent or suspension |
| | Viral | Dual HSV delivery<br>Adherent or suspension |
| BHK (mammalian-hamster) | Viral | Dual HSV delivery<br>Adherent or suspension |
| Sf9 (insect) | Viral | Single, dual, or triple transduction (baculovirus)<br>Stable integration of Rep and/or Cap sequences<br>Expansion within cell line or single transduction |
| Producer cell lines (various) | Plasmid/ integrated | Stable integration of some or all of the packaging components |

challenging. For instance, in some baculovirus-Sf9 systems, genetic instability of the ITR and packaging sequences can be observed in as little as 5 serial passages, leading to the potential for product differences including productivity and infectivity in both scale-up and long-term production within the same system [8]. This would require sufficient starting material to be generated to allow for low passages baculovirus stocks throughout the life of the program or risk material produced later in a program to perform significantly differently. In early-stage programs with limited resources, it is better to focus development efforts on more impactful process characterization, resulting in some of these scale-dependent product quality changes to hopefully be discovered earlier in the product lifecycle.

Current standard methods for AAV purification typically involve clarification of the harvest material, separation of the vector particles from the host cell contaminants and process reagents, enrichment for full capsids, and buffer exchange of enriched vectors into a stable matrix targeting the desired concentration for administration to patients. Ayuso *et al.*, reviews the challenges in establishing a platform AAV purification process for several AAV serotypes. For enrichment of full capsids, the more flexible steps for new or novel capsids, such as gradient ultra-centrifugation, often demonstrate poor scalability and reproducibility in a GMP environment [9]. In contrast, developing a universal chromatography-based enrichment method that works for different capsids or serotypes can be difficult due to the different charge or isoelectric points of the vectors and variable size of the packaged transgene. This could present a challenge at companies or contract manufacturing organizations producing and purifying a wide range of AAV serotypes or modified capsids. A universally applied protocol can show a broad range of enrichment from 2–8x more full capsids compared to the loaded material and serotype being purified [10]. This makes it challenging to achieve equal quality across multiple serotypes and/or transgene combinations without significant development and modifications to the purification and enrichment methods.

This difficulty in development can lead many groups to begin their pre-clinical and Phase 1 programs with a centrifugation-based purification method while a scalable, column-based purification method is developed and implemented in later stages of the clinical lifecycle. The variation in these polishing and enrichment steps can result in significantly different amounts of impurities including empty or partially filled capsids or contaminating host cell proteins or DNA. These differences often make showing product comparability from early to clinical studies difficult without a robust analytical package to compare not just overall product quality but the

impact of different packaged DNA and capsid content on infectivity and potency in the target population.

Together, these differences in production and purification methods and associated product quality highlight the need for more robust analytical methods that link processes changes to their impact on product quality and vector performance, especially at early stages in development. It is critical these methods become available to understand the impact of these process changes on vector performance as more programs and companies look to scale-up and commercialize their products. Understanding these differences is further compounded by the variation of capsid serotypes and modifications utilized, and the limited definition of critical quality attributes at early stages of development. As AAV gene therapy continues to mature, establishing how a company can make informed design decisions regarding process changes when analytical methods may not be available, or product quality attributes have not been identified, will need to be standardized to ensure vector performance is not impacted by production changes or by process scale-up.

## In-process hold steps & analytical testing

In early stages, limited studies have been performed to evaluate the impact of hold steps and formulations used in manufacturing processes and their impact on final product quality. Bee *et al.*, recently reported an increase in free DNA both after ion exchange and following each freeze and thaw cycle of the drug product as measured by a fluorescent dye free DNA assay [11]. While the absolute amount of DNA released was relatively low, this points to the importance of characterizing vector products at different storage temperature, time, and buffers for each hold step. The reasoning for increased release after ion exchange was theorized to be related to the low osmolality and higher pH. This is different than the suspected increase in free DNA during freeze thaw cycles which is attributed to capsid degradation, pointing to multiple mechanisms for quality differences carried through to the final drug product. Various AAV serotypes have also demonstrated differential capsid stability and transduction efficiency at lower pHs that was dependent on storage temperatures [12]. Since many affinity elution steps rely on low pH elutions, differences in elution buffers, neutralization buffers, neutralization protocols, and storage at this step should be evaluated critically. The ability to assess these differences can be further compounded by the fact that the accuracy of many current analytical methods are impacted by the buffers used during purification, or require a highly pure product to provide precise results about the vector. This limits the ability to characterize the sample in a representative state during in-process testing.

Methods for testing AAV and drug product critical quality attributes have been described and refined as data from more studies has been developed [13]. Testing AAV from in-process samples has relied on applying these analytical tools developed for purified drug product to a complex matrix with variable pH and salt contents, high amounts of host cell proteins, partially formed capsids, and residual plasmid DNA, limiting the reliability of the data reported. Given the large number of methods utilized to detect the same product attributes and the variations in what stage in the process they can be utilized, it can be difficult to define the impact of process changes (especially in upstream) to the final product quality. A recent study comparing multiple HPLC methods demonstrated the utility of a combination of analytical columns and measurement techniques including UV, light scattering, and intrinsic fluorescence to test DNA contamination, full and empty particle generation, and partially packaged capsids from unpurified, clarified lysate samples to purified products [14]. Additional methods have been developed that monitor a range product attributes with minimal sample manipulation by combine multiple methods currently used for AAV characterization into


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a single instrument allowing for assessment of capsid and genome titer, aggregation, and full and empty quantification [15]. Unfortunately, many of these methods still require relatively pure samples for this analysis. Continuing to advance methods such as these will decrease the variability observed from utilizing procedures qualified for purified, drug product vectors for in-process samples, or relying on translating results from multiple methods given the complex nature and intrinsic variability of the current analytical capabilities for AAV vector testing.

## DRUG PRODUCT FORMULATION & LONG-TERM STABILITY

Much of what is known to be critical for formulation of AAV vectors is derived from publications on AAV2 or other common serotypes, knowledge from the protein or monoclonal antibody fields, or the limited data available from the few commercial AAV products. Most AAVs are known to aggregate at capsid concentrations between 1–10 x $10^{14}$ capsids/mL and are especially prone to aggregation in low ionic strength buffers [16]. This study by Wright *et al.*, also demonstrated that different purification methods may co-purify process related impurities that could contribute to increased aggregation both in-process and in the final product, further demonstrating that the production and purification process can alter our understanding of drug product stability and should be taken into consideration with process changes for scale-up or commercialization. Some capsids have also shown pH dependent protease activity that can lead to cleavage of capsid proteins [17]. This highlights the criticality of early assessment of formulation buffers in combination with new serotypes or novel capsid modifications to ensure small pH or ionic changes that can occur in some formulation buffers do not result in significant product quality changes during storage, shipment, or handling of the product. Formulations for AAV drug products depend on the route of administration but are primarily composed of a buffering agent to maintain pH (often Tris or phosphate based), a salt to achieve a target ionic strength, and a poloxamer to reduce non-specific binding of the capsids to the tubes or vials [18,19]. AAV has been shown to be stable at <-70°C for 2 years, but decreases in *in vitro* and *in vivo* performance has been observed for several serotypes upon multiple freeze thaws and prolonged storage at +2–8°C [20–22]. With the increase stability in frozen conditions, some formulations also contain a cryoprotectant to reduce aggregation and increase capsid stability on freeze-thaw. While studies continue to be performed to allow for storage and shipment at lower temperatures, current best practices involve frozen formulations long term, with limited shelf life at +2–8°C following product thawing and preparation. This puts burden on the supply chain and clinical sites to maintain adequate cold storage and limits the shelf life of AAV products, presenting a hurdle to broad application of gene therapies.

## TRANSLATION INSIGHT

As the number of clinical trials continues to increase for AAV products, manufacturing practices have advanced to meet the product demands. Without a clear understanding of the link between different serotypes or capsid modifications, impact of production and purification methods, and establishment of defined critical attributes for vector performance, clinical studies have stalled trying to understand differences seen between pre-clinical, early- and late-stage clinical studies. Some of this can be attributed to:

- There are many variations in the production and purification processes used for manufacturing AAV vectors. Understanding of acceptable process parameters or critical process or product quality attributes are often based on similar production methods that may have been utilized for different serotypes, or productions of similar

products with varied process parameters. There is limited agreement is what process steps are critical for optimal AAV vector quality.

- Current analytical methods and standards are often developed and validated for purified drug product which makes understanding the impact of in-process changes on more nuanced product characteristics like partial genome packaging or capsid surface modifications difficult to determine. A better understanding of the product characteristics that have significant impact on clinical performance can help guide method development for understanding which process steps are critical for AAV vectors.

- Initiatives from industry and governmental agencies such as the development of reference standards for multiple serotypes and the establish of collaborative partnerships such at the Bespoke Gene Therapy Consortium are paving the way to better translate the breadth of data available on AAV vector product quality and performance. Multiple strategies have been presented and published to apply quality by design into the AAV manufacturing field and will continue to accelerate understanding of these gene therapy products.

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY CMC & QUALITY CONTROL

SPOTLIGHT

EXPERT INSIGHT

# The Chemistry Manufacturing and Controls (CMC) section of gene therapy-based INDs: overview in a changing landscape

Gabriela Denning

There are many product development considerations for human gene therapy investigational new drug (IND) applications to ensure the proper 'Chemistry, Manufacturing, and Controls' (CMC) is achieved. Unlike small molecule drugs or biologics intended for repeated dosing, gene therapies are designed to modify patient cells directly or indirectly (administration of genetically modified cells to patients) to confer long-lasting therapeutic effects. The complexity of gene therapy manufacturing and therefore control of the processes is continually evolving with the need for disease specific innovations as well as standards across the various gene delivery platforms. As data becomes available on the durability, efficacy, and safety of gene therapy treatments post-administration, new factors must be considered when designing transgene cassettes, gene delivery vehicles, and conditioning treatments for infusion. Such clinical outcomes ultimately affect manufacturing and the continual evaluation of CMC requirements. This article highlights regulatory considerations and new guidance available for gene therapy manufacturing in a continually evolving landscape.

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Gene therapies are genetically modifying drugs that confer a therapeutic effect via the transcription and/or translation of the transferred genetic material. The complexity of gene therapy manufacturing has led to a variety of regulatory guidances by the Food and Drug Administration (FDA) to ensure the proper control of the processes involved in final product manufacturing and characterization (refer to Table 1 for summary of Cell and Gene Therapy Guidances, with CMC-related guidances highlighted in bold). In addition, there are phase appropriate CMC considerations as clinical testing progresses to demonstrate the relationship between drug product quality attributes (lot-to-lot consistency) established through release criteria and clinical efficacy. The FDA continues to issue draft guidances and in 2020 finalized 6 Gene Therapy Guidances for Industry as well as added a new draft guidance. Each investigational gene therapy drug product needs to be evaluated for **safety, purity, potency** (strength of the investigational drug product), **identity, stability, and sterility** performed under current good manufacturing practices (cGMP) prior to patient administration. It is therefore crucial that during early-stage development, the applicable FDA guidance's issued by the Center for Biologics Evaluation and Research (CBER) are reviewed and taken into consideration starting with the recent 2020 Guidance for Industry: Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) which supersedes the guidance issued in 2008. The new guidance follows the CMC structure on the Common Technical Document (CTD) for INDs and therefore greatly facilitates evaluation and adaptation of the guidance. As greater clinical outcomes are evaluated from long-term gene therapy treatments, regulatory considerations must be taken which are informed by the safety and efficacy results.

It is important to recognize the inherent biological variability in gene therapy drug products that present challenges and limitations in controlling the manufacturing and quality. As such, both sponsor and regulators should establish a dialogue early during the development process to understand the disease specific considerations and allow the applicability of innovative research tools into the development process. The following are a few challenges related to CMC that should be taken into consideration during the development process to ensure that sufficient information (data) is provided in the IND application: Product identity assays should be established and assessed during pre-clinical development. Depending on the gene therapy, more than one assay may be needed to access identity. It is expected that purity of the final product may be mixed (i.e., viral vector heterogeneity such as empty vs. full AAV capsids and/or genetically modified or non-modified cell content in the final gene therapy drug product) and establishing the assays used to measure product identity and purity will ensure lot-to-lot specifications and strength of the product. Understanding final product purity is continually evolving as novel technologies are developed for the evaluation of viral vector particles before administration and are predicted to form part of the CMC release criteria testing in the future. Additional measures of purity should be taken into consideration to ensure that any impurities (either product or process-related) meet the acceptance criteria and safety criteria of the administered drug product.

Both identity and purity relate to one of the most critical characteristics of the drug product, potency. Potency or strength of the investigational drug product will allow for the measurement of the desired efficacy prior to administration. The establishment and validation of potency assays are expected to evolve with clinical stage of development and are intended to predict clinical efficacy. Most likely, innovative approaches/techniques will form part of the development of various potency assays that could involve both *in vitro* and/or *in vivo* methods and will help establish dose levels. For potency tests development refer to Final Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products


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**TABLE 1**

**FDA Cell and Gene Therapy Guidances.**

| Issue date | Title |
|---|---|
| 01/2021 | Human Gene Therapy for Neurodegenerative Diseases; Guidance for Industry |
| 01/2021 | **Manufacturing Considerations for Licensed and Investigational Cellular and Gene Therapy Products During COVID-19 Public Health Emergency; Guidance for Industry** |
| 01/2020 | Human Gene Therapy for Retinal Disorders; Guidance for Industry |
| 01/2020 | Human Gene Therapy for Rare Diseases: Guidance for Industry |
| 01/2020 | Human Gene Therapy for Hemophilia; Guidance for Industry |
| 01/2020 | **Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up; Guidance for Industry** |
| 01/2020 | Long-Term Follow-up After Administration of Human Gene Therapy Products; Guidance for Industry |
| 01/2020 | **Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs); Guidance for Industry (supersedes 2008 guidance)** |
| 01/2020 | Interpreting Sameness of Gene Therapy Products Under the Orphan Drug Regulations; Draft Guidance for Industry |
| 02/2019 | Expedited Programs for Regenerative Medicine Therapies for Serious Conditions; Guidance for Industry |
| 02/2019 | Evaluation of Devices Used with Regenerative Medicine Advanced Therapies; Guidance for Industry |
| 12/2017 | Regulatory Considerations for Human Cells, Tissues, and Cellular and Tissue-Based Products: Minimal Manipulation and Homologous Use; Guidance for Industry and Food and Drug Administration Staff |
| 11/2017 | Same Surgical Procedure Exception under 21 CFR 1271.15(b): Questions and Answers Regarding the Scope of the Exception: Guidance for Industry |
| 09/2017 | Deviation Reporting for Human cells, Tissues, and Cellular and Tissue-Based Products Regulated Solely Under Section 361 o the Public Health Service Act and 21 CFR Part 1271: Guidance for Industry |
| 09/2016 | **Recommendations for Microbial Vectors Used for Gene Therapy; Guidance for Industry** |
| 08/2015 | Design and Analysis of Shedding Studies for Virus and Bacteria-based Gene Therapy and Oncolytic Products; Guidance for Industry |
| 06/2015 | Considerations for the Design or Early-Phase Clinical Trials of Cellular and Gene Therapy Products; Guidance for Industry |
| 03/2015 | Determining the Need for and Content of Environmental Assessment for Gene Therapies. Vectored Vaccines, and Related Recombinant Viral or Microbial Products; Guidance for Industry |
| 03/2014 | IND Applications for Minimally Manipulated, Unrelated Allogeneic Placental/Umbilical Cord Blood Intended for Hematopoietic and Immunologic Reconstitution in Patients with Disorders Affecting the Hematopoietic System – Guidance for Industry and FDA Staff |
| 03/2014 | Guidance for Industry: BLA for Minimally Manipulated, Unrelated Allogeneic Placental/Umbilical Cord Blood Intended for Hematopoietic and Immunologic Reconstitution in Patients with Disorders Affecting the Hematopoietic System |
| 11/2013 | Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products |
| 12/2011 | Guidance for Industry: Preparation of IDEs and INDs for Products Intended to repair or Replace Knee Cartilage |
| 10/2011 | Guidance for Industry: Clinical Considerations for Therapeutic Cancer Vaccines |
| 01/2011 | Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products |
| 10/2010 | Guidance for Industry: Cellular Therapy for Cardiac Disease |
| 09/2009 | Guidance for Industry: Considerations for Allogeneic Pancreatic Islet Cell Products |
| 04/2008 | **Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)** |
| 08/2007 | Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products: Guidance for Industry |
| 03/1998 | Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy |

(issued in January 2011). A well validated potency assay with defined acceptance criteria is expected by the Biologics License Application (BLA). Sterility should be established at various points during manufacturing (in process and final drug product) and such procedures should meet the acceptability established in the United States Pharmacopeia (USP), FDA guidance and/or Codes of Federal Regulations (CFR). An aspect that is often ignored during development is drug product stability, and stability studies should be initiated

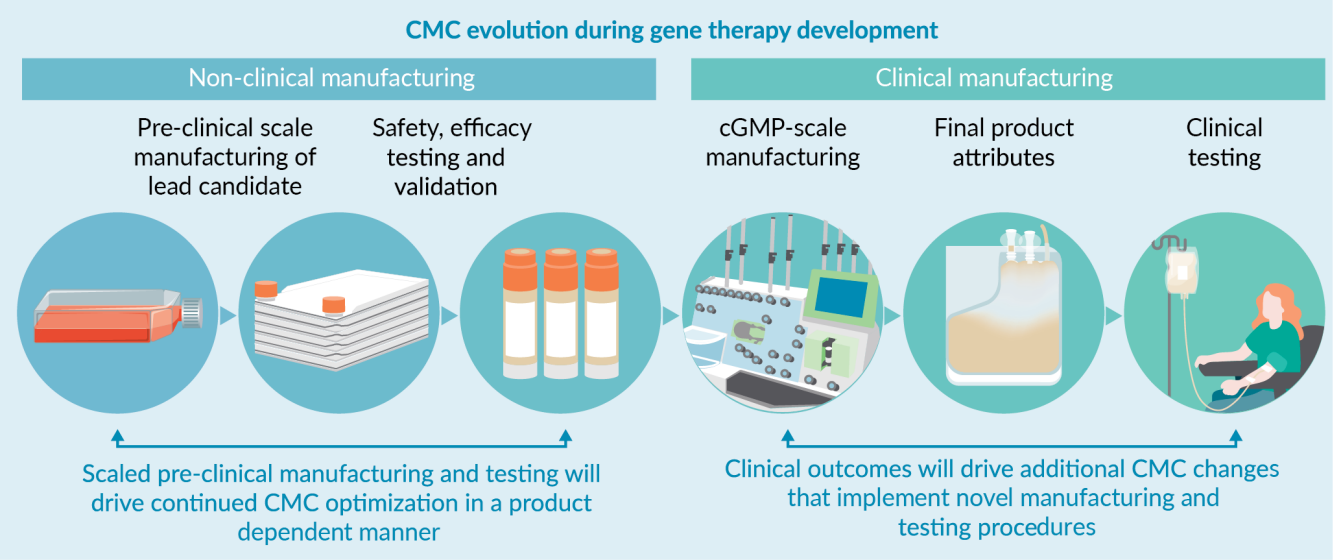
on final drug product at the expected storage conditions and post-thaw conditions prior to administration. Stability studies should also be performed on the individual components of the final drug product, such as viral vectors and nucleic acid intermediates that are mostly likely manufactured prior to final drug product manufacturing as is the case with *ex vivo* gene therapy drug products. Finally, safety will be established not only by the combination of the above drug product characteristics but also by toxicology testing. As gene therapy product manufacturing processes are established and scaled for clinical testing, changes in manufacturing facilities, procedures, and even transgene optimizations are expected. Such changes require comparability studies that should be discussed with regulatory agencies to avoid delays in approval and the need for repetitive studies when scientifically justified.

Each gene therapy product will present its own unique development and manufacturing challenges. The FDA has issued various disease specific guidance (Hemophilia, Neurodegenerative Diseases, Retinal Disorders, and Rare Diseases) that should be considered during drug product manufacturing and testing. Some of these guidances provide disease specific considerations for CMCs that should be factored into process development, although the guidances are not comprehensive standalone outlines. With great advances in cell and viral vector manufacturing, some aspects of gene therapy drug manufacturing are becoming more scalable with numerous CMO and CDMOs playing crucial roles in CMC development for gene therapy INDs. CDMOs are equipped with the expertise and established processes in place for collaborative development at escalating clinical scales and in many cases critical aspects of manufacturing already vetted by the FDA through drug master files (DMF). Establishing such interactions with CDMOs prior to IND-enabling studies will allow for successful technology transfer and collaborative CMC development plans. CMO and CDMOs are also able to meet the requirements for analytical procedures that facilitate release criteria testing and assay validations.

It is worth restating that tests and assays should evolve during clinical testing (Phase 1 through Phase 3) as greater understanding of the gene therapy drug product is attained. Full validation of analytical procedures is expected

## FIGURE 1

**Gene therapy product manufacturing advances through non-clinical testing and is driven by product dependent attributes.**




As manufacturing progresses to clinical stage, CMC changes will be driving by safety and efficacy outcomes.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for submission of BLA. Figure 1 highlights the relationship between non-clinical and clinical manufacturing driven by gene therapy product testing, the evolution of novel analytical tools, as well as clinical outcomes. As gene therapies continue to evolve, greater coherence can be achieved by the collaboration of various stakeholders in the field to address the many challenges posed by the development of these individualized therapies.

## TRANSLATIONAL INSIGHT

Increased understanding of viral vectors and final product drug quality attributes will continue to inform gene therapies for greater process control in an inherently variable biological process. Recent clinical outcomes clearly elucidate the need for greater process control that will ultimately affect the CMC gene therapy drug product development. As the field has greater understanding of the cellular mechanisms governing transgene expression post-administration, safety considerations must be taken to ensure that delivery (and viral vector integration) is properly controlled to limit off-target effects. New tools should be developed for greater control of the therapeutic payload into target cells on a patient-by-patient basis. Stakeholders will also benefit from the development of reference standards or reference materials used for quantitative assays in the testing of viral vectors and genetically modified cells drug products. The availability of AAV and lentiviral-based reference materials would facilitate the comparability between various gene therapy drug products, however product specific reference standard materials may need to be developed, qualified, and validated. As novel processes of CMC development for gene therapies are explored, sponsors are greatly encouraged to establish early relationships with the FDA to ensure early guidance and avoid delays in approvals.

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY CMC & QUALITY CONTROL

SPOTLIGHT

INTERVIEW

# Addressing challenges in characterizing novel AAV capsids and gene editing platform-driven therapeutics

**LAUREN M DROUIN** leads the Analytical Development group at LogicBio Therapeutics, where she supports the development and implementation of analytical methodologies needed to progress LB-001 and other pipeline products from preclinical development into the clinic and beyond. Current research interests include novel AAV capsid characterization and developing a robust understanding of the factors that influence potency of gene therapy products. Previously, Lauren worked at Voyager Therapeutics where she was responsible for analytical method development and overseeing CMC analytics operations for the Parkinson's Disease clinical program. Lauren received her PhD in Biochemistry and Molecular Biology from the University of Florida where she utilized molecular, biophysical, and structural techniques to characterize the AAV capsid for improved gene delivery.

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**Q** What are you working on right now?

**LMD:** LogicBio Therapeutics is a clinical stage genetic medicine company, pioneering gene editing and gene delivery platforms to address rare and serious diseases from infancy through adulthood. Our gene editing platform, GeneRide™, is a novel approach to using precise gene insertion and harnesses the cell's natural DNA repair process, which may lead to durable therapeutic protein expression levels. The GeneRide approach potentially offers a significant benefit over canonical gene therapy approaches, especially when targeting dividing cells such as those found in the liver, or in pediatric tissues, where canonical gene therapy vectors would be diluted as the child grows.

We recently dosed the first patient in our LB-001 Phase 1/2 clinical trial (SUNRISE) for pediatric patients with methylmalonic acidemia, which is a rare and life-threatening genetic metabolic disorder with no available pharmacological treatment options. We believe this is the first time an *in vivo* AAV-based genome editing approach has been delivered systematically to a pediatric patient, and we are excited about the potential to treat patients suffering from this serious disease.

In terms of what the analytical development team is working on at LogicBio, we are primarily focused on method development for upcoming gene editing and gene therapy programs, tech transferring existing methods for validation, and also supporting our process development team. In addition to these tasks, we have been working on characterizing novel AAV capsids that have been developed for improved transgene delivery to the liver as well as other tissues.

**Q** Tell us about the chief challenges and considerations in characterizing novel AAV capsids, both from your past experience and with LogicBio's own platform, sAAVy™.

**LMD:** sAAVy is an AAV capsid engineering platform designed to optimize gene delivery for treatments in a broad range of indications and tissues. Our first capsid produced from the platform, sL65, is currently being utilized to develop gene therapy candidates for the treatment of Fabry and Pompe diseases in collaboration with CANbridge Pharmaceuticals.

> "On the surface, AAVs appear to be relatively simple viruses but in fact, they pack so many functions into their small genome that just one minute change ... can sometimes knock out any number of steps in the virus life cycle."

Regarding the major challenges in working with novel AAV capsids, I think one of the primary concerns is to establish their manufacturability. On the surface, AAVs appear to be relatively simple viruses but in fact, they pack so many functions into their small genome that just one minute change to the inverted terminal repeats (ITRs) or to the


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capsid can sometimes knock out any number of steps in the virus life cycle. For instance, even minor changes to the viral protein (VP) sequence may affect capsid assembly and stoichiometric composition, stability and solubility, DNA packaging efficiency, etc. and can greatly impact overall yield and product quality.

Turning to analytical method development, we have several aspects to consider when we are characterizing a new capsid. Firstly, how well is the product purified using our standard manufacturing methods? Does it have the expected three VP bands or more, and is there anything off about them? What do the VP ratios look like? This information can give us insights into the basic properties of the capsid and can provide some advanced warning about any potential issues we may observe with infectivity. Beyond this, we need to consider how to titer the physical capsid – can we use an existing ELISA kit or do we need to develop an entirely new method? Finally, having an understanding of the surface charge and aggregation propensity of novel capsids can really help when we are developing our final product presentation in order to maximize our concentration, minimize aggregation, as well as to optimize the formulation buffer for long-term stability.

**Q** What are the main pros and cons of the current vector characterization toolbox in this regard?

**LMD:** Regarding the pros, many of the existing biophysical methods work quite well in characterizing the AAV capsid, if you know what you are looking for. For early characterization work, we employ methods that may not necessarily be utilized for product release but can provide basic biological information on the vector. And having a greater understanding of the capsid allows us to develop a more optimized manufacturing process, improve formulations, and enhance overall product stability.

In terms of the cons, I would say that *in vitro* potency assays don't always provide the full picture. We are often working with immortalized cell lines that have been passaged many times, and they don't necessarily represent our target tissue anymore. Furthermore, cell-based bioassays are highly variable. They can be difficult to interpret because of the complex mechanism of action of the viral vector drug product. Additionally, one single assay may not be adequate to assess both the gene expression and the biological activity of the product. In the end, this can make it challenging to establish correlation between *in vivo* and *in vitro* potency assays.

**Q** Staying with the analytical toolbox theme, can you comment on the challenges in introducing/integrating novel, high-throughput analytical tools, and how you approach these challenges?

**LMD:** Automation of certain assays can certainly enhance sample throughput and provide additional information on in-process manufacturing steps with a shorter turnaround time. It can also free-up our analysts from running mundane and repetitive assays, allowing them to focus on more complex method development, which is generally

more mentally stimulating. This being said, one of the biggest challenges in introducing these high-throughput analytical tools is maintaining high quality data that we can rely on. Time savings are worthless if we can't trust the data that is generated.

These novel tools, including liquid handlers, automated nucleic acid extraction, automated immunoassays, and robotic arms, must incorporate ease of use into their user interface without complicated programming being required. They must keep intra- and inter-assay repeatability high and variability low, whilst not requiring costly or highly specific or difficult to acquire consumables (we've seen quite a few problems with this in the last year of the COVID-19 pandemic). The initial cost of the equipment can also be prohibitive to small companies that are just starting out. I think offering options at different price points, or buildable or customizable models that can be added to as the company grows, may be appealing to all the new players in the cell and gene therapy field.

Internally, our approach to integrating these novel tools is very pragmatic. Firstly, we research the major competitors in the field. We speak with their technical specialists and begin to build out a spreadsheet breaking down the major pros and cons of the instrument. A few models are then selected for hands-on demos, after which we will put the techniques or assays head-to-head with traditional manual methods. If we find that the instrument can save analysts' time, enhance sample throughput, reduce assay variability, and/or increase the sensitivity or range of the assay, we will move to incorporate that instrument into our workflow.

It's a huge plus if the equipment manufacturers have platforms that are ready to go for gene therapy applications – also if their equipment is already being used by the big players in the CRO and CDMO space, which can allow for more efficient tech transfer of our internal methods.

**Q** How does LogicBio's proprietary genome editing platform, GeneRide, impact on the gene therapy product development side of things? What are the main considerations and priorities there from a CMC point of view?

**LMD:** Overall, I would say that there is little difference in the development of a GeneRide product versus a canonical gene therapy product from a CMC point of view. The biggest difference for us is in demonstrating GeneRide product potency, using either an *in vivo* or *in vitro* approach. Due to the fact we integrate directly into the albumin locus, the homology arm needs to be species-specific as does the transgene, and this can make correlating potency across different *in vivo* and *in vitro* models very challenging.

**Q** Let's go deeper on this very hot topic for gene therapy currently – potency assays. What more can you tell us about LogicBio's approach in this particular area, and your thoughts in general on how to approach this particularly complex and evolving aspect?


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"As the gene therapy field continues to evolve, I anticipate we will see increasing importance being placed on potency assays. We are beginning to see a shift from assessing potency of products *in vivo* and more towards *in vitro* methods. Scientists are already implementing automation in potency assays, which can enhance assay throughput and allow us to get a more complete picture of the effects of different steps of the manufacturing process on our product."

**LMD:** Gene therapy potency assays are notoriously challenging due to the rather complex mechanism of action of viral vectors. And for integrating GeneRide products, we have a few additional challenges that we must address. Firstly, we need to be able to distinguish our integrated transgene from the endogenously produced gene, and also from any episomal gene expression. Additionally, we have observed that treated cells tend to proliferate over untreated cells in a diseased tissue, demonstrating a selective advantage. Unfortunately, we are unable to recapitulate this exact process *in vitro*.

Our overall approach towards measuring potency for GeneRide products is to develop a matrix of assays. This means we will use a combination of assays that target different aspects of our product's mechanism of action in order to paint a bigger picture of its potency. For example, we may choose to evaluate product infectivity, gene expression, protein production, and/or the biological activity of the produced protein. Each assay will in turn provide a snapshot of information and lead to a synergistic increase in understanding of our product.

As the gene therapy field continues to evolve, I anticipate we will see increasing importance being placed on potency assays. We are beginning to see a shift from assessing potency of products *in vivo* and more towards *in vitro* methods. Scientists are already implementing automation in potency assays, which can enhance assay throughput and allow us to get a more complete picture of the effects of different steps of the manufacturing process on our product. Additionally, more conferences are popping up each year to address the growing area of gene therapy potency assays, and the regulatory agencies are closely monitoring all the latest advancements in this space – I think that it's likely we'll see additional guidance for sponsors in the next couple of years.

We have certainly come a long way in growing our knowledge of AAV vectors over the last ten years, but there is still a long way to go before we truly develop a robust understanding of AAV gene therapy products – such as we have with mAbs, for example.

**Q** Finally, can you summarize the key goals and priorities, both for yourself in your own role and for LogicBio as a whole, over the coming 12–24 months?

**LMD:** In the next couple of years, the analytical development team will be working towards demonstrating comparability between our previous and our next-generation manufacturing processes. New analytical methods that are coming online now will be validated alongside existing methods to support the manufacturing and release of future clinical material. In addition, extensive analytical characterization work will be required for forced degradation, and extractables and leachables studies.

On a personal level, I'm really looking forward to further building out the development capabilities of our team, as well as becoming more involved in organizations that provide guidance on potency assay development for gene therapy products.

For the company as a whole, our primary goal will be working towards demonstrating safety and efficacy of LB-001 in the clinic. We have also initiated multiple programs, both internally and through collaborations, for new gene editing and gene therapy projects over the past year. We will continue to advance these towards the clinic.

With GeneRide, we are working towards harnessing an important property, which is the ability of corrected cells to selectively expand in a damaged tissue, especially the liver. We expect to be able to treat many serious diseases with this technology.

With our gene therapy programs, we are using our first novel capsid from our sAAVy platform, sL65, which has properties we think will be clinically beneficial. We are also working to expand the sAAVy platform to develop capsids that target other organs in order to expand the use of GeneRide and gene therapy technologies outside of the liver. Our current focus is on pediatric disease because this is an area of high unmet need, and we believe our technology can address this underserved population.

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY CMC & QUALITY CONTROL

SPOTLIGHT

INTERVIEW

# Next steps in the evolution of AAV vector characterization technologies

**EDUARD AYUSO** is Chief Technology Officer at DiNAQOR (Zürich, Switzerland), a genetic medicine platform company focused on addressing severe inherited cardiac diseases. Dr Ayuso is an expert in the field of gene therapy using viral vector platforms, including adenoviral vectors, helper-dependent adenoviral vectors, and adeno-associated vectors (AAV), as well as their design, manufacture, and purification. With over two decades of academic and industry consulting experience, he has made significant contributions to the field of in vivo gene transfer in small and large animal models of diseases, as well as AAV vector development and analytics. Dr Ayuso previously served as Head of Innovative Vectorology at the French National Institute of Health and Medical Research (INSERM), and as the Scientific Director of the Translational Vector Core unit at University of Nantes. Since 2018, he has served as vice president of the French Society for Gene and Cell Therapy. He is also a member of the Translational Science and Drug Product Development committee of the American Society for Gene and Cell Therapy. Dr Ayuso earned his PhD in Biochemistry and Molecular Biology and his degree in Veterinary Medicine from Autonomous University of Barcelona.

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## Q Tell us about DiNAQOR's technology platform and R&D pipeline

**EA:** DiNAQOR is a genetic medicine platform company focused on addressing severe inherited cardiac diseases.

The DiNAQOR technology platform is built upon three pillars: We have the vector design and manufacturing piece. Then we have a platform for engineering heart tissue, which is a mini-heart derived from iPS cells that provides a disease model and more importantly, a testing tool to greatly accelerate development of new drugs in the cardiac space. It is one of our core technologies that we have recently implemented in our new facilities in Zurich. And third, we are developing a delivery device that allows local regional delivery of our vectors specifically to the heart.

These three technologies – vector design/vector production, engineered heart tissue technology, and local regional, organ-specific delivery – combine to advance and grow our gene therapy pipeline. Our lead candidate, an AAV-based gene therapy targeting cardiac myosin-binding protein-C (MYBPC3) cardiomyopathies, is in partnership with BioMarin Pharmaceutical, and we are working together to move it into the clinic. Further up the pipeline, we are targeting other monogenic cardiomyopathies.

## Q What are you working on right now?

**EA:** The main initiative we are working on is to bring our recently opened R&D facilities in Zurich up to full speed. That's for vector design, vector production for research purposes, and process development/analytical development.

In parallel to the R&D activities, we are working together with our Chief Product Officer to set up GMP facilities in the same location. Our research capabilities are ready but process development is a work in progress, and includes establishing all the operational processes relating to the new building where research production and GMP manufacture will be co-located. We will then work to set up the GMP facility itself, which will have a 26,000 square-feet footprint with clean rooms scalable to 500-liter capacity.

Finally, my unit is also working on a program for AAV capsid development, with the aim of delivering improved capsids for more efficient, safer gene therapy applications in the cardiac disease area.

## Q Reflecting upon your more than two decades of experience in the vectorology and vector manufacturing fields, what strikes you most today in terms of recent evolution in viral vector production – firstly, on the bioprocess side?

**EA:** The major bioprocess-related change we have seen in the last decade is the move from adherent cell culture to suspension culture, which has allowed more industrial approaches and increased production capacity.


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Today, it's all about process intensification, which is what will really make a difference in terms of industrialization. We can still improve in this area but overall, I think we have really made significant improvements since I started working on these vectors twenty years ago.

"Today, it's all about process intensification, which is what will really make a difference in terms of industrialization ... I think we have really made significant improvements..."

**Q** And how about on the bioanalytical side?

**EA:** I think there have also been impressive advancements on the bioanalytical side, with a number of technologies coming into the gene therapy space from other biotherapeutic fields (antibodies, proteins, vaccines, etc).

All of these technologies that weren't available at the beginning, including high-pressure liquid chromatography (HPLC), mass spectrometry, and next-generation sequencing, are making a difference. Sequencing in particular is driving a significant improvement in our understanding. The key next step will be to fully adapt these technologies to viral vectors, which are more complex than protein or antibody therapeutics, so that they ultimately have enough specificity and accuracy to provide a release testing method.

**Q** Can you expand on any specific examples where process analytical tools are being successfully incorporated and integrated into viral vector bioprocess devices or steps?

**EA:** Most of the efforts and examples to date are in improving process analytical tools for upstream bioprocessing. There are several examples I can cite. The first of these, and perhaps the easiest one, was to bring cell counting inline, which means we can now have very precise and timely measurements of cell concentration. This is an important parameter for intensification, but also for perfusion, eventually.

There is also metabolite analysis. I think this is a really important advancement because it allows us to closely monitor and optimize our strategies, allowing for more accurate fed-batch approaches and again, helping to establish perfusion approaches. This is key if you want to have a process that is really well controlled. You need to be able to analyze and to correct these parameters very rapidly.

So to sum up, in upstream bioprocessing, cell mass/cell counting and also biomass probes are important for controlling process intensification, while metabolite analysis is a key evolution that is increasingly being implemented.

**Q** What progress do you see in accelerating release testing for gene therapy products?

**EA:** I believe there is still a difference between release testing for early batches for Phase 1 trials and commercial product. In the early phases, the release package is relatively small and straightforward, because it is based on traditional methodologies that are well known to both manufacturers and regulators. However, we know that this is not enough. We need to have more characterization, and that's why the field is developing and adopting new analytical tools.

The new analytical tools will give us a much better understanding of the quality and safety of the drug. But first these new technologies have to be developed and implemented, and we then need to understand the new specification values before they can be validated in the regulatory and release testing context. Today, many of these new analytics are not yet part of release testing. They are part of characterization only, albeit extended characterization.

As these new methodologies mature and confidence in them grows, they will become release assays. And as I mentioned, most of the new bioanalytical technologies coming in have much quicker time to readout than those we use for release testing today.

**Q** What do you see as the key remaining challenges for the field in implementing novel bioanalytical tools and bringing them inline?

**EA:** I would come back to the fact that the bioanalytical tools entering the viral vector manufacturing space from biopharma must be adapted for both the different particle size and the much greater complexity of the product.

With AAV vectors, you have the protein component (the capsid) but you also have your DNA, and you need to be able to characterize them both. The complexity and the challenge is in finding methodologies that provide a very good understanding of each component.

On the DNA side, we have discussed the availability of potentially game-changing sequencing technologies, but these must be adapted very specifically to the product. To begin with, depending on your vector, you might be dealing with single-stranded or double-stranded DNA, and the sequencing technology must first be adapted to whichever is the case. And again, with these new technologies we don't yet know exactly what it is we are expecting to see. We have to do strong characterization work in order to be able to generate and recognize an accurate measurement. We are getting there, step by step, but there is still work to be done on the genome sequencing side, particularly at the single molecule level.

> "..there are more technologies coming in from the protein therapeutics field that are already proving to be very helpful, including HPLC and mass spectrometry. The challenge today is enhancing the accuracy of these methods."

Turning to characterization of the capsid, there are more technologies coming in from the protein therapeutics field that are


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already proving to be very helpful, including HPLC and mass spectrometry. The challenge today is enhancing the accuracy of these methods.

**Q** What gene therapy analytical tools would be on your wishlist for the future?

**EA:** We have huge numbers of particles in our vector preparations – $10^{15}$, $10^{16}$ – and most current methods require you to analyze what is a somehow heterogeneous population as a pool. For me, the greatest advance would be to allow analysis of both the capsid proteins and the genome at the single particle level.

On the genome side, you extract all the DNA and you characterize it as a whole. Even though there are now techniques that enable single genome analysis, it is still being done from this extraction. You don't really have one-to-one correlation.

With AAV in particular, the composition of individual particles varies at the capsid level, too. You have VP1, VP2, VP3, meaning the compositions that you analyze as a pool could be slightly different from particle to particle. This heterogeneity is currently difficult to analyze.

Technology is evolving towards the single particle level, but it is very challenging and we are not there yet.

**Q** What future evolution do you expect in the regulatory guidance around viral vector characterization and gene therapy QC – and how to prepare to meet the challenges this may present?

**EA:** I think we are still at the beginning, but we have already seen the first steps of evolution in trying to move away from the traditional methods of using adherent cell culture and serum. In general, the first key step is to avoid using products of human or animal origin, and I would say we are almost there.

In terms of characterization, I think technological and regulatory evolution go hand-in-hand. We introduce new therapeutic technologies and analytical methodologies, we characterize them, and we then provide the data to the regulators, who make decisions about the specifications of what we have to do moving forward based on those data. Full-empty capsid measurement, which has become a key regulatory request, provides a good example of this relationship at work.

At the moment, vector purity is a major point of focus for the regulators, so we have to work hard to remove any contaminants by upstream and downstream processing means. Of course, we need analytical technologies to be as accurate as possible in quantifying these contaminants.

Another challenging area that has come to the fore recently is how to quantify and measure vector potency. This is very difficult to achieve because every vector has a different objective or purpose, and this must be reflected in its potency assay(s). For this reason, you cannot really

standardize potency assays across sponsors and this remains a major challenge for the field. Furthermore, the regulator increasingly want potency assays to be in place earlier and earlier in development.

Moving forward, I think regulators will continue to ask for better quality and better characterization, and our next steps as an industry must be to develop the tools that can deliver the requisite insights into our products.

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# CELL & GENE THERAPY INSIGHTS

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INNOVATOR INSIGHT

# Chromatographic purification with CIMmultus™ Oligo dT increases mRNA stability

Matevž Korenč, Nina Mencin, Jasmina Puc, Janja Skok, Kristina Šprinzar Nemec, Anže Martinčič Celjar, Pete Gagnon, Aleš Štrancar and Rok Sekirnik

One of the major challenges of mRNA based vaccines has been their requirement for distribution and storage at extremely low temperatures, indicating that exposure of mRNA to suboptimal physico-chemical conditions can result in degradation and loss of potency; it is unclear whether this is due to instability of mRNA drug substance, or LNP-encapsulated mRNA, or both. In this study we compare the stability of model mRNA drug substance (eGFP, 995 nt) prepared by affinity chromatography with the stability of mRNA purified by precipitation. We show that both purification methods lead to highly pure mRNA drug substance, however, mRNA purified by chromatography remains stable for 28 days at 37°C, whereas mRNA purified by precipitation is subject to significant degradation under the same storage conditions. We conclude that chromatography eliminates elements and/or conditions with adverse impact on the quality of mRNA to a greater extent than precipitation method and that choosing appropriate purification strategy is crucial not only to achieve target purity but also to obtain a stable product with retained integrity.

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Strengths of mRNA technology were recently demonstrated by the extraordinarily rapid development and clinical success of two vaccines against SARS-CoV-2 and variants [1–3]. However, the challenges of global roll-out exposed limitations associated with (ultra)cold-storage requirements [4]. Moderna's Spikevax vaccine requires long-term storage between -15 and -25°C and BioNTech/Pfizer's Comirnaty vaccine between -60 and

-90°C [5]. Both mRNA vaccines use lipid nanoparticle (LNP) formulation of mRNA drug substance and it is unclear what factors lead to the different temperature requirements for long-term storage of the two vaccines - whether these arise from instability of mRNA drug substance, LNP-encapsulated mRNA ('drug product'), or a combination thereof [5]. A recent publication evaluated stability of LNP-encapsulated mRNA and demonstrated its stability at 25°C for 7 days, suggesting that encapsulation itself has a stabilizing effect on mRNA [6]; instability of mRNA vaccines could thus potentially stem from instability of mRNA drug substance itself.

With the proviso that mRNA stability can be highly sequence-dependent [7,8], it is possible that for a given sequence, impurity profile and/or purification approach, which could induce physico-chemical or mechanical stress, lead to instability of mRNA drug substance. Ribonucleic acids (RNA) are relatively unstable biomolecules, especially when compared to deoxyribonucleic acids (DNA). Degradation rates are very heterogeneous in solution because of the variety of local RNA conformations, which determine hydrolysis rates [9]. Hydrolytic cleavage of the phosphodiester bond is catalyzed by some metallic complexes, including with Mg2+, one of the critical cofactors required during production of mRNA [9], some reports even proposed that full protection from atmosphere is needed to prevent hydrolysis for long term storage [10]. mRNA stability is therefore highly dependent on the chemical environment to which it is exposed during production process.

An IVT reaction mixture contains a number of components, including enzymes, residual NTPs and DNA template, as well as small molecule additives used to improve mRNA yield (spermidine, Triton, etc.), that need to be removed during the manufacturing process. Traditional lab-scale purification methods are based on DNA removal by DNAse digestion followed by lithium chloride (LiCl) or ethanol precipitation, but these methods are chemically harsh, introduce toxic chemicals into the final product and are challenging to scale-up [11].

Chromatography offers a highly selective as well as scalable approach to purification, which when fully optimized delivers high purity of therapeutic agents. Due to its large size (1000 nt mRNA is approximately 400 kDa, nearly 3-times larger than an IgG), mRNA diffusion coefficient is low, rendering traditional chromatographic approaches, which depend on diffusive mass transport, less suitable as a purification tool for this therapeutic class [12]. Convective-flow purification media, such as monoliths, are more suitable for purification of such large biomolecules, providing higher binding capacity, faster purification cycles and lower shear [11,13–16]. For mRNA constructs containing a polyadenylic acid (PolyA) tail, the mRNA can be isolated from the IVT mixture in a pseudo-affinity mode, using affinity of chromatography support-immobilized poly-deoxythymidine for mRNA containing PolyA tail. For constructs that do not contain a polyA tail, charge or hydrogen bonding interactions can be employed [12].

In this study we evaluated the contribution of purification methodology to quality of mRNA drug substance, using stability as an index. Two purification approaches were evaluated, both expected to yield highly pure mRNA: RNA extraction kit was chosen as a standard RNA purification tool used in many laboratories working with RNA, and monolithic chromatography media was selected for their ability to support rapid high-resolution separation of very large molecules in a low-shear environment [11,13–16]. Stability of mRNA at 37°C (optimal temperature for activity of RNAses), 4°C (typical refrigeration temperature), -20°C and -80°C was assessed by a selection of standard physicochemical analytical techniques used for mRNA drug substance quality attribute assessment (UV spectroscopy (A260 nm) for content determination, agarose gel electrophoresis for molecular mass and RNA integrity assessment, chip capillary electrophoresis for quantification


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of fragmentation) [4–6, 17], and two HPLC methods which are reported for the first time: quantification of poly-adenylated mRNA using CIMac™ Oligo dT and purity assessment of mRNA by CIMac PrimaS™. Freeze-thaw stability was also assessed after each purification approach using the same set of analytical methods. For the full materials and methods, please refer to the supplementary data for this article.

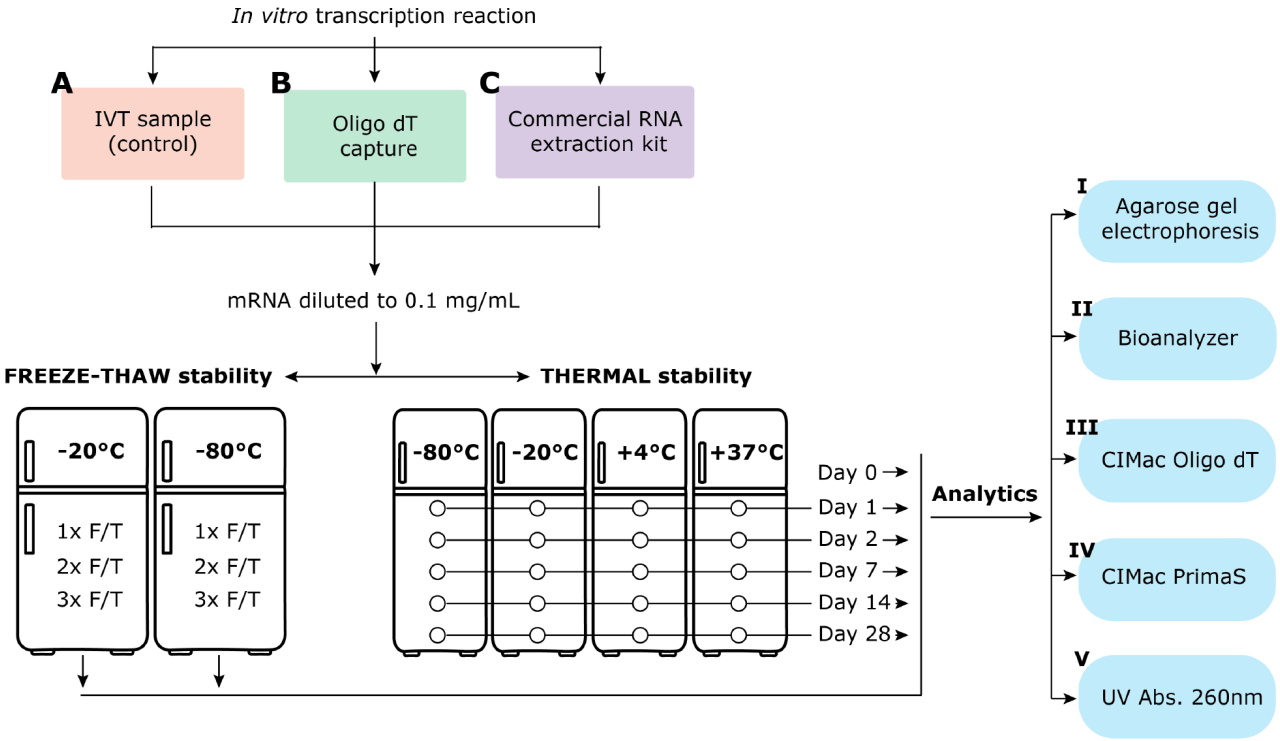
## RESULTS & DISCUSSION

After IVT production of mRNA from a plasmid encoding for eGFP (950 nucleotides) and poly-adenosine tail (45 nucleotides) according to a standard IVT protocol (Figure 1), the reaction mixture was either diluted in $ddH_2O$, purified using affinity chromatography (CIMmultus™ Oligo dT), or purified using a standard RNA extraction kit. Purified mRNA was incubated at defined storage conditions until stability time-points. Note that capital letters label purification approaches and roman numerals label analytical methods in all figures presented.

mRNA at expected molecular size (995 nt) was observed by AGE and BioAnalyzer for all samples. Oligo dT eluate showed a minor band at 2000 nt by both AGE and BioAnalyzer, corresponding to a dimeric form of mRNA, which disappeared with denaturing the sample by heating it at 70°C for 5 min (Supplementary Figure 1). For diluted IVT, analytical affinity chromatography (CIMac™ Oligo dT) revealed the expected flow-through peak, corresponding to UV-absorbing IVT reaction components (nucleotides, enzymes), and the elution peak, corresponding to polyadenylated RNA. Peak area corresponded to expected mRNA concentration (0.1 mg/mL). mRNA purified by affinity chromatography and extraction kit only showed elution peak at expected concentration and no flow-through peak. Similarly, CIMac PrimaS™ chromatogram demonstrated the presence of nucleotide-like reaction components

**FIGURE 1**

mRNA production, purification and stability testing scheme.



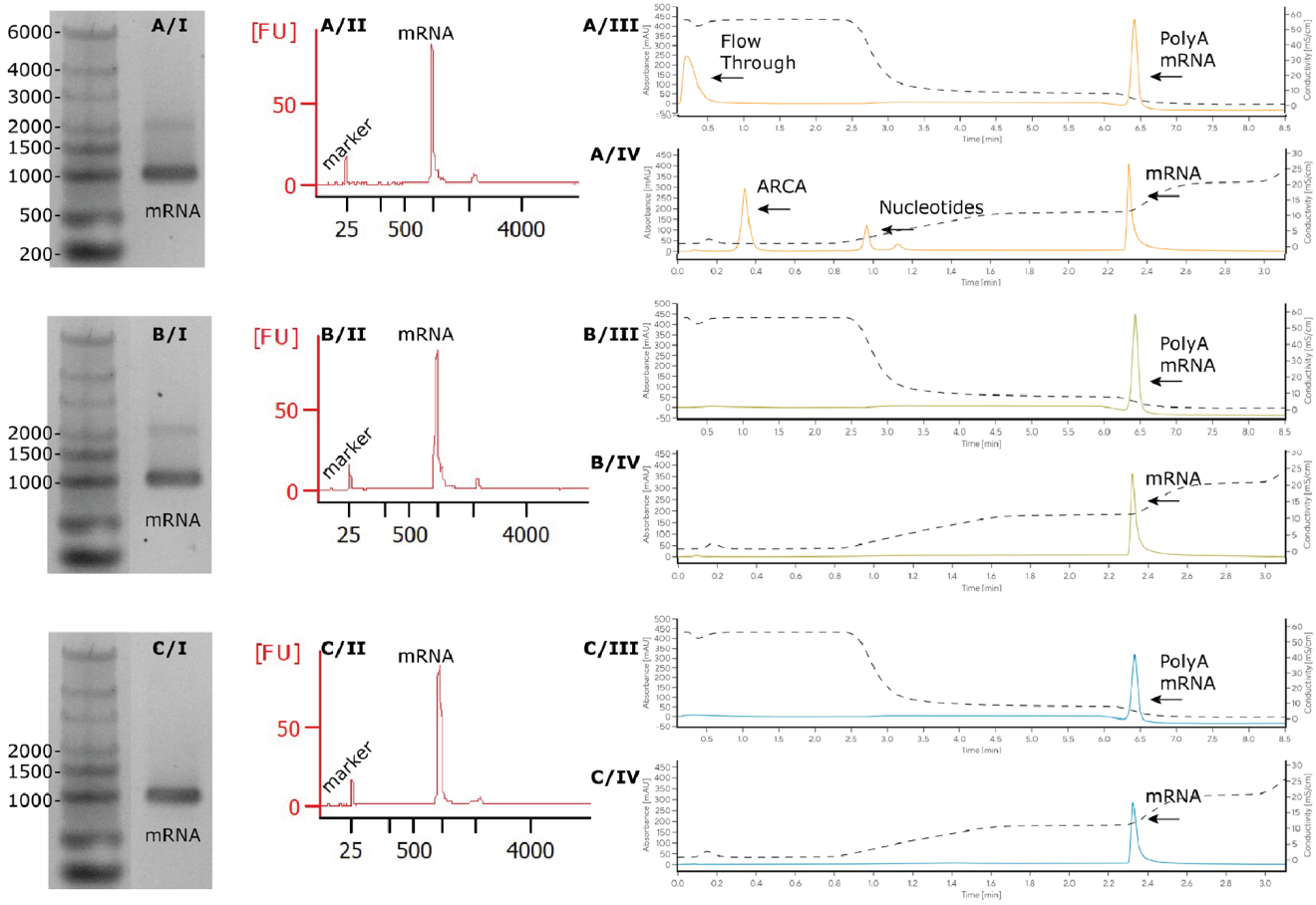
(capping reagent and residual NTPs) and RNA in the IVT sample, but only RNA in samples purified by affinity chromatography and extraction kit (Figure 2), suggesting that both purification methods yield highly pure, poly-adenylated RNA.

Stability of mRNA prepared according to the three methods was then evaluated at different temperatures over a period of 28 days, and freeze-thaw stability at -20°C/-80 was evaluated for up to three freeze-thaw cycles (Figure 1).

All three preparation methods resulted in mRNA that was stable at -80, -20 and 4°C for up to 28 days, and up to three freeze-thaw cycles at -20°C and -80°C (Figure 3) as assessed by electrophoretic methods (AGE and BioAnalyzer, Figure 4), and chromatography (Figure 5). Incubating mRNA at 37°C, however, resulted in significant degradation of mRNA produced by extraction kit or dilution, but not when purified chromatographically. Purity of extraction kit- purified material decreased in a time-dependent fashion; it was less than 20% by day 14 and 6% by day 28 (as determined by BioAnalyzer, Figure 4 (C/II), Supplementary Figure S2). Similarly, smearing of RNA band at 1000 nt was observed by agarose gel electrophoresis which resulted in no observable band at 1000 nt by day 28 (Figure 4 (C/I). In comparison, when mRNA purified by CIMmultus™ Oligo dT was incubated at 37°C, it retained 80% integrity (as determined by BioAnalyzer) by day 14 and

## FIGURE 2

**Electrophoretic and chromatographic properties of mRNA produced by A) IVT, and purified by B) Oligo dT and C) extraction kit. I) Agarose gel electrophoresis, II) BioAnalyzer, III) CIMac Oligo dT, IV) CIMac PrimaS.**




FT: flow-through, ARCA: anti-reverse cap analog,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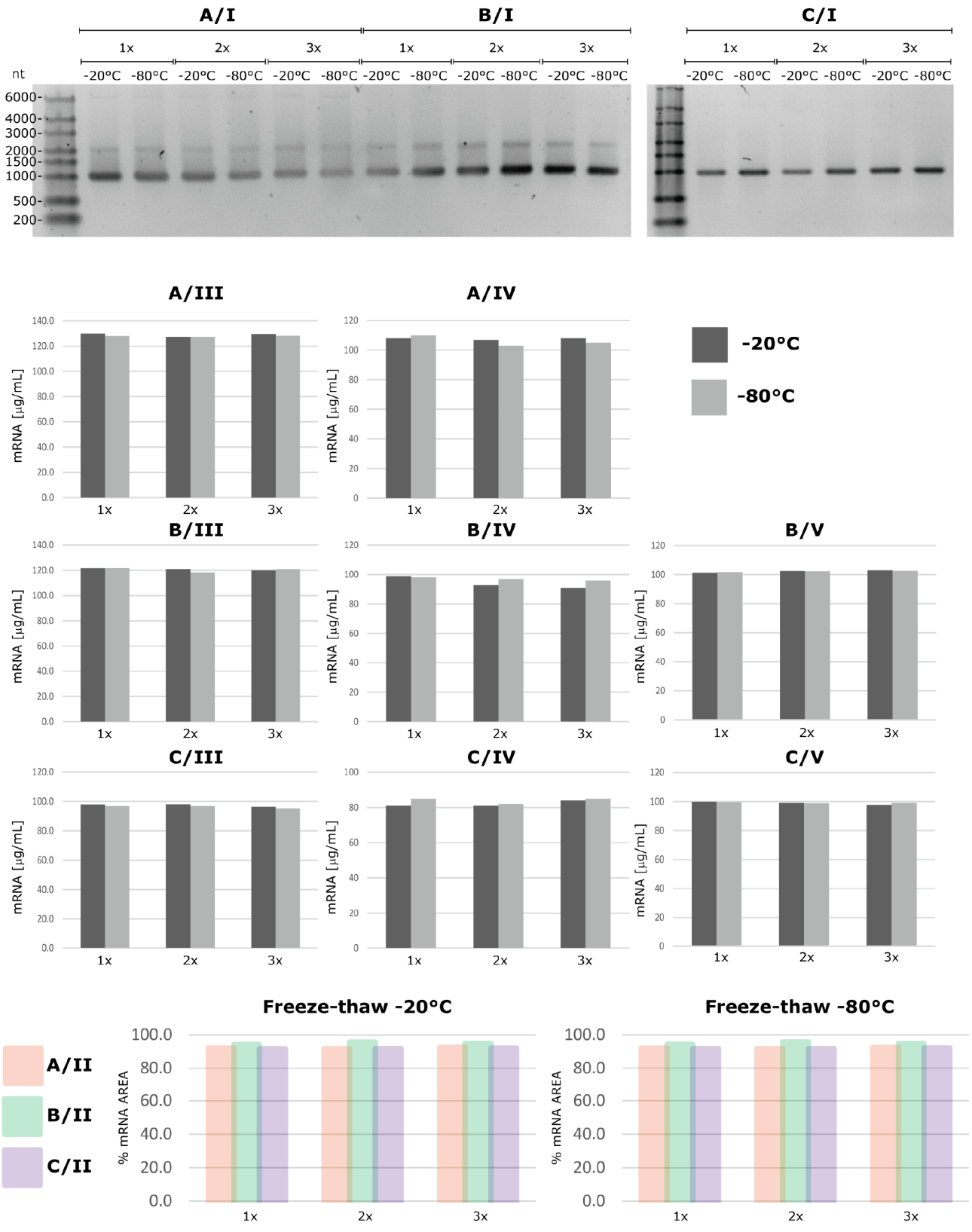
## FIGURE 3

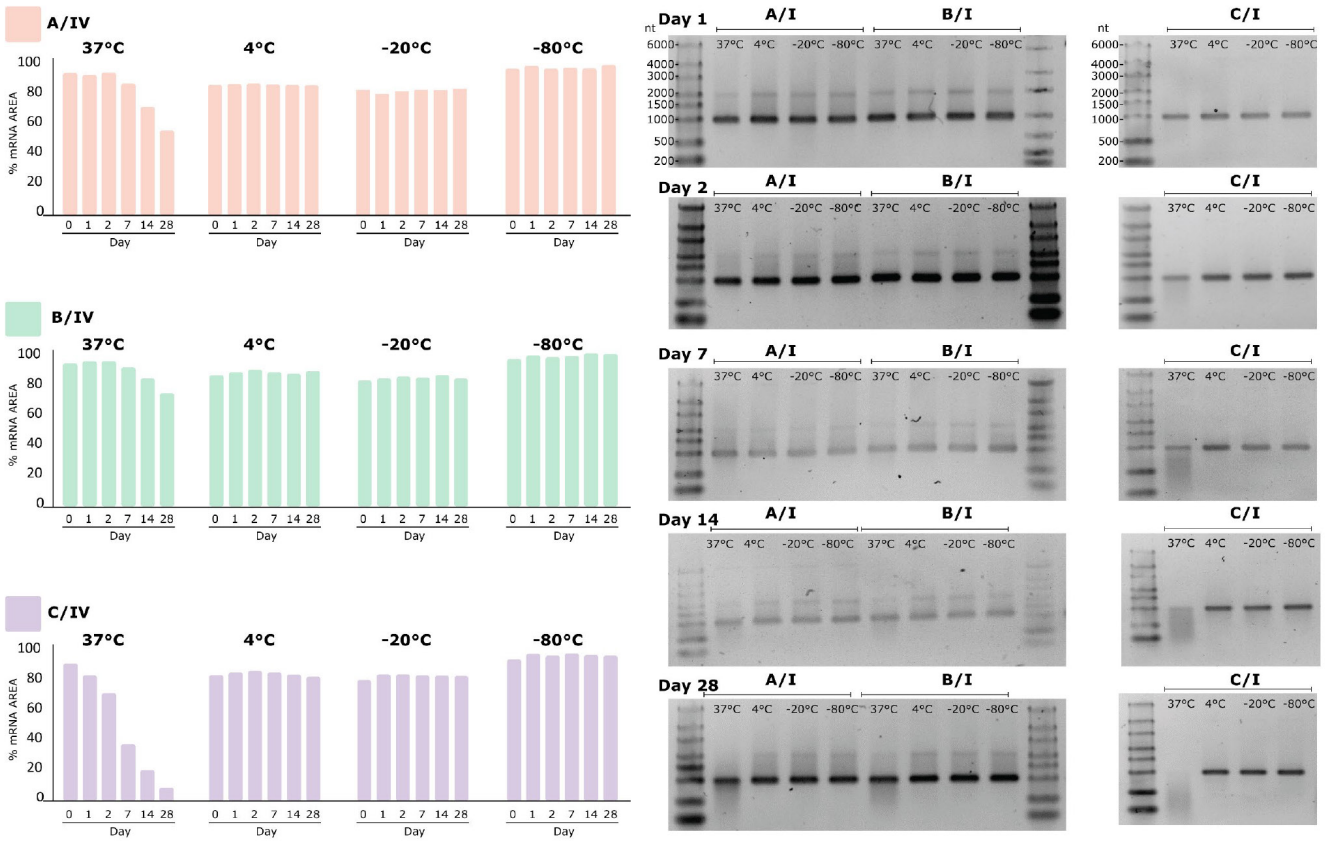
Freeze-thaw stability of mRNA.




**I)** Agarose gel electrophoresis, **II)** BioAnalyzer, **III)** CIMac Oligo dT, **IV)** CIMac PrimaS and **V)** UV (260 nm, right).

## FIGURE 4

**Thermal stability of mRNA at -80/-20/4/37° for up to 28 days.**




**Left:** BioAnalyzer; and **right:** Agarose gel electrophoresis of mRNA prepared by **A)** IVT (diluted), **B)** Oligo dT-purified and **C)** extraction-kit purified.

72% by day 28 (Figure 4 B/II, Supplementary Figure S2); only barely detectable smearing of agarose gel band was observed by day 28.

Chromatographic analysis by CIMac™ Oligo dT (labeled with roman number III in all figures) was in close agreement with electrophoretic results. The content of mRNA prepared by all three methods did not differ significantly for incubation temperatures of -80/-20/4°C, but a significant decrease in content (from starting 90 µg/ml to 38 to µg/ml by day 28) was observed for mRNA purified by extraction kit, but not other methods, when incubated at 37°C for up to 28 days (Figure 5 C/III). CIMac PrimaS™ analysis, which resolves mRNA from NTPs, capping reagent and plasmid, resulted in mRNA peak intensity which did not change with incubation time or temperature (Figure 5C/IV). Interestingly, analysis of peak corresponding to residual ARCA capping reagent (retention time of 0.4 min, Figure S3) in diluted IVT sample indicated a shift to 0.8 min observed at 37°C from day 1 onwards, suggesting possible degradation of the reagent. Although IVT reactions are seldomly carried out beyond 3-6 hours, stability of reactants under IVT conditions (e.g. 37°C) should nonetheless be evaluated to avoid potential safety issues due to incorporation of degradation products into nascent mRNA.

As expected, UV absorbance of stability samples resulted in no apparent difference in content as measured by absorbance at 260 nm even when other analytical methods showed severe degradation (Figure 5 (B/V and C/V)). Content by UV is one of the critical quality attributes assessed for release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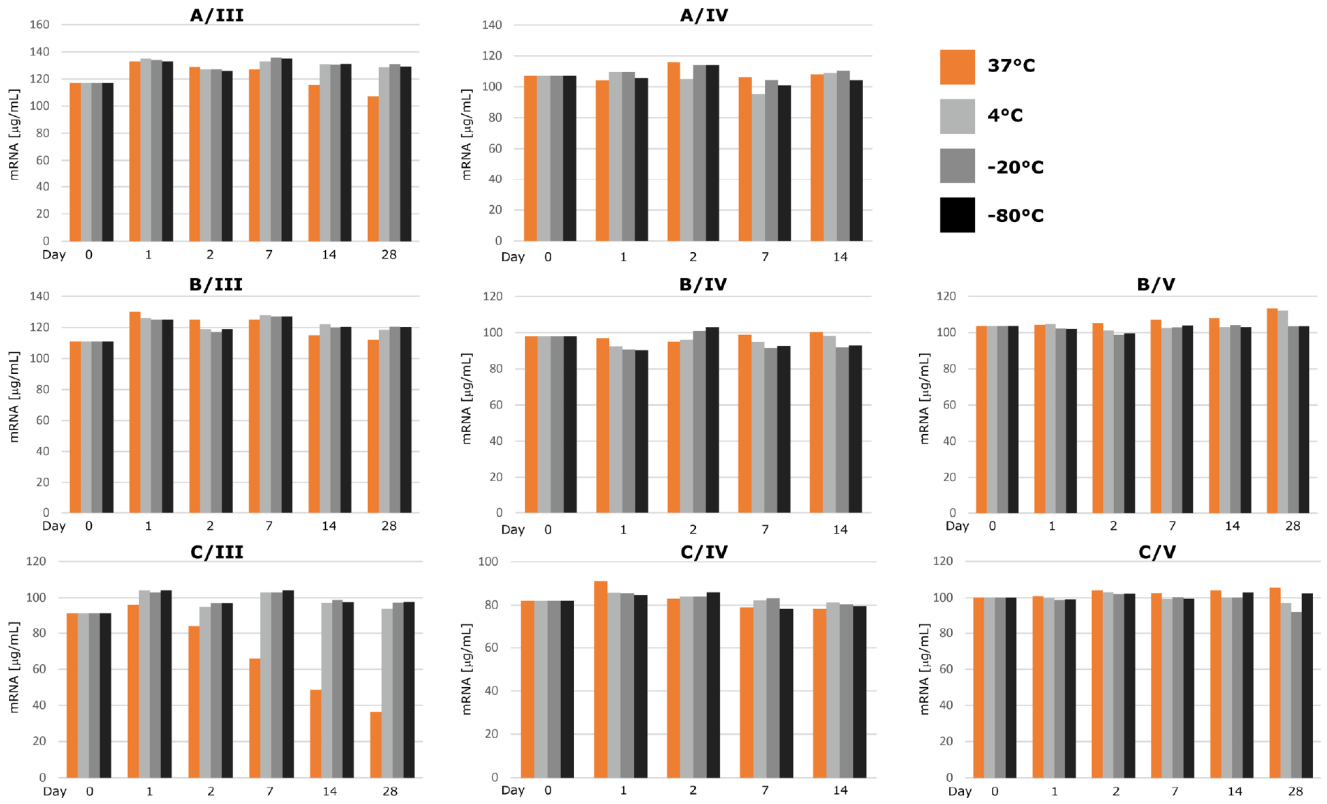
## FIGURE 5

HPLC and UV analytics of mRNA stability.




**III)** CIMac Oligo dT, **IV)** CIMac PrimaS, **V)** UV absorbance at 260 nm (Nanodrop). mRNA was prepared by **A)** dilution of IVT, **B)** CIMmultus Oligo dT and **C)** RNA extraction kit.

of mRNA vaccines [5]; our results point to the need to evaluate the UV results in conjunction with other purity methods, as it is not indicative of content of intact target molecule.

## CONCLUSIONS

In this study, we compared stability of mRNA drug substance (995 nt) prepared by chromatographic method using CIMmultus Oligo dT column versus a precipitation technique using commercial RNA extraction kit. mRNA purified by chromatography was shown to remain stable for 28 days at 37°C, whereas purification by precipitation led to significant degradation of mRNA at 37°C, detectable by electrophoretic (AGE and Bio-Analyzer), chromatographic (CIMac™ Oligo dT), but not UV-spectrophotometric method. Although follow-up studies on a wider range of mRNA sizes and modalities are warranted in the future, our results demonstrate the need for careful selection of purification strategy during development of mRNA therapeutics, which should be based on consideration of long-term stability impact, as well as scalability and compatibility with GMP standards.

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## AUTHORSHIP & CONFLICT OF INTEREST

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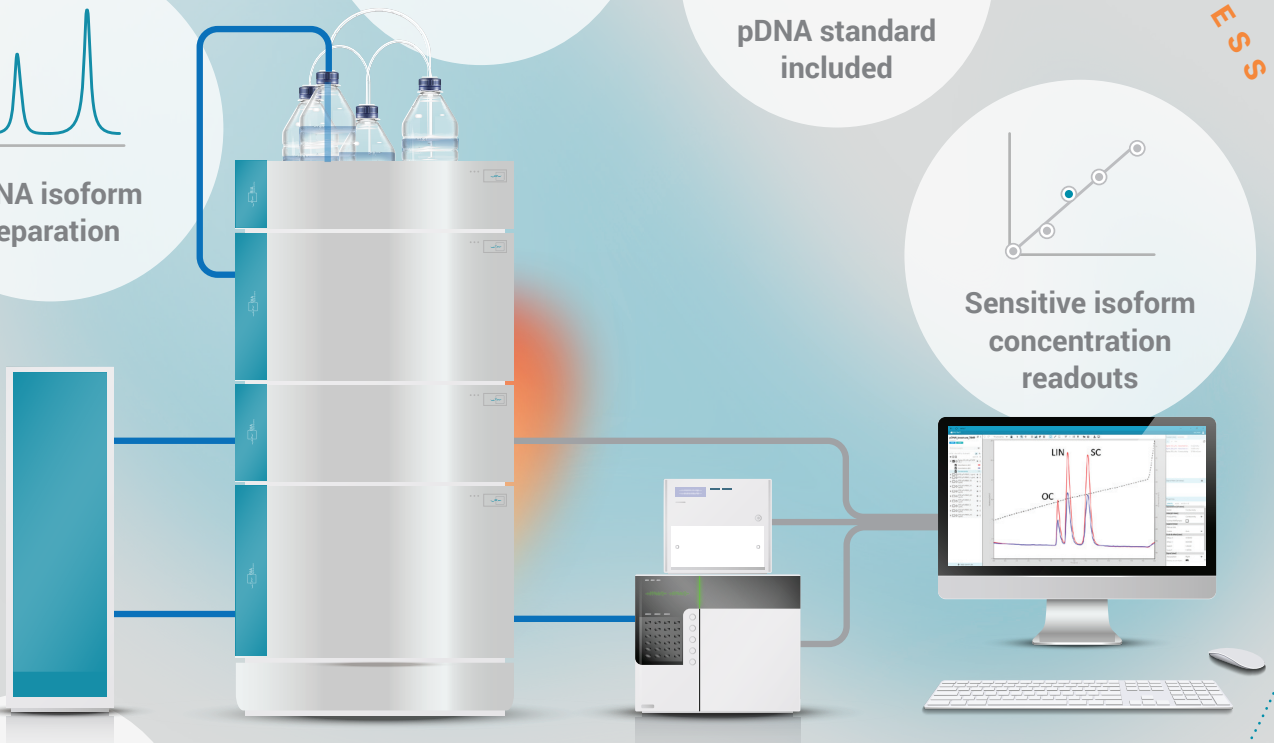


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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

# Practical application of cell counting method performance evaluation and comparison derived from the ISO Cell Counting Standards Part 1 and 2

Yongyang Huang, Jordan Bell, Dmitry Kuksin, Sumona Sarkar, Laura T Pierce, David Newton, Jean Qiu & Leo Li-Ying Chan

The increased utilization of cells in biomanufacturing and as therapeutic products over the last decade has prompted the development and publication of two ISO Cell Counting Standards, ISO 20391 – 1:2018 and ISO 20391 – 2:2019 to provide guidance on general principles relating to cell counting and to establish an approach to evaluate the quality of cell counting methods. In this work, we demonstrate the practical implementation of the experimental protocol outlined in ISO Cell Counting Standard Part 2 and a Bland-Altman comparative analysis to evaluate performance and comparison of cell counting methods. We compare two cell types, two image cytometry instruments, and two fluorescent stains, calculating the precision, coefficient of determination ($R^2$), and a proportionality index (*PI*) parameter to evaluate cell counting method performance. In addition, the cell counting results are directly compared to evaluate bias between two cell counting methods. The protocol is suitable for evaluating and comparing the performance of multiple cell counting methods to select for downstream assays.

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## INTRODUCTION

In the recent decade, cell and gene therapies have drastically improved their efficacy and have become essential players in cancer treatment [1,2]. With the approval of two chimeric antigen receptor (CAR) T cell therapies by the U.S. Food and Drug Administration (FDA) in 2017, the numbers of clinical studies and tests on new and novel cell therapy products have also surged [3–5]. Typically, cellular therapies require genetic modification of the immune cells (i.e. T cells, NK cells) collected from patients, culture expansion, and re-introduction of the final products back into the patients. Therefore, it is critical to provide accurate cell counting for the administration of proper dosages, which may otherwise lead to inefficacy or induce unwanted autoimmune responses in patients undergoing therapeutic treatments [6–8].

In the 21st Century Cures Act, the United States Congress has also recognized the importance of standardization for streamlining development, quality assurance, and facilitating regulatory approval of cell and gene therapy products [9]. In the "Synergizing Efforts in Standards Development for Cellular Therapies and Regenerative Medicine Products" workshop held by the FDA on March 31st, 2014, cell counting and viability measurement assurance were identified as opportunities for standards development [10,11]. ISO has since published two cell counting standards, "ISO 20391-1:2018 Biotechnology – Cell Counting – Part 1: General Guidance on Cell Counting Methods" and "ISO 20391-2:2019 Biotechnology – Cell Counting – Part 2: Experimental Design and Statistical Analysis to Quantify Counting Method Performance", which can serve as guidance for researchers working in the field of immunotherapy and adoptive cell therapy, where both require high quality and robust cell counting measurements for biologics and cell products [12,13].

Derived from general concepts described in ISO Cell Counting Standard Part 1, we propose 6 key factors that can provide guidance on the selection of cell counting methods and improve the quality of the cell counting measurements:

1. Determine the intended use of the cell counting result (e.g. cell count for normalization of bioassays, cell therapy dosing, post-tumor digestion for single cell-based transcriptome analysis, mouse tissue processing for cytotoxicity assays, or isolation of human PBMCs for immunophenotyping analysis, etc.)
2. Investigate to understand cell sample composition (e.g. various cell types, particle debris, chemical impurities, and suspension medium), as well as the morphological appearances of the cells under microscopy
3. Understand the assay principles and select the appropriate cell counting assay, such as total, live and dead cell count, viability, or cell population analysis
4. Investigate the capabilities and select the appropriate cell counting systems, where the system consists of reagents, consumables, instrument, and software algorithms, as well as assay performance criteria (i.e. precision, range, linearity, etc.)
5. Treat each cell counting method as a whole process, including sampling, diluting, and staining, which are critical for proper sample preparation
6. Provide continuous operator training, in order to ensure consistent cell counting results

It is also realized that the cell counting needs for cell and gene therapies are broad due to a wide range of biological sample types with various formulations and bioprocessing steps, which are complex, dynamic, and heterogeneous. Because there are currently no reference materials for live mammalian cells that are certified for cell concentration, the accuracy parameter outlined in the ICH Harmonised Tripartite Guideline – Validation of Analytical Procedures: Text and Methodology Q2 (R1) cannot be readily applied, thus increasing the challenge and difficulty of validating the accuracy of cell counting [14–16].


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Therefore, the ISO cell counting standards can serve as a valuable tool to evaluate and select cell counting methods that are fit-for-purpose, in order to increase confidence in the cell counting results.

## DEVELOPMENT OF THE PROTOCOL

We have employed the guidance from ISO 20391 – 2:2019 Biotechnology – Cell Counting – Part 2 and utilized information from the ICH Q2 (R1) to develop an appropriate protocol to evaluate the performance of selected cell counting methods. The ICH Q2 (R1) guidance document presents multiple parameters for the validation of analytical methods, such as robustness, linearity, detection range, limits of detection (LOD), limits of quantitation (LOQ), precision (repeatability, intermediate precision, reproducibility), and accuracy. It is important to note that since there is no reference material to provide a reference value for cell concentration, the evaluation of the accuracy parameter needs to be indirectly assessed by orthogonal comparative methods. The ISO Cell Counting Standard Part 2 describes a detailed protocol to simultaneously evaluate precision (repeatability), coefficient of determination ($R^2$), and proportionality.

Utilizing the ISO Cell Counting Standard Part 2 document, we have identified several key parameters that can quickly and sufficiently assess the performance of cell counting methods [17,18]. In this work, we will focus on an experimental protocol derived from the ISO Cell Counting Standard Part 2, which evaluates the coefficient of determination ($R^2$ value), precision (repeatability – coefficient of variation [CV]), and proportionality index (*PI*) of a cell counting method. The proportionality index is a metric introduced in the ISO Cell Counting Standard Part 2 that quantifies the degree to which a cell counting method conforms to the principle of proportionality, where it is expected that cell counts will scale proportionally with dilution. The principle of proportionality is a fundamental property of any cell counting method, and any deviation from proportionality would indicate a systematic or non-systematic error resulting in a loss of measurement accuracy. To more directly evaluate systematic deviation from proportionality, which is an indicator of loss of accuracy, the *PI* is calculated by fitting a proportional model to the dilution series data, then summarizing residuals based on smoothed data, thus reducing the influence of random variation on the evaluation of proportionality [17]. There are several approaches to calculate *PI*, where some *PI* metrics may be more relevant based on the fit-for-purpose need of the cell counting method. Some metrics penalize more for outliers, while others weigh errors evenly across the dilutions or allow more contribution by higher cell concentrations. In this work, we utilized the *PI* model published previously from the National Institute of Standards and Technology (NIST) [17]. It should be noted that sources of systematic error which are proportional to sample dilution will not be detected with this approach. For example, if debris are mixed with the cell suspension and falsely identified as cells, concentration of both cells and debris would be proportionally reduced with dilution, and the false counts would not affect the proportionality. In order to demonstrate the appropriate usage of the proposed experiments, these protocols were tested using various image cytometry systems from Nexcelom Bioscience LLC. (Lawrence, MA).

## BLAND-ALTMAN COMPARATIVE ANALYSIS METHOD

Comparative analysis methods can be employed to compare the performance of different cell counting methods. While the lack of reference material precludes the direct measurement of cell counting accuracy, comparison of orthogonal methods may serve as a viable alternative. It is also often desirable to determine how closely the results of one method will agree with another, such as when an instrument is

upgraded after many years in the lab. One useful method is the construction of a Tukey mean-difference plot, also known as a Bland-Altman plot [20–22]. The Bland-Altman analysis results in the calculation of a bias (with corresponding confidence interval) between two methods, indicating which method counts higher or lower on average and by how much. The analysis also provides an estimate of how well the two methods are expected to agree for a single sample. Here we modify the dilution series experimental design described in ISO Cell Counting Standard Part 2 document to collect data appropriate for a Bland-Altman analysis while also meeting the standards requirements for calculating CV, $R^2$, and *PI*.

Usually, Bland-Altman plots consist of absolute differences between two measurements plotted against their mean. In the case of cell counting, variance is not constant for different concentrations, but is generally proportional to the number of cells counted [17]. For Bland-Altman analysis to be useful in such an application, the data can be transformed to achieve roughly constant variance across a range of concentrations. In this protocol, we use percent differences rather than absolute differences to achieve more uniform variance.

The Bland-Altman analysis method produces three metrics of comparison:

1. The bias between two methods, which is the mean of the differences.
2. The limits of agreement (LoA), which are a multiple of the standard deviation of the differences.
3. The confidence interval (CI) of the bias, which is a multiple of the standard error of the mean of the differences.

The bias describes the average difference between measurement results obtained via the two methods. Due to biological variation in the samples and variability in the measurement process for both methods, it is impossible to predict exactly how much the measurement of any single sample will differ between the two methods. However, when measurements of many samples are averaged, a bias – even a slight one – may become clear. The bias may be interpreted as one method measuring higher or lower than another on average, though the difference between methods when measuring a single sample may vary widely.

The limits of agreement describe how widely these differences may vary. When added to and subtracted from the bias, the LoA define a range within which the difference between the measurements from two methods of a single sample is expected to be found. In this protocol, we use the limits of agreement that approximate a 95% confidence interval (1.96 × standard deviations) for a normal distribution. This is a sufficient approximation for our purposes and 72-measurement sample size. If fewer measurements are acquired, confidence intervals calculated from the appropriate t distribution (rather than the Normal distribution) are advised. If the percent differences between the results from the two methods follow a normal distribution, we can expect that 95% of the differences will fall within one LoA from the value of the bias. In reality, the values will not be strictly normal, but the approximation is useful for evaluating subsequent measurements [23]. If more statistical rigor is required, tests for normality can be applied, and the confidence intervals can be more exactly calculated [24]. Depending on the variation observed between samples relative to the variation between replicate measurements from each sample, it may be helpful to include random effects terms typically included in analysis of hierarchical experiments. In this work, we were not concerned with the sample-to-sample variation in the proposed experiments.

The confidence interval of the bias provides the approximate uncertainty for the calculated bias value and suggests a range within which the true value of the bias between the two cell counting methods is likely to be found. Unlike the LoA, this confidence interval narrows with an increased


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number of samples measured. If the 95% confidence interval is larger than the absolute value of the bias (i.e. the CI brackets the value 0), the method comparison has not demonstrated a statistically significant bias between the two methods (at $\alpha = 0.05$ significance level). With enough samples, even a very slight bias may be confidently measured. A slight bias is often negligible compared to sample variation. Researchers should consider how a cell count is being used in order to determine acceptable levels of bias in their case.

Before proceeding with Bland-Altman comparative analysis for cell counting, researchers should:

1. Determine the range of cell concentration values for which comparison between the two methods is desired;
2. Determine what values of the bias and LoA are acceptable for their application,
3. Select cell samples that are representative of the population for which the comparison is desired and the range determined in step 1; and
4. Measure each sample using the different cell counting methods, taking care that the sample does not change between measurements (minimal delay between measurements, proper mixing, etc.) [25].

A higher number of paired measurements can reduce the uncertainties of the bias and LoA, e.g. the confidence interval of the bias narrows with more measurements. Researchers should determine the precision they require, and increase the number of paired measurements accordingly – we suggest a minimum of 20 paired measurements be used as a starting point. Finally, it is possible that either the bias or the variation will vary with cell concentration. In such a case, the bias and LoA obtained for the entire group of data may not be representative of how the two methods compare over a narrower range of concentrations. It may be useful to perform Bland-Altman analysis on smaller subsets of data.

## APPLICATIONS OF THE METHOD

The cell counting method performance evaluation and comparison protocols can be applied to research, analytical method development, process development, and preclinical or clinical trials. In addition, the method can be applied to a plethora of research fields requiring the usage of cells such as cellular and gene therapy, immuno-oncology and immunotherapy, cell line development and biologics production, virology and infectious disease, regenerative medicine, toxicology, food science, and even renewable energy. The quality of cell counting results is critical for a wide range of cell types used in the research fields mentioned above. These cell types can include primary cells such as human or mouse whole blood, cord blood, bone marrow aspirate, adipose tissue, hepatocytes, PBMCs, leukapheresis sample, platelets, tumor or tissue digests are typically used. In addition, bacteria and yeast cells are often used to generate biologics or used for beverage production.

## EXPERIMENTAL DESIGN

The cell counting method performance evaluation proposed here consists of a dilution series experiment and comparative analysis for multiple methods. The experimental design is demonstrated using CHO-S and Jurkat cell lines fluorescently stained with acridine orange and a green nuclear dye. Two cell counting systems are compared: the Cellaca MX High-Throughput Cell Counter (Cellaca MX) and the Celigo Image Cytometer (Celigo). It is important to note that ISO Cell Counting Part 2 requires users to assess pipetting error contributions to dilution integrity to establish confidence in dilution and sampling. Here, we conducted a pre-evaluation of pipetting error, which will not be described in this protocol. It is also important to investigate the stability of the target cell sample prior to conducting the experiment in order to avoid drift in concentration and viability during the assay time frame. The stability of

the Jurkat and CHO cells used in this work have been previously tested and showed no noticeable trends (Supplementary Figure 1).

The dilution experiment consists of a 6-point concentration series of the target cell types, where each concentration is independently produced from the original stock (rather than the other dilutions) to reduce a propagation of dilution error that can affect proportionality. The dilution series should span the typical concentration range of the target cell samples in order to evaluate the performance of the cell counting method in the specified range.

Three replicate samples are generated per concentration, and each replicate sample is measured 4 times per cell counting method so that each method provides a total of 12 measurements per concentration and a total of 72 measurements in a 6-point concentration series. The measurements are used to calculate the coefficient of determination ($R^2$), precision (repeatability – Coefficient of Variation, CV), and proportionality index (*PI*) parameters for each cell counting method. It is important to note that the tested Jurkat and CHO cells were stained with acridine orange and Nuclear Green dye to measure only the total cell concentration in this work.

For performance comparison between two cell counting methods, the Bland-Altman method is applied. Like the proportionality measurement, the comparison results are valid only for the intended use of the specific methods (cell type, assay type, exact instruments, etc.), and only for the range of cell concentrations included in the test. Therefore, it is vital to first define the exact methods, test conditions, and range of cell concentrations over which comparison is desired. For most accurate results, the Bland-Altman analysis should include as many measurements as possible, encompassing the sources of variation that are expected for the normal operation of the cell counting method, such as multiple operators, reagent lots, and cell culture flasks. Each point on the Bland-Altman plot is obtained by using both cell counting methods to measure a single sample. The sample should be carefully mixed to ensure homogeneity before portions are taken for measurement with each cell counting method. Measurements should be made with minimal lag time between them, simultaneously if possible. If the experiment described above is performed with the same tubes of cells using both methods at the same time, Bland-Altman analysis may be performed with the resulting data. If desired, tighter confidence intervals on the calculated bias or less uncertainty on the Limits of Agreement can be obtained by supplementing the data with more samples. Concentrations spanning the selected concentration range should be represented roughly equally in the samples used.

## EXPERTISE NEEDED TO IMPLEMENT THE PROTOCOL

In general, the expertise required to implement the cell counting method performance evaluation is proper training by an expert user in the operation of the cell counting systems. In addition, the users should be trained on sample preparation to ensure consistent performance of the dilution, sampling, and staining steps of the cell counting process.

## LIMITATIONS

Accuracy is one of the most critical parameters for the validation of an analytical method, however, it cannot be directly applied to most cell counting methods. Since there are limited live cell reference standards, it is challenging to assess the accuracy of a cell counting method. Therefore, proportionality is an alternative parameter to assess accuracy relative to dilution fraction, which serves as the internal control, as well as utilizing orthogonal methods for comparison.

It should be recognized that $R^2$ values calculated over a range of concentrations are strongly dependent on the range chosen. A larger range of linear data results in an $R^2$ value closer to 1. If comparison between $R^2$


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values is to be attempted, it is important that the range for the two calculations be the same. In addition, it should be noted that the proportionality index as defined here is not normalized to the number of dilution fractions and the number of biological replicates per dilution fraction. It is required that the same experimental design be used if *PI* is to be meaningfully compared between methods.

## MATERIALS

### Documentation materials

- ISO 20391-1:2018 Biotechnology – Cell Counting – Part 1: General Guidance on Cell Counting Methods
- ISO 20391-2:2019 Biotechnology – Cell Counting – Part 2: Experimental Design and Statistical Analysis to Quantify Counting Method Performance

### Biological materials

- Chinese Hamster Ovary (CHO-S) cell line (Gibco, #11619012)
- Jurkat, Clone E6-1 cell line (ATCC, TIB-152™)

### Growth medium & supplements

- CD CHO Medium (1X) (Gibco, #10743011)
- GlutaMAX-1 (100X) (Gibco, #35050061)
- HT Supplement (100X) (Gibco, #11067030)
- RPMI Medium 1640 (1X) (Gibco, #11875093)
- Fetal Bovine Serum (FBS) (Access, #A19023)
- Antibiotic Antimycotic Solution (100X) (Sigma-Aldrich, #A5955-100ML)

### Fluorescent staining reagents

- ViaStain™ AOPI Staining Solution (AOPI, Nexcelom Bioscience, CS2-0106-5mL)
- ViaStain™ AO Staining Solution (AO, Nexcelom Bioscience, CS2-0108-5mL)
- ViaStain™ Total Cell Nuclear Green (Nuclear Green, Nexcelom Bioscience, CS1-V0008-1)

### Other reagents & chemicals

- Phosphate Buffered Saline (PBS) powder (Sigma-Aldrich, #P38135)
- HyClone™ Water, Cell Culture Grade (Endotoxin-Free) (GE Health, #SH3052903)

### Equipment

- Tissue culture hood (Forma Scientific, ClassII A/B3 BSC)
- Cell culture incubator (Thermo, Forma 370)
- Plate rocker (Boekel, RockerII 260350)
- Automatic pipettor (Fisherbrand™ Pipet Controller, #FB14955202)
- Manual pipettors (P10, P100, P1000) (VWR, 1-10UL, 10-100UL, 100-1000UL)
- Centrifuge (Eppendorf, 2702)
- Cellometer Spectrum and operating laptop computer (Spectrum, Nexcelom Bioscience)
- Cellaca MX High-Throughput Automated Cell Counter and operating laptop computer, concentration range of 1 x $10^5$ – 1 x $10^7$ cells/mL (Nexcelom Bioscience)
- Celigo Image Cytometer and operating desktop computer (Nexcelom Bioscience)

### Disposable instruments

- T-75 $cm^2$ flask (USA Scientific, CC7682-48)
- 15-mL centrifuge tube (Greiner Bio, 188271)

- Serological Pipets 5 mL, 10 mL, 25 mL (USA Scientific, #1075-0110, #1071-0810, #1072-5410)
- Pipette tips (P10 and P1000) (VWR, 7320561, 83007-380)
- Pipette tips (P200) (USA Scientific, 11111210)
- Microtubes 1.5 mL (VWR, 89000028)
- Microtubes 0.5 mL (CellTreat, 229440)
- Cell counting slides (Nexcelom Bioscience, CHT4-SD100-002)
- Cellaca MX High-throughput Automated Cell Counter Plates (Cellaca MX plates, Nexcelom Bioscience, CHM24-A100-001)

## REAGENT SETUP

### Phosphate-Buffered Saline (PBS) solution

Prepare the PBS solution by mixing 5 L of H2O with 1 packet of PBS powder to generate a solution of 0.01M PBS at pH 7.4 with NaCl at 0.138 M and KCl at 0.0027 M.

### CHO-S cell culture medium

Prepare CHO-S medium (500 mL) with the CD CHO Medium (1X) and supplement with 5 mL of the GlutaMAX-1 (100X) and 5 mL of the HT Supplement (100X).

### Jurkat cell culture medium

Prepare Jurkat medium (500 mL) with the RPMI Medium 1640 (1X) and supplement with 10% FBS (50 mL) and 5 mL of the Antibiotic Antimycotic Solution (100X).

### ViaStain™ AOPI Staining Solution

The acridine orange (AO) and propidium iodide (PI) staining solution is already prepared to the correct concentration before staining at 1:1 with the cells.

### ViaStain™ AO Staining Solution

The acridine orange (AO) staining solution is already prepared to the correct concentration before staining 1:1 with the cells.

### ViaStain™ Total Cell Nuclear Green staining solution

Prepare a 2X staining solution (10 µM) by mixing PBS and the Nuclear Green stock solution at 5 mM. Pipette 10 mL of PBS into a 15-mL centrifuge tube and add 20 µL of the Total Cell Nuclear Green stock solution. Close the 15-mL centrifuge tube and invert 10X to mix the staining solution before use.

## EQUIPMENT SETUP

### Cellometer Spectrum

Connect the Cellometer Spectrum to the operating laptop computer via the USB cable and plug in the power cord. Turn the instrument power on from the back side and then open the Cellometer Spectrum analysis software. In the Cellometer Spectrum software, select the "AOPI Viability Assay_S5" default assay type for cell counting.

### Cellaca™ MX High-Throughput Automated Cell Counter

Connect the Cellaca MX to the operating laptop computer via the USB cable and plug in the power cord. Turn the instrument power on from the back side and then open the Cellaca MX analysis software. In the Cellaca MX software (v1.2), select the "MX04.0_AOPI_LiveDead" default assay type for cell counting.


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### Celigo® Image Cytometer

Turn on the Celigo power on the front and open the Celigo analysis software. Navigate to the top right and click on 'Administration' and then select 'Manage Plate Profiles'. After the "Plate Profile Management" window opens, click on the 'Import' button and select the plate profile for Cellaca 12 × 2 plate. Return to the home screen for image acquisition and analysis.

## PROCEDURE

### Maintenance of CHO-S cells

Timing: 20 – 30 min for passaging the cells and measuring their concentration and viability.

1. Passage the CHO-S cells when they are between 2 to 4 × $10^6$ cells/mL. Allowing the cells to grow above that concentration may decrease cell division as well as decrease viability due to insufficient nutrients in the media.
2. Warm the CHO-S cell culture medium at 37°C for 15 min in the incubator or in a water bath at 37°C for 5 min before passaging.
3. Under the biosafety cabinet, use a 10 mL pipette, pipette up and down at least 10 times to break up the cell clumps, and create a homogenous cell suspension in the T-75 flask.
4. Remove 200 µL of cells from the T-75 flask and transfer into a 1.5 mL microtube before the cells have had a chance to settle.
5. Obtain a CHT4-SD100 cell counting slide and peel off the protective plastic film on the top and bottom, and place the slide on a Kim-Wipe.
6. Mix 20 µL of CHO-S cell sample and 20 µL of AOPI within a 0.5 mL microtube.
7. Pipette 20 µL of stained cell sample into one chamber on the cell counting slide.
8. Insert the cell counting slide into the Spectrum and select the "AOPI Viability Assay_S5".
9. Measure the cell concentration and viability.
10. Based on the measured concentration, calculate the ratio of cells to new media that is needed in order to achieve a concentration of 2 × $10^5$ cells/mL.
11. Remove the calculated cell volume from the flask and replace with an appropriate amount of warmed CHO-S cell culture medium.
12. Place the passaged flask back onto the plate rocker inside the 8% $CO_2$ incubator at 37°C.
13. Monitor the growth of cells daily, and continue to passage as needed (usually 3 times a week).

### Maintenance of Jurkat cells

Timing: 20–30 min for passaging the cells and measuring their concentration and viability.

14. Passage the Jurkat cells when they are between 1 to 2 × $10^6$ cells/mL. Allowing the cells to grow above that concentration may decrease cell division as well as decrease viability due to insufficient nutrients in the media.
15. Warm the Jurkat cell culture medium at 37°C for 15 min in the incubator or in a water bath at 37°C for 5 min before passaging.
16. Under the biosafety cabinet, use a 10 mL pipette, pipette up and down at least 10 times to break up the cell clumps, and create a homogenous cell suspension in the T-75 flask.
17. Remove 200 µL of cells from the T-75 flask and transfer into a 1.5 mL microtube before the cells have had a chance to settle.
18. Obtain a CHT4-SD100 cell counting slide, peel off the protective plastic film on the

top and bottom, and place the slide on a Kim-Wipe.

19. Mix 20 µL of Jurkat cell sample and 20 µL of AOPI within a 0.5 mL microtube.

20. Pipette 20 µL of stained cell sample into one chamber on the cell counting slide.

21. Insert the cell counting slide into the Spectrum and select the "AOPI Viability Assay_S5".

22. Measure the cell concentration and viability.

23. Based on the measured concentration, calculate the ratio of cells to new media that is needed in order to achieve a concentration of $2 \times 10^5$ cells/mL.

24. Remove the calculated cell volume from the flask and replace with an appropriate amount of warmed Jurkat cell culture medium.

25. Place the passaged flask back inside the 5% $CO_2$ incubator at 37°C.

26. Monitor the growth of cells daily, and continue to passage as needed (usually 3 times a week).

## Stock cell sample preparation from cell culture

Timing: 15 min for collecting the cells from cell culture flasks, 5 min for cell counting and viability analysis, and 10 min for adjusting cell sample concentration if necessary.

27. Collect a stock of CHO-S and Jurkat cell sample separately into a 15-mL tube from cell culture following aseptic techniques.

28. Obtain a CHT4-SD100 cell counting slide, peel off the protective plastic film on the top and bottom, and place the slide on a Kim-Wipe.

29. Pipette 20 µL of the cell sample using a P100 pipettor into a 0.5 mL microtube.

30. Pipette 20 µL of the AOPI and add to the 0.5 mL microtube.

31. Aspirate the mixture of cells and AOPI up and down at least 5 times.

32. Pipette 20 µL of the stained cells into one chamber on the cell counting slide.

33. Insert the cell counting slide into the Spectrum and count the stained cells to generate cell count and viability.

34. Adjust the stock cell sample concentration to ~$5 \times 10^6$ cells/mL for both CHO-S and Jurkat cells.

    a. Decrease the concentration by dilution in cell media.

    b. Increase the concentration by centrifugation and resuspend in cell media.

35. Repeat steps 28–33 to ensure the concentration is adjusted to ~$5 \times 10^6$ cells/mL.

## Sample preparation & cell counting preparation for cell counting methods performance evaluation & comparison

Timing: 15–30 min with a single, manual pipette for sample preparation. 15–20 min for incubation of cell samples mixed with Nuclear Green (Figure 1). ~10 min per Cellaca MX plate with a single, manual pipette for cell counting preparation.

**CRITICAL:** Under the guidance of ISO Cell Counting Part 2, an initial accuracy validation experiment of pipetting volume using the experimental pipettors is necessary to increase sampling confidence. Such validation can be performed with a sensitive and well-calibrated laboratory balance and a fluid of known density, but the procedure will not be described in this protocol. Directly dilute the cells to generate independent dilution samples instead of serial dilution to reduce the propagation of pipetting error that can affect proportionality.

36. Obtain the prepared stock CHO-S cell and Jurkat cell samples at the highest


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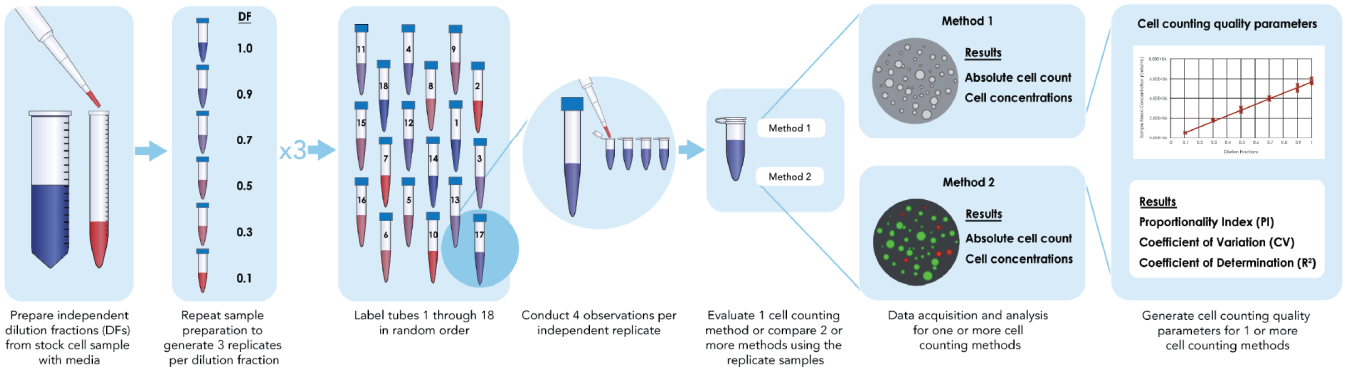
## FIGURE 1

**Cell sample preparation and measurement process diagram.**




Procedure sequence from left to right: **(1)** Collect your target cell sample and prepare different concentrations with specific dilution fractions using cell media. **(2)** Repeat this process to generate 3 replicates for each dilution fraction. **(3)** Label each tube in random order from 1–18. **(4)** Prepare and measure each tube 4 times with each selected cell counting method. **(5)** Analyze the images with each cell counting method to generate cell counting results. **(6)** Utilize the cell counting results to generate proportionality index (*PI*), coefficient of variation (CV), and coefficient of determination ($R^2$).

concentration for the intended use and range (~5 × $10^6$ cells/mL).

37. Prepare other samples from the stock of CHO-S and Jurkat cell samples at 0.1, 0.3, 0.5, 0.7, 0.9 and 1.0 dilution fractions (DFs) independently (Table 1).
    a. Prepare replicate samples with PBS or cell culture media.

38. Pipette 120 µL of CHO-S or Jurkat stock cell sample into the 1st microtube for the 1.0 DF sample.

39. Pipette 108 µL of CHO-S or Jurkat stock cell sample into the 2nd microtube and add 12 µL of PBS for the 0.9 DF sample.

40. Pipette 84 µL of CHO-S or Jurkat stock cell sample into the 3rd microtube and add 36 µL of PBS for the 0.7 DF sample.

## TABLE 1

**Dilution fractions and the corresponding volumes preparation for cell sample and PBS.**

| DF | Cell volume (µL) | PBS volume (µL) |
|---|---|---|
| 1.0 | 120 | 0 |
| 0.9 | 108 | 12 |
| 0.7 | 84 | 36 |
| 0.5 | 60 | 60 |
| 0.3 | 36 | 84 |
| 0.1 | 12 | 108 |

41. Pipette 60 µL of CHO-S or Jurkat stock cell sample into the 4th microtube and add 60 µL of PBS for the 0.5 DF sample.

42. Pipette 36 µL of CHO-S or Jurkat stock cell sample into the 5th microtube and add 84 µL of PBS for the 0.3 DF sample.

43. Pipette 12 µL of CHO-S or Jurkat stock cell sample into the 6th microtube and add 108 µL of PBS for the 0.1 DF sample.

44. Repeat Steps 38–43 two more times to generate a total of 3 replicate samples at each DF, where a total of 18 tubes of cell samples are generated.

45. Pipette 120 µL of AO staining solution into the 1st microtube of each DF sample to make a 1:1 mixed sample. After this step, a total of 240 µL cell sample is prepared in the 1st microtube at each DF.

46. Invert the 0.1 DF microtube 10 times to ensure uniform mixture.

47. Transfer 50 µL from the mixed 0.1 DF microtube into the A1 loading well on the 1st Cellaca MX plate. Repeat the transfer 3 more times into the A2 – A4 loading wells of the 1st Cellaca MX plate.

48. Repeat Step 46–47 for the 1st microtubes of the remaining DFs (0.3, 0.5, 0.7, 0.9, and

## TABLE 2

**Cellaca plate map for cell samples at different DFs.**

| Plate 1 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 | 0.3 | 0.5 | 0.5 | 0.5 | 0.5 |
| B | 0.7 | 0.7 | 0.7 | 0.7 | 0.9 | 0.9 | 0.9 | 0.9 | 1.0 | 1.0 | 1.0 | 1.0 |
| **Plate 2** | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 | 0.3 | 0.5 | 0.5 | 0.5 | 0.5 |
| B | 0.7 | 0.7 | 0.7 | 0.7 | 0.9 | 0.9 | 0.9 | 0.9 | 1.0 | 1.0 | 1.0 | 1.0 |
| **Plate 3** | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 0.1 | 0.1 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 | 0.3 | 0.5 | 0.5 | 0.5 | 0.5 |
| B | 0.7 | 0.7 | 0.7 | 0.7 | 0.9 | 0.9 | 0.9 | 0.9 | 1.0 | 1.0 | 1.0 | 1.0 |

1.0) samples into the remaining loading wells on the 1st Cellaca MX plate, following the plate map shown below. After this step, the 1st Cellaca MX plate is prepared (Table 2).

a. Randomize Step 46–48 if applicable. This is suggested by ISO Cell Counting Standard Part 2 in order to minimize the systematic time-dependence effects on the proportionality index and other metrics of the cell counting measurement process quality.

49. Repeat Step 45–48 for the 2nd and 3rd replicate samples at different DFs to prepare the 2nd and 3rd Cellaca MX plates.

a. Prepare each Cellaca MX plate right before the image acquisition, instead of preparing all Cellaca MX plates at the beginning, to minimize the time gap between sample preparation and image acquisition.

50. Repeat Steps 36–49 and stain with 120 µL of Nuclear Green.

a. Incubate the Nuclear Green-stained cell samples for 15–20 min at room temperature. Incubation time can be reduced at 37 °C.

## Image acquisition & analysis for each cell counting method

Timing: Scanning and analysis are 6 min per plate for the Cellaca MX and 5–10 min per plate for the Celigo.

51. Load the 1st Cellaca MX plate into the Cellaca MX after preparation.

52. Select the "MX04.0_AOPI_LiveDead" default assay type for cell counting in the Cellaca MX software for cell samples stained with AO staining solution. For cell samples stained with Nuclear Green, increase the FL1 exposure time by 50–100%. Check the fluorescent intensity of the nuclear green stained cells in the preview images before image acquisition.

53. Use the default analysis parameters for counting cells in the captured Cellaca MX bright field and FL1 fluorescent images. Export the concentration data.

54. Transfer the 1st Cellaca MX plate to the Celigo. Select the plate profile for Cellaca MX plates. Use the default experiment setting for image acquisition and analysis. Export total cell counts from the captured fluorescence images.

55. Repeat 51–54 for the 2nd and 3rd Cellaca MX plates.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## Cell counting method performance evaluation

Timing: ~30 min to calculate and analyze the parameters for performance evaluation for one cell line and one stain.

56. Calculate the cell concentration using the total cell counts from the Celigo exported data and multiply by a factor of 1383.979, which is the conversion ratio based on the counted volume and dilution factor from staining with AO and Nuclear Green.

57. Calculate the mean concentration $M_{Ai}$ acquired with method A (Cellaca MX) from a total number of $n_{Ai}$ replicate measurements for sample i using **Equation 1**.

[1] $M_{Ai} = \frac{\sum_{r=1}^{n_{Ai}} M_{Air}}{n_{Ai}}$, where $M_{Air}$ is the concentration acquired with method A for sample *i* during replicate measurement *r*.

58. Calculate the mean concentration $M_{Ak}$ acquired with method A (Cellaca MX) for dilution fraction k ($DF_k$) using **Equation 2**.

[2] $M_{Ak} = \frac{\sum_{i \in DF_k} n_{Ai} M_{Ai}}{\sum_{i \in DF_k} n_{Ai}}$

59. Calculate the variance of concentration $var_{Ai}$ acquired with method A (Cellaca MX) from a total number of $n_{Ai}$ replicate measurements for sample i using **Equation 3**.

[3] $var_{Ai} = \frac{\sum_{r=1}^{n_{Ai}} (M_{Air} - M_{Ai})^2}{n_{Ai} - 1}$

60. Calculate the pooled variance of concentration $var_{AK}$ acquired with method A (Cellaca MX) for $DF_k$ using **Equation 4**.

[4] $var_{Ak} = \frac{\sum_{i \in DF_k} (n_{Ai} - 1) var_{Ai}}{\sum_{i \in DF_k} (n_{Ai} - 1)}$

61. Calculate the pooled standard deviation of concentration $\sigma_{Ak}$ acquired with method A (Cellaca MX) for $DF_k$ using **Equation 5**.

[5] $\sigma_{Ak} = \sqrt{var_{Ak}}$

62. Calculate the pooled CV, $CV_{Ak}$ acquired with method A (Cellaca MX) for $DF_k$ using equation **Equation 6**.

[6] $CV_{Ak} = \frac{\sigma_{Ak}}{M_{Ak}}$

63. Repeat 57–62 to calculate the mean concentration $M_{Bk}$, the pooled standard deviation of concentration $\sigma_{Bk}$ and the pooled CV acquired with method B (Celigo) for $DF_k$.

64. Use mean concentrations ($M_{Ai}$, $M_{Bi}$) from all samples at 6 different DFs to generate a concentration series for both Cellaca MX and Celigo. Perform a proportional fit with the concentration series for each method using the iteratively reweighted least squares (IRLS) model. Set the weights of the least squares proportional to the reciprocal of the variances, which can be estimated by mean concentrations under the assumption of a quasi-Poisson distribution that the variances of cell concentrations are proportional to their respective mean concentrations ($var_{Ai} = \varphi M_{Ai}$), where φ is a scalar estimated from the experimental data that cancels out when used in the weighting of every least squares term [17]. Re-run the model fitting by updating weights using predicted values of the mean concentrations until the proportional fit is optimized. Generate a list of predicted values of mean concentrations ($\widehat{M}_{Ai,ideal}$, $\widehat{M}_{Bi,ideal}$) from the IRLS model.

65. Determine the coefficient of determination ($R^2$ value) from the IRLS model for method A (Cellaca MX) using **Equation 7** [26,27]. Use the same method to determine the $R^2$ value for method B (Celigo).

[7] $R_A^2 = 1 - \frac{residual\ sum\ of\ squares}{uncentered\ total\ sum\ of\ squares} = 1 - \frac{\sum_i (M_{Ai} - \widehat{M}_{Ai})^2}{\sum_i M_{Ai}^2}$

66. Perform a fit with the concentration series for each method (Cellaca MX, Celigo) using a higher-order polynomial model as a flexible model. Set the order of the polynomial to be the number of DFs minus 1. Generate a list of predicted values of mean concentrations

($\widehat{M}_{Ai,flex}$, $\widehat{M}_{Bi,flex}$) from the polynomial model.

67. Determine the proportionality index (*PI*) based on the smoothed sum of absolute scaled residuals ($PI_A^{SAbsSR}$, $PI_A^{SAbsSR}$) for both Cellaca MX and Celigo using **Equation 8** following previous publication [17,18],

[8] $$PI_A^{SAbsSR} = \sum_i abs\left(\frac{\widehat{M}_{Ai,flex} - \widehat{M}_{Ai,ideal}}{\widehat{M}_{Ai,ideal}}\right)$$

68. Apply the Bland-Altman method to compare the performance between two cell counting methods.

69. We utilized an internally developed software application derived from the ISO Cell Counting Standard Part 2 and Bland-Altman comparative method to automatically calculate the coefficient of determination, precision, proportionality index parameters, as well as the Bland-Altman analysis parameters (bias, LoA, the CI of the bias).

## Bland-Altman comparative method: data calculation

Timing: ~30 min to analyze and plot the Bland-Altman comparison data for one cell line and one stain.

70. Calculate the percent difference $Y_i$ between the measurement $M_{Ai}$ acquired with method A and the measurement $M_{Bi}$ acquired with method B for each sample i using the **Equation 9**, only if the samples are paired between method A and B.

[9] $Y_i = \frac{M_{Ai} - M_{Bi}}{X_i}$, where $X_i$ is the sample mean given by $X_i = \frac{M_{Ai} + M_{Bi}}{2}$

a. If measurements $M_{Air}$ and $M_{Bir}$ from replicate r are paired, calculate the percent difference $Y_{ir}$ between the measurement $M_{Air}$ acquired with method A and the measurement $M_{Bir}$ acquired with method B for each replicate r of sample i using the **Equation 10**.

[10] $Y_{ir} = \frac{M_{Air} - M_{Bir}}{X_{ir}}$, where $X_{ir}$ is the sample mean given by $X_{ir} = \frac{M_{Air} + M_{Bir}}{2}$

71. Calculate the bias from method A to method B ($Bias_{AB}$) by averaging the $Y_i$ values using **Equation 11** or by averaging the $Y_{ir}$ values using **Equation 12**

[11] $Bias_{AB} = \frac{1}{N}\sum_i Y_i$, where N is the number of samples (for paired samples, unpaired replicates, i.e. for each sample, different replicates are measured with each method).

[12] $Bias_{AB} = \frac{1}{N}\sum_{i,r} Y_{ir}$, where N is the total number of replicate measurements (for paired samples with paired replicates, i.e. for each sample, the same replicates are measured using both methods).

72. Calculate the LoA by multiplying 1.96 to the mean for percent differences determined in step 70 using **Equation 13** or **14**. LoA are defined as the one-sided 95% confidence interval for a single sample.

[13] $$LoA = 1.96 * \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(Y_i - Bias_{AB})^2}$$

where N is the number of samples (paired samples, unpaired replicates).

[14] $$LoA = 1.96 * \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(Y_{ir} - Bias_{AB})^2}$$

where N is the total number of replicate measurements (paired samples, unpaired replicates).

73. Calculate the CI of the bias using **Equation 15**.

[15] $CI_{Bias} = \frac{LoA}{\sqrt{N}}$, where N is the number of samples (for paired samples without paired replicates) or the total number of


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replicate measurements (if both samples and replicates are paired).

## Bland-Altman comparative method: graphical representation

74. Plot a single point on the Bland-Altman diagram for each sample, with $X_i$ (sample mean) on the horizontal axis and $Y_i$ (percent difference) on the vertical axis.

75. Plot a horizontal line that crosses the vertical axis at the value of $Bias_{AB}$ calculated in step 71.

76. Plot two additional horizontal lines that cross the vertical axis at the values of $Bias_{AB} + LoA$ and $Bias_{AB} - LoA$, where the LoA is calculated as described in step 72. These lines define a range of values for the expected percent difference between the two methods for a single sample.

77. Plot two additional horizontal lines at the values $Bias_{AB} + CI_{Bias}$ and $Bias_{AB} - CI_{Bias}$. This range provides a sense of the uncertainty on the bias value itself.

78. Examine the plot and note any concentration-dependence in either the bias or variation.

## TROUBLESHOOTING

Follow the troubleshooting Table 3 to optimize the experiments and output.

## TIMING

1. Step 1–26, maintenance of CHO-S and Jurkat cells: 20–30 min for passaging the cells and measuring their concentration and viability per cell line.

2. Steps 27–35, stock cell sample preparation from cell culture: 15 min for collecting the cells from cell culture flasks, 5 min for cell counting and viability analysis, and 10 min for adjusting cell sample concentration if necessary.

3. Steps 36–50, sample preparation and cell counting preparation for cell counting methods performance evaluation and comparison: 15–30 min with a single, manual pipette for sample preparation, 15–20 min for incubation of cell samples mixed with Nuclear Green, and ~10 min per Cellaca MX plate for cell counting preparation.

4. Step 51–55, image acquisition and analysis: 6 min per plate for the Cellaca MX and 5–10 min per plate for the Celigo.

5. Step 56–78, performance evaluation and Bland-Altman comparison analysis: 1 h per cell line per staining solution.

## ANTICIPATED RESULTS & DISCUSSION

Two cell lines (CHO-S, Jurkat), two dyes (AO, Nuclear Green), and 2 cell counting methods were evaluated to demonstrate the application of cell counting method performance evaluation and Bland-Altman comparative analysis. Figure 2 shows the mean and pooled CV, respectively, of the 6-point concentration series of Jurkat cells stained with AO using both cell counting systems. Table 4 shows the numerical results for the mean and pooled CV. The concentration range measured in the experiment was ~$5 \times 10^5$ to ~$6 \times 10^6$ cells/mL. Both Cellaca MX and Celigo have pooled CVs ranging from 1.8–7.6% for all replicates per concentration.

Based on the measurements in Table 4, the coefficient of determination and proportionality index can be calculated via regression analysis. Figure 3 shows the proportional fits of the 6-point concentration series as a function of dilution fractions for both Cellaca MX and Celigo. Results for each parameter are shown in Figure 4 and Table 5. Both Cellaca MX and Celigo show comparable values of coefficient of determination ($R^2$ values)

**TABLE 3**

**Troubleshooting table.**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 62, 63 | CV is too large at one or a few DFs | Sampling or pipetting error | ▶ Properly mix and pipette samples following ISO cell counting standard |
| | | Counting errors due to clumps | ▶ Adjust the counting parameters<br>▶ Remove the outliers if severe counting errors are observed |
| 67 | Poor Proportionality | Propagation of pipetting error | ▶ Directly dilute to generate independent dilution samples instead of serial dilution to eliminate the propagation of pipetting error |
| 68, 70–78 | A large bias between two cell counting methods | Sample variation (i.e. different stocks of samples) | ▶ Use the same stock of cell samples for both cell counting methods<br>▶ If possible, use the same cell sample in the same piece of consumable to conduct cell counting comparison<br>▶ Test the stability of the cell sample for concentration and viability for the duration of the assay. If a trend is observed, then the results may be invalid |
| | | Sample condition change (e.g. photobleaching, sample dry-out) | ▶ Practice cell counting performance evaluation and comparison experiments<br>▶ Use presets in the software<br>▶ Finish image acquisitions in a short time duration |
| | | Cell counting analysis variation (e.g. declumping) | ▶ Adjust the imaging and counting parameters in the software to ensure that cells are counted properly |
| | | Instrument comparison | ▶ Ensure the exact instruments are compared in repeated experiments |
| | | Instrument calibration | ▶ Ensure both instruments are well calibrated and data acquisition and analysis parameters are optimized before use. |

and proportionallity indices (*PIs*) from the proportional fits in this cell counting method evaluation. No significant differences are observed between Cellaca MX and Celigo for both $R^2$ and *PI* values.

Next, the Bland-Altman method is applied to compare the performance between Cellaca MX and Celigo cell counting methods. Figure 5 shows a representative Bland-Altman plot between Cellaca MX and Celigo. In this plot, a positive percentage indicates a higher concentration for Celigo measurements. Each point in the plot represents a pair of measurements determined by both cell counting methods. Results of concentration bias, 95% confidence interval and standard deviation of the bias between Cellaca MX and Celigo are shown in Table 6. A bias of ~ -1.5% (n = 72 replicate measurements) indicates that concentration measured by Celigo is ~1.5% lower than Cellaca MX in this cell counting method comparison. Since the value of 0 lies outside the confidence interval of the bias, it is concluded that the concentration difference observed between these two cell counting methods is significant ($p < 0.05$), despite being relatively small. The bias exhibits a slight dependence on cell concentration in this case. An additional paired measurement made using two methods under the same conditions would be expected to fall within the range defined by the LoA's in approximately 95% of cases.

A summary of the cell counting performance evaluation and comparison results between Cellaca MX and Celigo using different combinations of targeted cell lines and staining solutions is shown in Table 7. Concentration biases are -2–6% for the paired cell counting methods in these four cell counting


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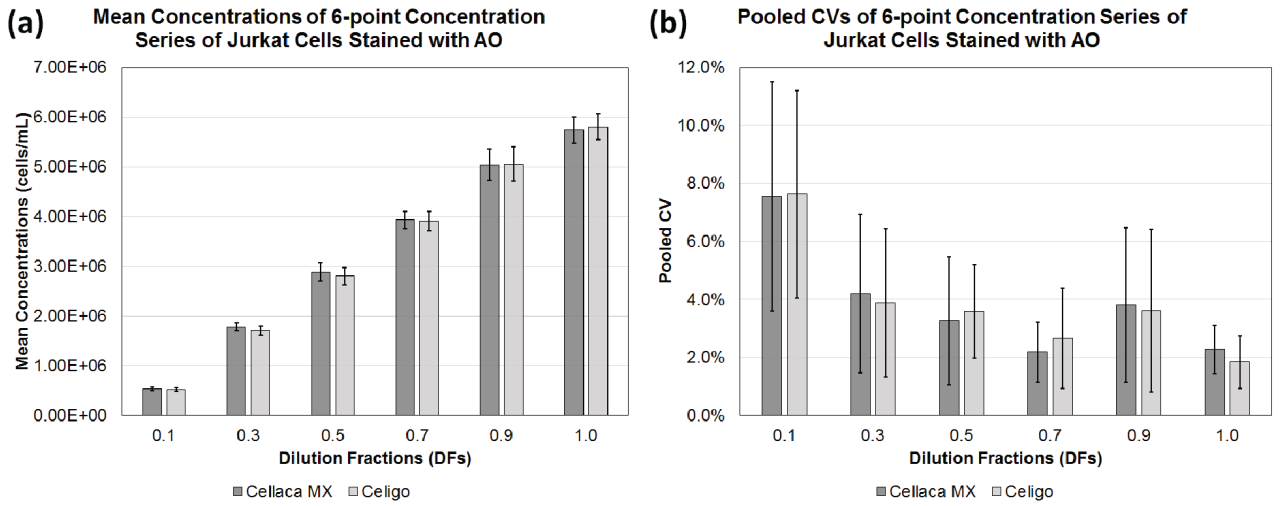
## FIGURE 2

**Cell counting results for (a) mean concentrations and (b) pooled CV of 6-point concentration series of Jurkat Cells Stained with AO.**




The mean concentrations and CV values measured by Cellaca MX and Celigo were highly comparable at each dilution fraction. It is clear that as concentration decreased, the pooled CV increased, likely due to the Poisson Noise (Random Error) at lower concentrations.

methods. Because each cell-stain combination was treated independently, the results were not combined, and the False Discovery Rate (FDR) or Family-Wise Error Rate (FWER) were not calculated. Overall, we have conducted multiple practical experiments following ISO Cell Counting Standard Part 2 with Celigo and Cellaca MX, and the results presented here are in the expected range of the cell counting measurement quality.

The ISO Cell Counting Standard Part 2 enables cell and gene therapy researchers to conduct experiments to evaluate and compare the quality of cell counting measurement processes. In this practical application of the standard, we demonstrated the evaluation

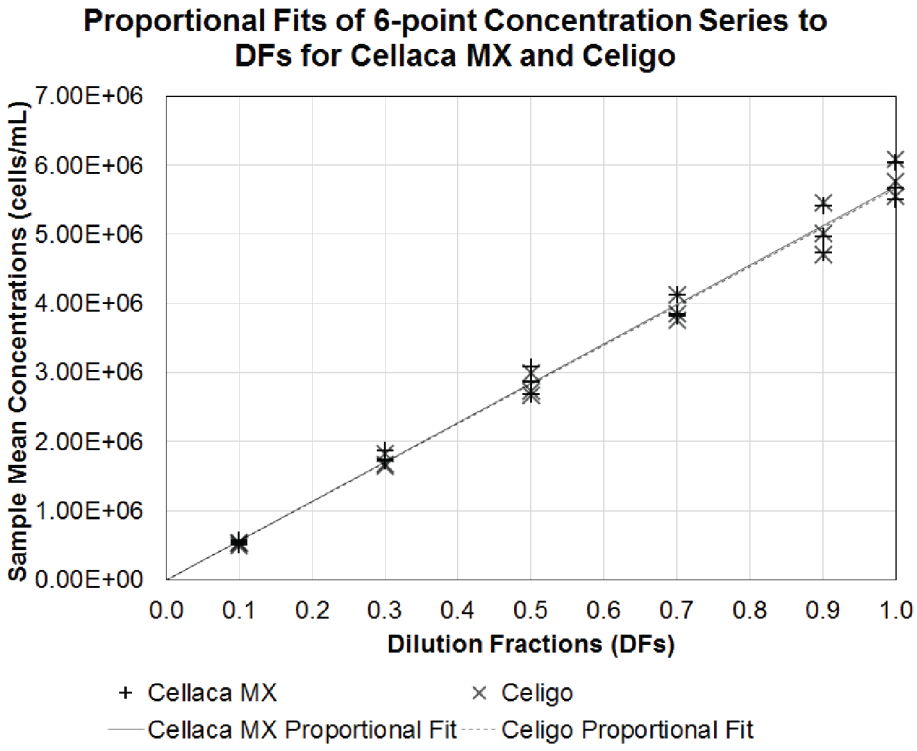
## TABLE 4

**Calculated mean concentration and pooled CV for each dilution fraction using ISO Cell Counting Standard Part 2.**

| Cell counting method | DF | n | Mean (cells/mL) | Pooled CV (%) |
|---|---|---|---|---|
| Cellaca MX | 0.1 | 12 | 5.37E+05 | 7.5 |
| | 0.3 | 12 | 1.78E+06 | 4.2 |
| | 0.5 | 12 | 2.88E+06 | 3.3 |
| | 0.7 | 12 | 3.93E+06 | 2.2 |
| | 0.9 | 12 | 5.05E+06 | 3.8 |
| | 1.0 | 12 | 5.74E+06 | 2.3 |
| Celigo | 0.1 | 12 | 5.21E+05 | 7.6 |
| | 0.3 | 12 | 1.71E+06 | 3.9 |
| | 0.5 | 12 | 2.80E+06 | 3.6 |
| | 0.7 | 12 | 3.91E+06 | 2.7 |
| | 0.9 | 12 | 5.06E+06 | 3.6 |
| | 1.0 | 12 | 5.80E+06 | 1.8 |

**FIGURE 3**

Linear regression fitting of the 6-point concentration series as a function of dilution fractions (DFs), which shows distribution of concentration measurements for Cellaca MX and Celigo at each dilution fraction.




of the Cellaca MX and Celigo using Jurkat and CHO cells tailored to the bioprocessing and cell therapy communities. The users of the ISO Cell Counting Standard Part 2 may utilize the protocol to evaluate one or more cell counting methods. For the evaluation of one method, one can perform the experiments to establish a baseline for each of the quality parameters, where this baseline can be further monitored with different operators, instruments, processes, etc. For comparison of cell counting methods, the quality parameters can be compared via bootstrap analysis or replicate studies, and the difference or bias between the methods can be determined using the Bland-Altman comparative analysis. It is also important to note that the quality parameters obtained from the ISO Cell Counting Standard Part 2 are specific to the measurement process evaluated (e.g. operators, instruments, cell sample properties, etc.), thus the robustness of the measurement process should also be evaluated in order to extend the findings of the study to similar measurement processes.

## FREQUENTLY ASKED QUESTIONS

1. Can we reduce the number of replicate samples and measurements?
   a. Yes, to an extent; the minimum recommended experimental design consists of at least 4 target dilution fractions, 3 replicate samples, and 3 replicate measurements.
   b. Quality indicators from experimental designs that do not meet these recommendations should be interpreted with caution and may require additional studies to properly evaluate proportionality and precision.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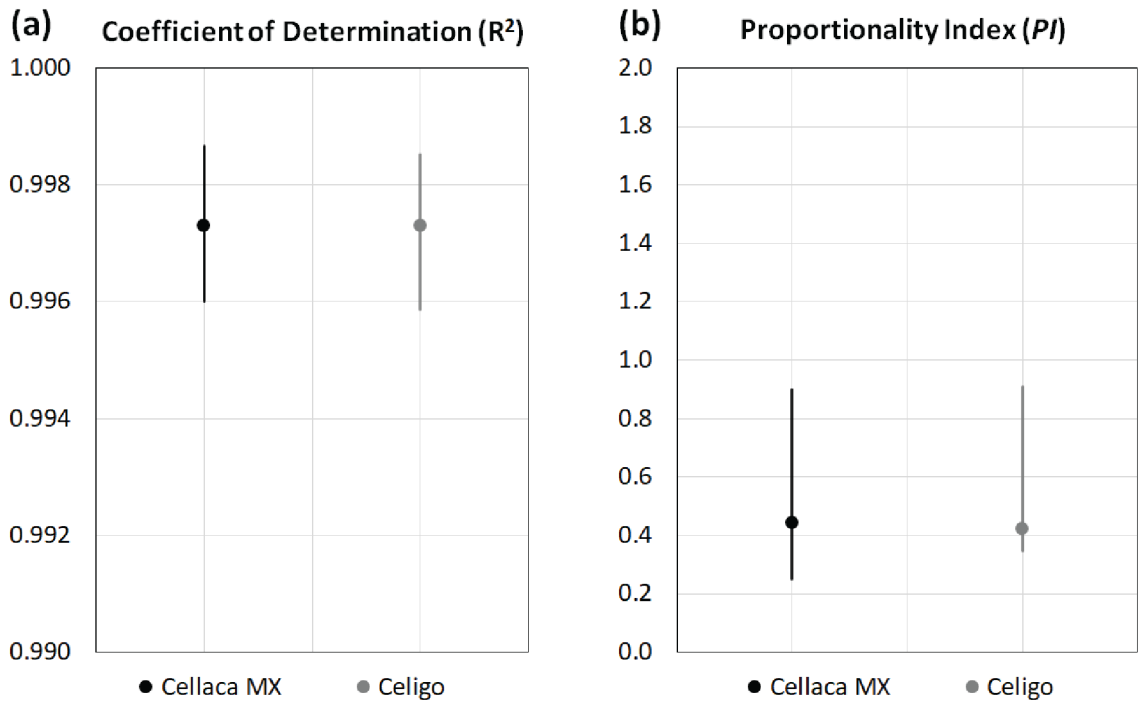
## FIGURE 4

**Comparison of the $R^2$ and *PI* values between Cellaca MX and Celigo. The $R^2$ and *PI* values are both highly comparable.**




c. For example, if an experimental design has only 2 replicate measurements, the evaluation of CV directly from this experimental design and statistical analysis may not be appropriate, however, evaluation of proportionality can still be conducted.

   i. In this case, a second experiment with more replicate measurements of fewer samples and/or fewer dilution fractions may be conducted to more directly address precision of the method.

2. What is the cell concentration range we should use?

   a. It should be fit-for-purpose for the typical range of cell concentrations you intend to evaluate for your cell type.

3. Should we check the pipettors?

   a. Always use professionally calibrated pipettors

   b. Performing a check on pipettors will increase confidence in the results

   c. ISO 20391-2 also suggest an approach for generating measured dilution fractions, where the mass of solution pipetted while generating the fractions is used to calculate a more accurate measured dilution fraction value for use in the analysis of $R^2$ and *PI*. In this case, small errors in pipetting can be accounted for in the proportional model fit.

4. What can the results tell us?

   a. The quality indicators provide a means to quantify and compare the quality

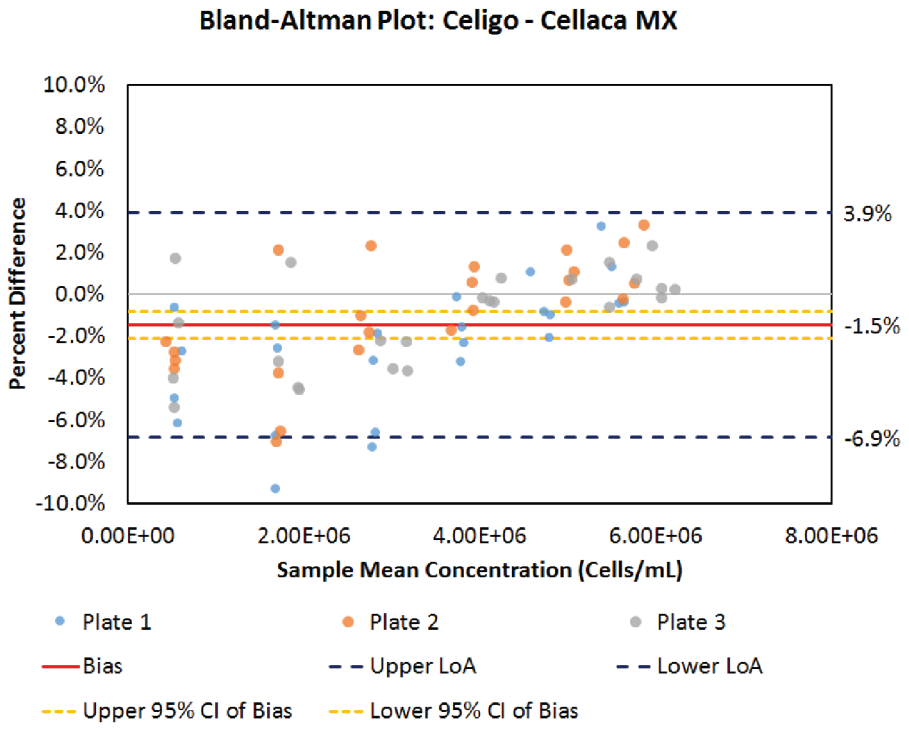
## TABLE 5

**Calculated R-square and *PI* values for performance evaluation.**

| | R-Square | *PI* |
|---|---|---|
| Cellaca MX | 0.997 | 0.44 |
| Celigo | 0.997 | 0.42 |
| Significance | No | No |

## FIGURE 5

Bland-Altman plot between Cellaca MX and Celigo cell counting methods.




The calculated percent differences show an increasing trend as the concentration increases.

## TABLE 6

Bland-Altman comparative analysis results between Cellaca MX and Celigo.

| Bias | Limit of agreement | CI of bias |
|---|---|---|
| -1.5% | -6.9% to 3.9% | -2.1% to -0.8% |

## TABLE 7

Summary table of cell counting performance evaluation and comparison results.

| Cell line | Staining solution | Cellaca MX | | | Celigo | | | Bias | LoA | 95% CI of bias | Signif-icance of bias |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $R^2$ | Pooled CV range (%) | *PI* | $R^2$ | Pooled CV range (%) | *PI* | | | | |
| Jurkat | Nuclear green | 0.998 | 3.8% to 6.1% | 0.30 | 0.997 | 3.7% to 7.4% | 0.37 | 3.4% | -6.0% to 12.8% | 2.3% to 4.5% | Y |
| Jurkat | AO | 0.997 | 2.2% to 7.5% | 0.44 | 0.997 | 1.8% to 7.6% | 0.42 | -1.5% | -6.9% to 3.9% | -2.1% to -0.8% | Y |
| CHO | Nuclear green | 0.998 | 3.4% to 5.8% | 0.40 | 0.999 | 2.7% to 6.8% | 0.35 | 5.6% | -3.4% to 14.6% | 4.5% to 6.7% | Y |
| CHO | AO | 0.998 | 2.7% to 7.0% | 0.44 | 0.996 | 2.7% to 6.4% | 0.35 | 5.1% | -2.4% to 12.5% | 4.2% to 5.9% | Y |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of cell counting methods based on principles that are fundamental to counting: precision and proportionality

b. The ISO Counting Standard Part 2 analysis makes no assumptions about the true cell count and can make conclusions about method quality in the absence of a reference material or reference method.

c. The Bland-Altman comparative analysis will indicate the percent difference between 2 methods.

d. These approaches do not indicate or compare the accuracy of the cell counting methods.

e. Cell counting method selection should be made based on the quality of the method and on what is fit-for-purpose for your measurement needs.

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## AUTHORSHIP & CONFLICT OF INTEREST

***Contributions:*** *Leo Li-Ying Chan and Jean Qiu conceived the study and experimental design. Dmitry Kuksin performed the cell culture and provided the Jurkat and CHO-S cells for the experimentation. Jordan Bell and Yongyang Huang developed software application for the ISO Cell Counting Standards Part II parameters and the Bland-Altman analysis method. Yongyang Huang conducted the cell counting method performance evaluation and comparison experiments. Sumona Sarkar and Laura T Pierce contributed to the development of cell counting standardization, which this protocol was derived from. David Newton contributed to the development and description of the statistical analysis. Leo Li-Ying Chan, Yongyang Huang, Jordan Bell, Sumona Sarkar, and Laura T Pierce wrote the manuscript.*

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***Disclosure and potential conflicts of interest:*** *Yongyang Huang, Jordan Bell, Dmitry Kuksin, Jean Qiu, and Leo Li-Ying Chan declare competing interests. The application of the cell counting method performance evaluation and comparison employed cell counting systems and reagents from Nexcelom Bioscience LLC.*

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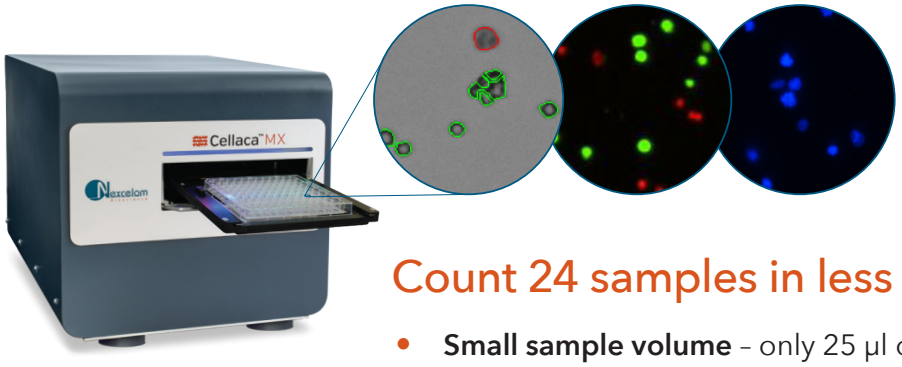
# Cellaca™ MX

## High-throughput Automated Cell Counter




## Count 24 samples in less than 3 minutes

- **Small sample volume** – only 25 µl of cell sample required
- **Analyze complex samples** – designed for cell lines as well as complex and messy samples including whole blood, peripheral blood, T cells, and bone marrow
- **Autofocus** – fast autofocus prior to analysis
- **Cell based assays** – apoptosis, protein expression (including GFP and RFP), and reactive oxygen species (ROS)
- **Automation ready** – robotic integration ability with optional API
- **21 CFR Part 11 ready** – optional add-on that includes audit trail, user access control, and digital signature

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Nexcelom Bioscience

# Achieving cost-effective, scalable high-titer AAV production

**Chao Yan Liu,** Senior Manager, Cell Biology, Thermo Fisher Scientific

**This poster describes the Gibco™ AAV-MAX Helper Free AAV Production System, a scaleable, high-titer adeno-associated virus (AAV) production system, which encompasses cell culture, plasmid transfection, and viral vector production steps in upstream bioprocessing.**

*Cell & Gene Therapy Insights* 2021; 7(9), 1147 • DOI: 10.18609/cgti.2021.152

The system consists of a newly developed clonal viral cell line (Viral Production Cells 2.0), viral production medium, AAV-MAX transfection kit, Viral-Plex™ complexation buffer, and AAV-MAX lysis buffer (Figure 1). AAV-MAX uses triple transfection technology, with three recombinant (r)AAV expression plasmids transfected into HEK293 producer cells to generate the AAV virus particles. The research-use version of the system is now available, with a cell therapy-grade version coming in 2022.

## SYSTEM PERFORMANCE

We looked at genome titers at the time of harvest for five different AAV serotypes and found consistently high titers of around 1 × $10^{11}$ Vg/ML (Figure 2). Cell density was around 4.5 × $10^{6}$/mL and viability was between 70 and 80% at the time of harvest. This compares favorably with the existing LV-MAX lentiviral production system.

## COMPETITIVE BENCHMARKING

We compared the AAV-MAX system with the popular PEIpro™ and PEI MAX and found that the AAV-MAX system produced 4 to 6 times more virus than PEI-mediated systems (Figure 3).

## CUSTOMER CASE STUDIES

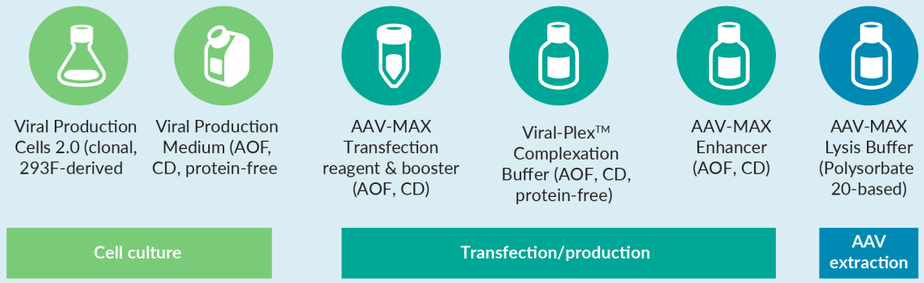
Early-access customers have reported outstanding system performance, with high viral titers achieved across serotypes and production scales (Figure 4).

## SCALEABILITY

We have tested the system in multi-well plates of every size, shake flasks from 120 mL to 2 L, and bioreactors up to 3 L. Across all scales, consistent and comparable viral titers were achieved. We worked with collaborators to test larger-scale bioreactors (50 L and 3,000 L) and they achieved similar results.

In summary, AAV-MAX is a scaleable high-titer, simplified AAV production workflow, with excellent performance across a range of serotypes and production scales.

**Figure 1. Components of the AAV-MAX production system.**




AOF: Animal origin free; CD: Chemically defined.

**Figure 2. AAV genome titer.**

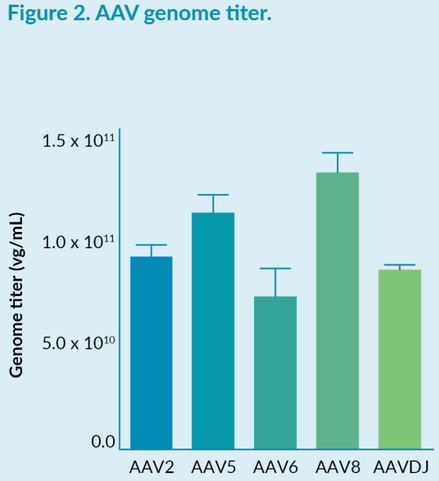



**Figure 3. Comparing AAV-MAX and PEI-mediated systems.**

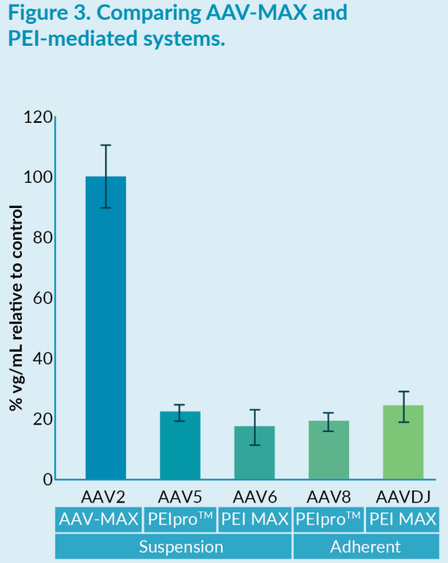
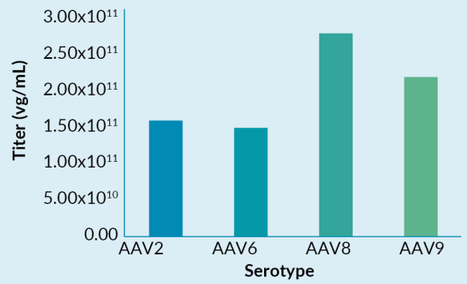


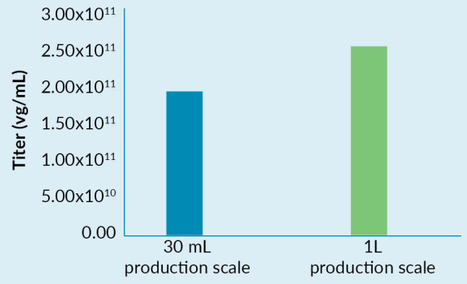

**Figure 4. Customer data showing high titers across multiple serotypes (top) and different production scales (bottom).**

**High titers across multiple serotypes**




External data (100mL in shake flask)

**High titers across production scales**




External data



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# Vector Channel

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# VECTOR CHANNEL: Upstream Bioprocessing

September 2021
Volume 7, Issue 9

## INNOVATOR INSIGHT



## COMMENTARY



## FAST FACTS

VIEWPOINT

# Overview of major AAV production platforms

**Jote Bulcha**

1073–1076

CELL & GENE THERAPY INSIGHTS

UPSTREAM BIOPROCESSING


INNOVATOR INSIGHT

# Scalability comparison between 50 and 500 liter stirred tank bioreactor for production of rAAV viral vector

**Todd P Sanderson, Timothy Erlandson, Nathan Hazi, Anne MacIntyre, Benjamin I Ingersoll, Michael McLaughlin, Steven Wesel & Phillip B Maples**

Viral vectors are a new class of biologics which facilitate gene transfer and modification in living cells, potentially treating a multitude of conditions with genetic causes. Scalable manufacturing technologies are critical to ensuring these cutting-edge medicines can be produced in sufficient quantities to meet the needs of process development, clinical trials, and ultimately commercial manufacturing [1]. As viral vector-based products have only relatively recently received regulatory approval, public information on scalable optimization of these processes is very limited. Abeona Therapeutics is a gene therapy company developing novel gene replacement therapies for rare inherited diseases. These conditions can impact development and limit both quality of life and/or life expectancy [2]. These transformative medicines can be used to replace a defective gene with a functional copy, silence a defective gene or even directly edit genes [3,4]. We evaluated the Pall Allegro™ STR bioreactor family as an rAAV vector production platform and evaluated the scalability of the PEI-mediated transfection manufacturing process for rAAV at the 50 L and 500 L working volume. Process scalability was evaluated based on cell growth, metabolic profile, and vector production. This testing demonstrates that control of key process parameters enables a scalable vector production process between the 50 L and 500 L scale using Allegro STR single use bioreactors.

*Cell & Gene Therapy Insights* 2021; 7(9), 1025–1033

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Viral vectors based on AAV are the vector of choice for many gene delivery applications [1]. These vectors are recombinantly produced virus-like particles (VLPs) based on a type of non-pathogenic parvovirus called adeno-associated virus. These vectors deliver the therapeutic gene to the patient or isolated patient cells [5].

One of the most common production methods used for rAAV production is transient transfection of cultured human cells [6]. This process relies on the introduction of several types of plasmid DNA into the cells to induce vector production. Transfection complex is produced by combining negatively charged DNA and positively charged transfection agent. These two reagents interact to form small particles of transfection complex with a neutral charge that can be introduced into the cell culture vessel or bioreactor and absorbed by the cells. Once the cells recover, they start expressing the viral genes and produce and package the vector.

These manufacturing processes are dependent on time consuming procedures and expensive raw materials [1]. Scalable manufacturing processes are critical to providing the quantities of vector needed to bring these potentially life-saving treatments to waiting patient populations. Many gene therapy manufacturing processes rely on culturing HEK293 cell lines (or derivative AAV293 cell lines), and several early and current forms of production culture these cells on an adherent substrate [7].

Here we evaluate the performance of an rAAV transient transfection production process in the 50 L and 500 L Allegro™ STR bioreactors (Figure 1). The scale-up strategy

**FIGURE 1**

**The Allegro™ STR Bioreactor Family**

**TABLE 1**

**Equipment and materials used in the study.**

| Equipment | Manufacturer | Model/part no. |
|---|---|---|
| Allegro STR 50 Bioreactor | Pall Corporation | STR 50-JC110-R-SU |
| LAUDA Integral T 2200 | LAUDA | L002242 |
| Allegro STR 500 Bioreactor | Pall Corporation | STR 500-JC110 |
| LAUDA VC 10000 | LAUDA | S190003372 |
| Nova Flex 2 Bioanalyzer | Nova Biomedical | T08310040 |
| Vi-Cell XR | Beckman Coulter | 30527950 |
| pH probe InPro3253/225/pt1000 | Mettler Toledo | 52200966 |
| DO probe InPro 6800/12/220 | Mettler Toledo | 52002569 |
| Allegro 50 L Biocontainer | Pall Corporation | 6412-0927L |
| Allegro 500 L Biocontainer | Pall Corporation | X6412-0891S |
| **Materials** | **Manufacturer** | **Model/part/serial no.** |
| Suspension AAV293 Master Cell Bank | Abeona | N/A |
| FreeStyle F17 media | Thermo Fisher | A1383504 |
| GlutaMAX 100X | Thermo Fisher | 35050061 |
| 100X/10% Pluronic F-68 | Thermo Fisher | 24040032 |
| DENERASE, 5MU | c-LECTA | 20804-5M |
| PEIpro | PolyPlus Transfection | #115-100 |
| pHelp, pAAV, pGOI Plasmids | Aldevron | |


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**TABLE 2**

**Shake flask seed train parameters.**

| Parameter | Target |
|---|---|
| Media | FreeStyle F17 + 4 mM GlutaMAX |
| Incubator $CO_2$ setpoint (%) | 5 |
| Incubator temperature (°C) | 37 |
| Incubator humidity (%) | Ambient with water reservoir |
| Shaker speed for flask sizes < 3 L (rpm) | 120 |
| Shaker speed for 3 L shake flasks (rpm) | 72 |
| Shaker orbit (mm) | 19 |

utilized constant power per unit volume (P/V) while maintaining scalable gas flow (vvm) for both sparge rate and gas overlay.

## MATERIALS & METHODS (Table 1)

### Seed Train

Suspension-adapted AAV293 cells were thawed from cryopreservation and cultivated in a 125 mL shake flask (30 mL working volume) using Freestyle™ F17 media supplemented with 4 mM GlutaMAX™ (Thermo Fisher). Growth and incubator conditions are shown in Table 2. One day post-vial thaw, the culture was expanded to a 250 mL flask at 60 mL working volume. Viable cell density and viability were monitored using a Vicell™ XR (Beckman). Cells were maintained between 0.2 and 2.0 x $10^6$ cells/mL during cell expansion. The culture volume was scaled-up a total of 6 passages until three 3 L flasks were used to inoculate an Allegro STR 50 bioreactor at a working volume of 20 L.

### N-1 bioreactor

The Allegro STR 50 bioreactor was used as the N-1 bioreactor by inoculating at a density of 0.2 x $10^6$ cells/mL at a 20 L initial volume and expanded to 50 L two days later. A summary of the N-1 culture process parameters is shown in Table 3. The viable cell density was adjusted daily as the growth rate was slightly higher than anticipated. This was done to prevent the cells from growing above 2.0 x $10^6$ cells/mL for transfection on a specific pre-planned day.

### Bioreactor production

The same inoculation strategy was used for production in the STR 500 as the N-1

**TABLE 3**

**N-1 process parameters.**

| Parameter | STR 50 (20 L working volume) | STR 50 (50 L working volume) |
|---|---|---|
| Basal medium | FreeStyle F17 + 4 mM GlutaMAX + 0.1 % Pluronic F-68 | |
| Working volume (L) | 20 | 50 |
| Power input P/V (W/m$^3$) | 30 | |
| Agitation (rpm) | 65 | 88 |
| Air sparge flowrate (L/min) | 0.1 | |
| Overlay flowrate (L/min) | 0.2 | |
| pH | Pre-conditioned with 10% $CO_2$ – no active control post-inoculation | |
| Dissolved oxygen (%) | 40 | |
| Temperature (°C) | 37 | |
| Inoculation cell concentration | 0.2 x $10^6$ cells/mL | |

## TABLE 4

**Production bioreactor operating parameters.**

| Parameter | STR 500 (PRE feed-up) | STR 500 (POST feed-up) | STR 50 (PRE feed-up) | STR 50 (POST feed-up) |
|---|---|---|---|---|
| Basal medium | FreeStyle F17 + 4mM GlutaMAX + 0.1 % Pluronic F-68 | | | |
| Initial working volume (L) | 250 | 475 | 25 | 47.5 |
| Power input P/V (W/m$^3$) | 30 | | | |
| Agitation (RPM) | 72 | 90 | 70 | 88 |
| Agitation direction | Downflow | | | |
| Air flowrate (L/min) | 0.5 | 1.0 | 0.1 | 0.1 |
| Overlay flowrate (L/min) | 2.0 | 2.0 | 0.2 | 0.2 |
| pH | Pre-conditioned with 10% $CO_2$ – no active control post-inoculation | | | |
| Dissolved oxygen (%) | 40 | | | |
| Temperature (°C) | 37 | | | |
| Initial cell density | 0.2 x 10$^6$ cells/mL | | | |

## TABLE 5

**PID settings for production.**

| Parameters | Setpoint | P | I | D | Dead-band |
|---|---|---|---|---|---|
| DO | 40 | 5 | 500 | 0 | 0 |
| Temperature | 37 | 20 | 500 | 0 | N/A |

passage in the STR 50. A uniform inoculation pool of 275 L was prepared in the STR 500. 25 L was then transferred to a new STR 50 vessel. This resulted in both vessels inoculated at half capacity with the same cell density. The vessels were then expanded to the full working volume after 24 hours. The culture was continued for 2 days until the viable cell density (VCD) reached the target transfection density of ~1.0 x 10$^6$ cells/mL.

The process control parameters and PID settings are shown in Tables 4 & 5.

The process parameter scaling strategy utilized for this comparison is to scale-up based on constant power per unit volume and normalized gas flow. Because of minor geometry difference between the impellers of the two vessels, similar power per unit volume outputs are achieved using slightly different rotational rates.

Transfection complex was prepared by diluting appropriate amounts of each plasmid DNA and PEIPro™ into cell culture media, combining the reagents and allowing the mixture to incubate for 15 minutes before addition to the bioreactors. This process was performed separately for each bioreactor.

DENARASE™ (30 U/mL, c-LECTA) was added to the bioreactor 24 hours post transfection. The culture was then continued with daily monitoring and harvested when the viability fell to <20%.

A summary of the production process conditions is shown in Table 6 below.

## TABLE 6

**Transfection and DENARASE parameters.**

| Parameter | Target |
|---|---|
| VCD at transfection (10$^6$ cells/mL) | 1.0 |
| Target time of addition completely added post-mix (minutes) | 15 |
| Target DENARASE addition timing (hours post transfection) | 24 |
| Target DENARASE activity in culture (U/mL) | 30 |
| Target amount of media to dilute DENARASE in for STR (mL/L) | 1 |
| Media used for DENARASE addition | F17 + 4 mM GlutaMAX |


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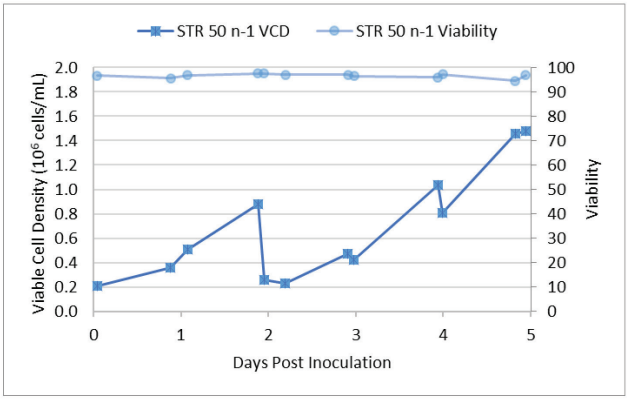
## Analytical methods

Aliquots were collected for vector production analysis starting 7 days post-transfection. Samples were centrifuged at 500 g for 5 min and clarified supernatant was stored at -20°C.

Viral vector physical titer was measured using droplet digital polymerase chain reaction (ddPCR) assay using the Biorad QX200 droplet System with the Auto DG droplet generator. The PCR primer/probe (IDT) combination targeted an amplicon contained in the gene of interest of the rAAV transfer genome.

**FIGURE 2**

**N-1 viable cell density.**




## RESULTS

## N-1 bioreactor

Suspension adapted AAV293 cells were recovered from cryopreservation as described in the methods section. The culture was expanded for 6 passages before sufficient biomass was generated to inoculate the N-1 bioreactor at 40% capacity.

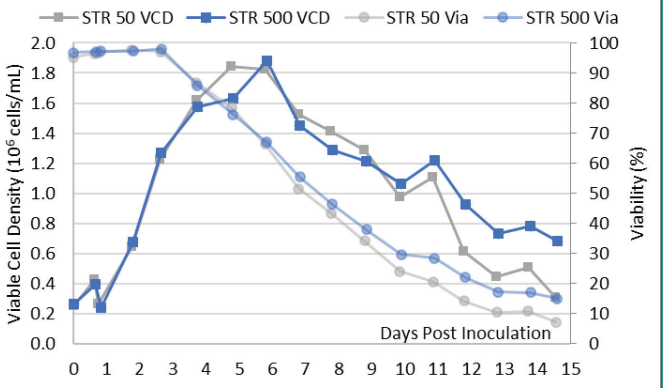
After 2 days of culture, cells were removed, and the volume was adjusted to 100% capacity. Minor volume adjustments (media addition and culture removal) were performed each day to compensate for slightly faster than anticipated cell growth. The faster growth rate observed in the STR 50 than shake flask may have been a result of the bioreactor providing a better controlled environment.

Very good growth and viability were observed in the N-1 culture. The VCD and viability trends are shown in Figures 2 & 3.

The N-1 bioreactor was harvested on day 5 to inoculate the production STR 500 and control vessel, STR 50.

**FIGURE 3**

**Cell growth and viability in the STR 500 and STR 50 bioreactors.**

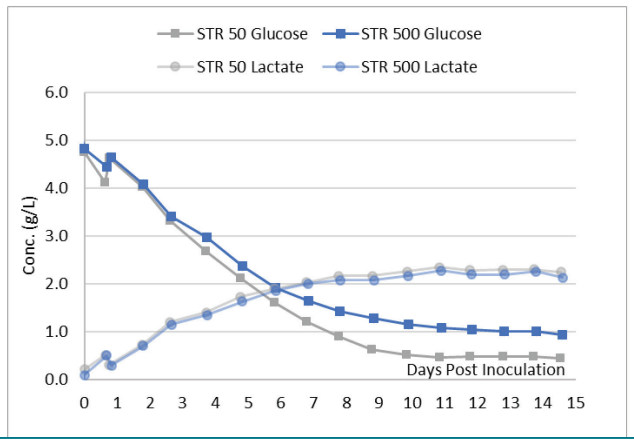



## Production bioreactor

As described in the methods section, a parallel STR 500 and STR 50 were inoculated with a uniform cell culture bolus. The VCD

**FIGURE 4**

**Glucose and lactate profiles in the STR 500 and STR 50 bioreactors.**



and viabilities of these cultures were measured daily and shown in Figure 3.

These trendlines show near identical cell growth and viability between both the STR 50 and STR 500 cultures up until transfection on day 3. After transfection, there is a drop in viability between the two vessels while the viable cell density continued to increase. Both cultures reached a maximum viable cell density of ~1.8 x $10^6$ cells/mL. The STR 50 culture showed a slightly lower viability in the second half of the culture.

Nutrient and metabolite analyses were also performed daily. The glucose and lactate profile of the cultures are shown in Figure 4 and the offline pH and online DO data are shown in Figure 5.

The data in Figure 4 shows the STR 50 consumed slightly more glucose than the STR 500. The VCD data in Figure 3 indicates a slightly higher viable biomass in the STR 500. Slight differences in transfection efficiency between the two vessels could explain these slight differences in VCD and metabolic profiles.

After initial bioreactor conditioning and inoculation, there was no active pH control of the vessels. The pH at both scales trended together, however the STR 50 had slightly lower pH throughout the run. There were no noticeable differences between $pCO_2$ levels between the two scales indicating that the aeration strategy was effective at maintaining a similar bioreactor environment (data not shown).

The DO trendlines shown in Figure 5 showed the bioreactors were able to maintain one-side DO control at the 50% setpoint.

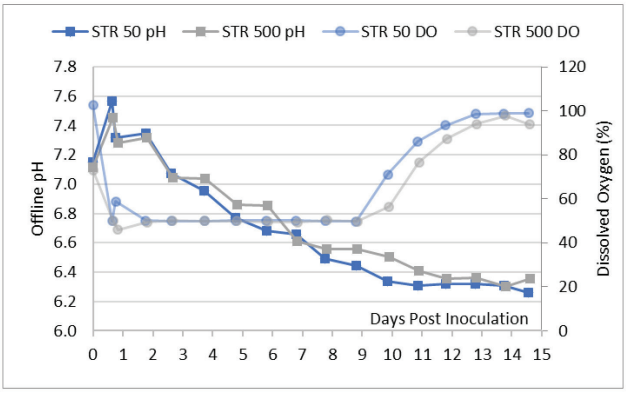
To maintain control of the DO at this setpoint, the bioreactor adjusts the $O_2$ sparge rate. Figure 6 shows the normalized $O_2$ sparge rate, (vSG) for the two cultures. The overall $O_2$ sparge profiles of the cultures are very similar with slightly more $O_2$ consumed in the STR 50 after transfection

When the culture was analyzed for product titer, the productivity between the two scales was within 10–20% with slightly higher titers observed in the STR 50. The titer data collected during the run is shown in Figure 7.

The data shows rAAV titer increases throughout the culture with maximum titer being observed at harvest. Final product titers were 4.3 x $10^{10}$ gc/mL and 4.8 x $10^{10}$ gc/

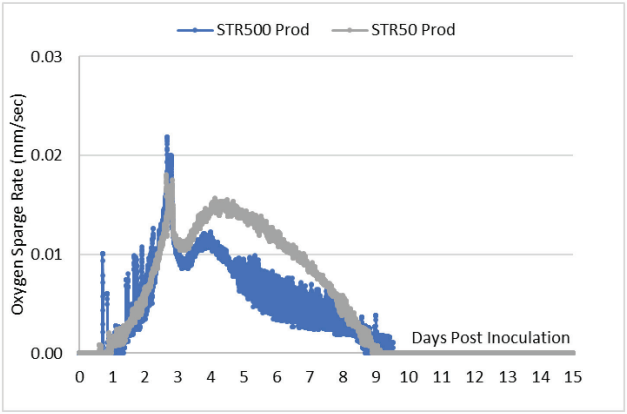
## FIGURE 5

pH and DO trends.



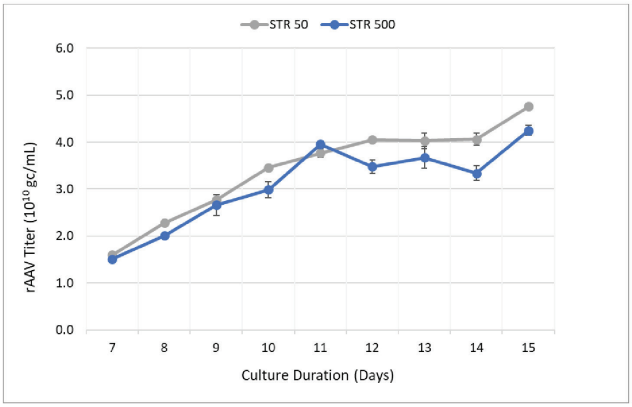

## FIGURE 6

Normalized $O_2$ sparge.




## FIGURE 7

Vector production during culture.





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mL for the STR 500 and STR 50 respectively. Error bars represent standard deviation of 8 replicates (2 assay dilutions with 4 technical replicates each). The two cultures had final harvest titers within ~10% indicating a scalable process.

This scalable bioreactor performance resulted in cultures with similar growth profiles, viability and viral vector productivity. This scalability is realized when utilizing Pall's recommended scale up strategy across the Allegro STR family

## CONCLUSIONS/DISCUSSION

Scalable upstream technologies are critical to enable the manufacturing capacity needed to bring gene therapy treatments with large patient populations to market. Optimal bioreactor performance can be achieved when the bioreactor is able to provide a controlled, uniform environment so that each cell can realize its full productivity potential.

The data presented here demonstrates that the Allegro STR 50 and STR 500 bioreactors are appropriate for rAAV production and that they produced similar bioreactor environments at both the 50 L and 500 L scales. Some minor differences in metabolic profile were observed after transfection. The root cause was not identified but was likely related to slightly different efficiencies of plasmid transfection.

## TRANSLATIONAL INSIGHTS

This work demonstrates scalability of this transfection-based production process between the 50 L and 500 L scale. Production at the 500 L scale is critical to providing sufficient vector for clinical trials and may be sufficient for full manufacturing capacity for certain indications, but for many others, further scale-up to 1,000 L and 2,000 L will be required.

Pall's single-use bioreactors are available up to 2,000 L. There are a number of other technologies in the industry which are being utilized to further increase vector productivity. Improvements to expression systems with improved packaging efficiency and development of producer cell lines are a couple technologies being evaluated to further increase vector yields [7].

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## AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

UPSTREAM BIOPROCESSING


COMMENTARY/OPINION

# Enabling development of commercial-ready lentiviral vector manufacturing processes using stable producer cell lines

Peter Archibald & Anthony Shillings

The manufacture of lentiviral vectors (LVVs) is currently a bottleneck for the development of cell and gene therapies. Typical upstream and downstream processes are often low-yielding, unscalable, involve many manual operations, and are poorly characterized. In this article, we describe the potential that LVV stable producer cell lines offer when used in upstream processes based upon suspension culture systems, and the implications that this can have on downstream unit operations. We also describe potential areas for innovation in order to develop LVV manufacturing processes suitable for commercial supply of potentially transformative cell and gene therapy medicines.

*Cell & Gene Therapy Insights* 2021; 7(9), 981–993

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## INTRODUCTION

For autologous cell and gene therapies, the production of viral vectors can often be one of the most expensive components of the supply chain and is frequently on the critical path for clinical development programs. Lentiviral vectors (LVVs) are commonly used for these therapies due to their ability to deliver sequences that can stably integrate into the

host cell genome of dividing and non-dividing cells, as well as their broad tropism.

Currently, the industry standard for LVV manufacturing processes involves vector production via transient transfection of plasmid DNA into a host cell line, with downstream processing involving multiple depth filtration, chromatography, and tangential flow filtration steps. Host cell lines cultured in adherent cell culture systems are often used for upstream LVV processes, although significant progress has been made in the adaptation of host cell lines to suspension culture. LVV production through transient transfection of suspension cultures has typically yielded infectious titers of >1 x $10^6$ TU/mL [1–3], with infectious titers of up to 1 x $10^8$ TU/mL reported more recently after optimization of transfection conditions [4]. Suspension-based processes have been shown to reduce the cost of goods for LVV manufacturing by up to 90% compared to adherent systems [5]. However, downstream processing steps have also been found to contribute significantly to the cost of goods for LVV batch manufacture [5], and the introduction of suspension processes has presented new challenges for LVV downstream processing due to the increase in processing volumes.

In recent years, the development of stable LVV packaging and producer cell lines has also accelerated. Historically, it had been shown to be feasible to generate stable lentiviral vector packaging or producer cell lines; however, the infectious titers achieved at harvest were often low (<1 x $10^7$ TU/mL) [6–9]. The toxicity of constitutively expressed vector packaging or transgene sequences has also made development of cell lines suitable for LVV manufacturing challenging [10,11]. More recently, a number of groups have reported the generation of suspension-adapted LVV stable producer cell lines (PCLs) capable of producing infectious titers of >1 x $10^7$ TU/mL upon induction [12,13]. For example, at GlaxoSmithKline plc (GSK), Bacterial Artificial Chromosomes (BACs) encoding for LVV packaging and transgene sequences have been used for stable PCL development [12], and international patents WO2017/089307 and WO2017/089308 have been granted for this technology. This BAC technology allows for the rapid generation of stable PCLs through the transfection of a single DNA construct and offers the potential to significantly reduce the time required for cell line development campaigns. The use of such cell lines for upstream manufacturing processes has the potential to simplify operations and supply chains for LVV manufacturing, which will be discussed further in this article. The introduction of stable PCLs in upstream LVV processes also has significant implications for downstream processing, which will also be described in subsequent sections.

## CONSIDERATIONS FOR LVV UPSTREAM PROCESSING

Upstream processes utilizing transient transfection for production of third-generation LVVs typically require four plasmid DNA constructs encoding for packaging components, envelope, and transgene sequences. Batches of each of these plasmid DNA constructs must be manufactured to the required quality for clinical or commercial supply and may require bacterial cell banks to be generated to ensure supply continuity (Figure 1). This is in addition to the generation of manufacturing cell banks for the host cell line (often HEK293 derived), for which a two-tiered master cell bank (MCB) and working cell bank (WCB) system is often used, and which must be suitable for GMP LVV batch manufacturing. Although the packaging plasmid batches and manufacturing cell banks can be utilized for the manufacture of LVVs encoding for various therapeutic transgenes, sourcing these starting materials requires significant expenditure, and also adds supply chain complexity. The manufacture of these starting materials is typically outsourced to contract manufacturers and is dependent upon their available manufacturing capacity, which may result in extended timelines given the number of starting material batches required. Batches


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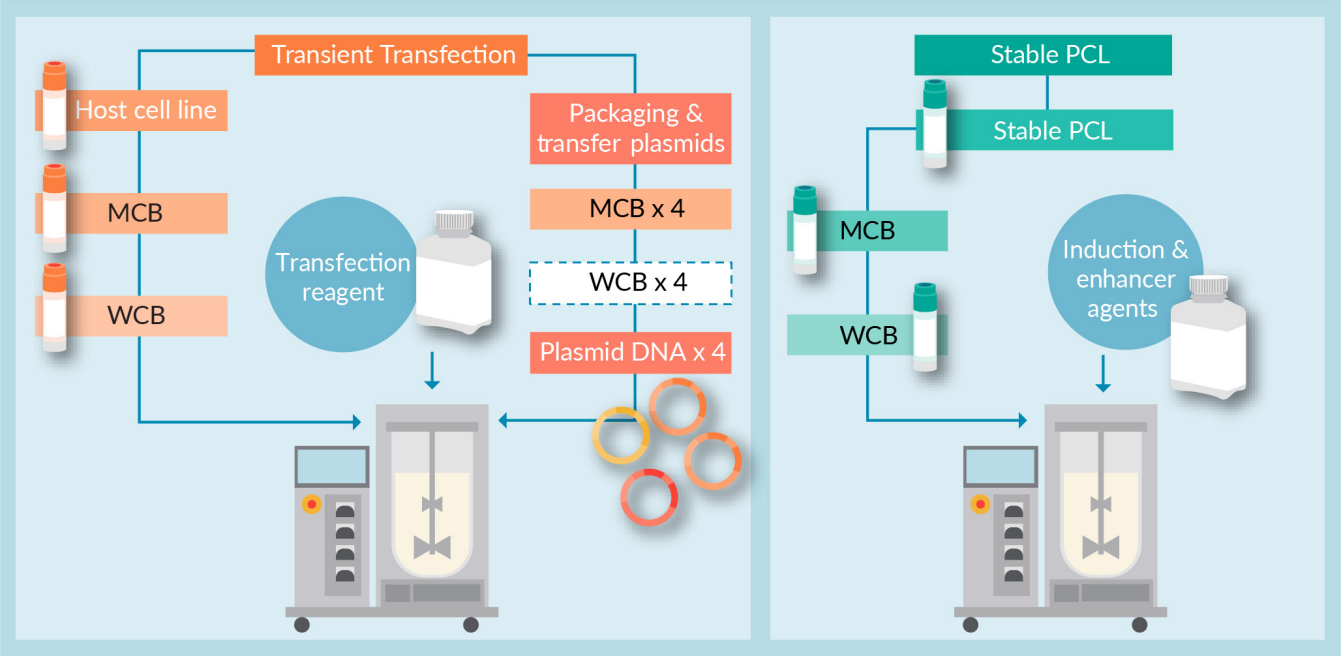
**FIGURE 1**

Raw & starting materials for transient transfection & stable PCL-based upstream LVV processes.




of these starting materials must also be well characterized with a good understanding of their manufacturing process demonstrated for clinical and commercial supply, which can add time and cost to the development lifecycle. Variability in the manufacturing, quality, and stability of these materials also has the potential to impact LVV batch quality and process robustness.

Two-tiered cell banks should also be established for each stable PCL used for LVV manufacturing, as these are specific to each LVV transgene. However, the sourcing of plasmid DNA batches is not required for processes using stable PCLs. Given the transgene specificity of these cell lines, a cell line development campaign is required to generate each LVV stable PCL, with each requiring significant time (approximately 6–9 months) and resource to complete. Currently, there is limited experience across the industry in cell line development for LVV stable PCLs, and further understanding of how different therapeutic transgenes impact the generation of LVV stable PCLs, and which stable PCL attributes impact LVV manufacturing, is required.

In addition to the sourcing of starting materials, suitable raw materials must be procured for upstream LVV processes. Viral vector production based upon adherent cell culture systems typically utilizes cell culture media containing animal-derived components (e.g., serum), whereas animal-derived component-free (ADCF) or chemically-defined (CD) media formulations are now being used for viral vector production in suspension systems. Additionally, for transient transfection-based processes, transfection reagents are required. Various types can be used to efficiently deliver plasmid DNA into host cells, for example, cationic lipid-based or calcium phosphate transfection [14], and these vary in their applicability for large-scale suspension processes. In recent years, polymer-based transfection reagents (e.g., polyethylenimine) have been shown to be suitable for transient transfection and viral vector production [3], and can be sourced at a suitable quality for GMP manufacturing. A number of proprietary transfection reagents are now also available; however, purchasing these can contribute significantly to the cost of goods for viral vector batches when procuring for large-scale

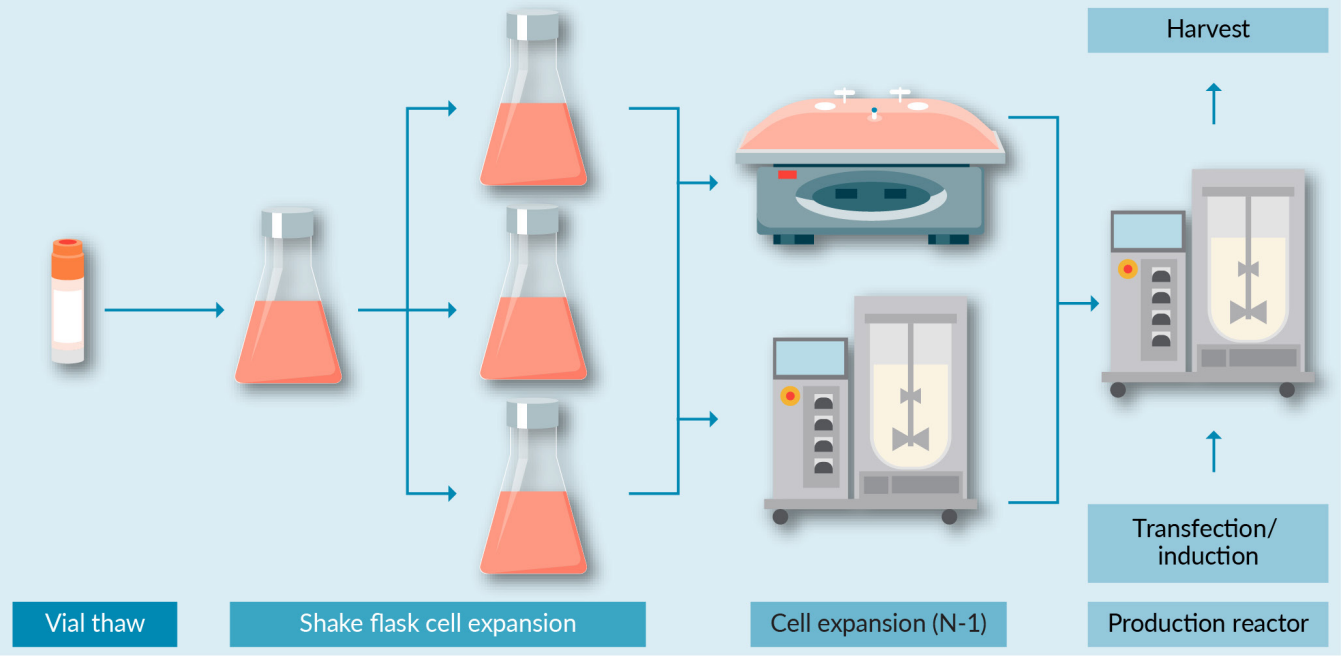
manufacturing. Additionally, demonstration of suitable clearance of these raw materials through downstream processing is often required, and the development of methods to quantify the concentration of such materials can be challenging depending upon the components of the chosen transfection reagent.

In contrast, the sourcing of raw materials for upstream LVV processes based upon stable PCLs can be more straightforward. Tetracycline-inducible systems (Tet-on) are often used in LVV stable PCLs, which require the presence of tetracycline or doxycycline to activate gene expression and viral production [12,15,16]. These reagents are typically inexpensive, non-proprietary, and can be sourced to pharmacopeial grade. Similar to transfection reagents, the clearance of induction reagents through downstream processing should also be evaluated. However, the risk of using such reagents may often be low due to low concentrations required in the upstream process, the significant removal of these reagents through the downstream process which is likely observed, and the grade at which these reagents can be sourced given their use as antibiotics in medicine. Considering this, it is probable that the concentration of these reagents at the end of downstream processing would be significantly below the permissible daily exposure (PDE) for humans. For *ex vivo* cell and gene therapies, additional clearance of these reagents is also likely to occur during processing of patient cells, in which LVV transduction and numerous media replacement steps often occur, and therefore the patient risk is likely to be further reduced. The genetic stability of stable PCLs should be evaluated during process development to determine whether maintenance of antibiotic selection in culture is required. The use of antibiotics in LVV manufacturing processes can pose significant challenges for materials procurement, facility control, and waste disposal. Therefore, establishing the genetic stability of stable PCLs without antibiotic selection is advantageous.

LVV manufacturing processes typically begin with the thaw of a cryopreserved WCB vial, seeding of cells in an adherent or suspension format, and cell expansion over multiple passages to generate a sufficient volume of cells for inoculation of the production vessel at the desired scale and cell density (Figure 2).

## FIGURE 2

**Typical unit operations for LVV upstream processes using suspension cell culture systems.**





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These steps are applicable to transient transfection or stable PCL-based processes. Typical suspension process seed train unit operations can be applied for stable PCLs, including cell bank thawing and cell culture in formats such as shake flasks, rocking motion bioreactors, and stirred tank bioreactors. Additionally, the utilization of single-use technologies for upstream unit operations in biologics manufacturing processes has simplified operations and reduced microbial contamination risk in manufacturing, and these technologies are now being applied for viral vector manufacturing.

Optimization of seed train parameters is required for host cell lines and stable PCLs in order to achieve the desired cell viability and viable cell number for inoculation of the production vessel, and to minimize seed train duration. For stable PCLs, the growth and metabolic profile of the clone should be considered. In the event that the selected stable PCL exhibits extended population doubling times, poor cell recovery post-thaw, or low cell viabilities during cell expansion, this optimization becomes critical. Such characteristics may be associated with the process of clonal selection during cell line development campaigns, or the constitutive expression of vector packaging sequences or transgene sequences [10,11]. As described previously, the genetic stability in culture of each stable PCL must also be demonstrated to establish a limit of *in vitro* cell age (LIVCA) sufficient for the number of population doublings required for LVV batch manufacture at scale. Chen *et al.* [12] have demonstrated that this may be feasible with LVV stable PCLs, and have generated cell lines that maintain functionality and genetic stability over more than 20 population doublings in the presence of antibiotic selection. This would be sufficient to culture 1 x $10^7$ viable cells from a single cryovial over multiple passages and to inoculate a 5,000L bioreactor at 2 x $10^6$ viable cells/mL.

For LVV production at scale, the utilization of stable PCLs holds significant advantages over transient transfection. Transient transfection-based processes typically require the formation of complexes of the required plasmid DNA constructs with the chosen transfection reagent/s. In addition to the potential variability introduced by this process and the use of these components, this step can introduce challenges during process scale-up. Development of an effective complex formation step at scale requires optimization of various parameters relating to mixing dynamics (e.g., mixing/addition method, time, temperature, etc.) and the complex components (e.g., plasmid DNA-transfection reagent ratios, complex formation medium, complex volume, etc.), followed by verification of the suitability of this process step at scale. Characterization of plasmid complexes and determination of complex formation efficiency can prove challenging, given the limited availability of established analytical methods linking complex attributes to transfection efficiency, or to be suitable for rapid determination of transfection efficiency when using LVVs encoding for a therapeutic transgene.

In contrast, when using LVV stable PCLs based upon tetracycline-inducible systems, the induction of LVV production can allow for more straightforward manufacturing operations. This step involves only the addition of an induction reagent and any enhancers or supplements, which are typically added within 24 hours of induction, followed by vector harvest approximately 48 hours post-induction when peak titer is achieved. A number of parameters for the induction step and LVV production phase can be optimized to maximize productivity and minimize process residuals concentrations, including cell density at induction, inducer/enhancer concentration, and harvest time. The development of control strategies for production bioreactor parameters, including pH, dissolved oxygen, temperature, and agitation speed, can also maximize yield and process robustness. When defining these process parameters, the growth characteristics, metabolic profile, and LVV production kinetics of the stable PCL should be considered. Given the relative simplicity of the

induction unit operation, LVV production using stable PCLs can easily be applied to large-scale manufacturing, for example in stirred tank bioreactors at hundreds or thousands of liters scale, and can streamline process scale-up. The use of stable PCLs can also reduce equipment requirements, compared to those required for complex formation, and reduce the risk of microbial contamination, given the fewer manual operator manipulations and number of materials required as compared to a complex formation step. Additionally, stable PCLs offer the potential for reduced variability between batches and greater process robustness given the monoclonality of these cell lines, and the lack of the variable transient transfection step.

Although significant progress has been made in the generation of high producing LVV stable PCLs and the scale-up of LVV upstream processes, a number of other opportunities also exist to reduce the cost of goods per LVV batch, improve the quality of each batch, reduce batch to batch variation, and to develop further process understanding. Developments in upstream LVV processes could help to achieve these through increases in process yield, reduction in batch durations, reduction in residuals concentrations at harvest and carryover into downstream processing, and implementation of process analytical technologies (PAT).

Process intensification offers the potential to reduce batch duration and consequently reduce the required facility time per batch, resulting in an increase in manufacturing capacity and reduced operational costs. Also, minimizing the number of passages in cell culture flasks by generating high cell density and volume cell banks suitable for thaw and subsequent inoculation of a bioreactor system in a closed manner, would not only reduce the risk of microbial contamination but also reduce process duration. The implementation of perfusion-based systems, such as alternating tangential flow filtration (ATF) or tangential flow filtration (TFF), have also been shown to substantially increase viable cell densities and product yield for the manufacture of monoclonal antibodies. Recently, the introduction of such technologies has been evaluated for the production of lentiviral vectors from HEK293 stable PCLs, with an increase in cumulative yield of more than 1 log achieved in a perfusion process ($8 \times 10^{10}$ TU/L) compared to a batch process ($6.9 \times 10^{9}$ TU/L) [13]. Therefore, the implementation of perfusion processes has the potential to substantially decrease the cost of goods associated with LVV manufacturing, as well as increasing process yield from smaller batch sizes. Introduction of perfusion technology in the production bioreactor step is likely to be more applicable to upstream processes utilizing stable PCLs, due to lower specific productivities which can be observed after transient transfection at higher cell densities [2,4] and the amount of plasmid required for high cell density transient transfection [17]. Furthermore, the implementation of perfusion processes offers the possibility of developing continuous upstream and downstream processes. However, this is yet to be established for LVV manufacturing with significant challenges to be overcome. For example, prolonging the maintenance of high viability cell cultures and LVV production during upstream processing to enable continuous processing presents a hurdle, particularly if cytotoxic sequences are constitutively expressed by the stable PCL. Furthermore, the implementation of suitable technologies for continuous cell retention and LVV harvest is required, and the impact of potentially greater contaminant levels from perfusion/continuous upstream processes upon downstream processing must be considered.

The development of novel media formulations and feeding strategies offers the potential to improve cell growth, maintain cell viability, and improve LVV production in batch, fed-batch, and perfusion processes. In recent years, a number of chemically-defined, commercially-available media formulations have been developed and optimized for viral vector production. However, these have often focused on viral vector production via transient transfection, with the delivery of


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plasmid DNA into a host cell line. Understanding of the metabolic profile of each LVV stable PCLs is required to develop optimized media formulations and feeding strategies for these cell lines. Numerous studies have been performed to understand the metabolism of HEK293 cells during virus production and to identify target metabolites for supplementation (e.g., lipids, cholesterol, sugars, vitamins) [18–21]. Additionally, a number of small molecules are known to enhance lentivirus production in HEK293 cell lines, for example Histone Deacetylation inhibitors and caffeine [22–24]. The continuation of such experiments to target the identification of novel feeds and LVV production enhancers for stable PCLs could further increase infectious titers and reduce LVV manufacturing cost of goods. For each candidate LVV production enhancer identified, the molecule's safety profile should be considered, the clearance of the molecule through downstream processing evaluated, and the procurement of material suitable for use in a GMP manufacturing facility investigated.

The engineering of host cell lines used to generate stable PCLs also represents an area where significant advances could be made to maximize cell viability and LVV productivity, and minimize the presence of contaminants during upstream processing. For example, through the knockdown of pro-apoptotic genes [25], through transgene repression [26], through modulation of viral budding pathways [27], and through secretion of nucleases [11].

The development of process analytical technologies for the characterization of transient and stable PCL upstream processes for LVV production will also be critical for process optimization and monitoring of critical quality attributes. The implementation of such at-line or online technologies could allow for monitoring of viable cell density, monitoring of the accumulation or depletion of key metabolites, optimization and automation of feeding strategies, and quantification of contaminant levels through upstream and downstream processing. Additionally, these tools could allow for quantification of the number of infectious and non-infectious virus particles, and the optimization of the method and timing of harvest in order to maximize LVV yield. Capacitance, or dielectric spectroscopy, is a technology that could be applied to overcome some of these challenges, as it has been shown to be suitable for monitoring cell growth and virus production kinetics [28]. Raman spectroscopy, at-line high-performance liquid chromatography (HPLC), and at-line mass spectrometry also represent potential solutions. These developments would represent a step change in process control for LVV manufacturing.

## CONSIDERATION FOR LVV DOWNSTREAM PROCESSING

For downstream processing of LVVs, the average overall yield from manufacturing is estimated to be ~31% [29]. This high attrition of transducing units (TU) across a process can be attributed to a number of factors, such as relatively low stability and limited physiological range of LVV. Additionally, the challenge of achieving batch-to-batch consistency in downstream processing is often seen when scaling up to large batch sizes (200–500L). The requirements to ensure product quality and to reach the required infectious titers remain the primary influencers in developing a robust LVV downstream process. The use of stable PCLs for LVV production offers a number of advantages and challenges in addressing these issues.

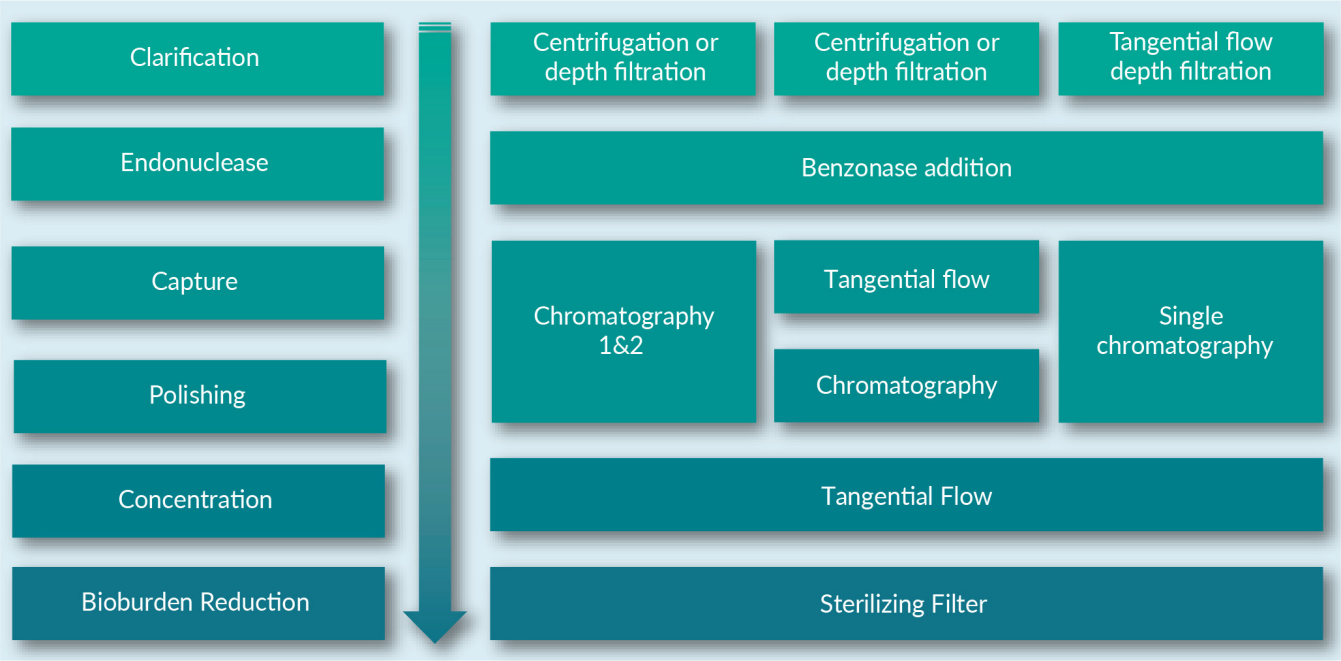
To increase overall titers of the process, there is often a driver from the upstream process for higher cell density cultures (e.g., in perfusion mode) and a subsequent increase of TU into the downstream process. However, when processing such large volumes of LVV harvest material, the requirement to minimize processing time and shear stress has one of the biggest impacts on process yield. This is largely due to the relative instability of the lentivirus particle, in part because of the fragility of the membrane envelope which

necessitates a narrow physiological window for separation techniques. Typically, downstream unit operations are performed at pH range of 7+/-1 [30], in an environment of low osmolarity and low temperature to maintain the stability of the virion. It is the effect of temperature which is one of the main inhibitory factors in maintaining stability of the vector, with processing usually maintained below 12°C to maintain vector integrity. Even with the narrow considerations for processing, stability of the vector is still a concern as evidenced by typical completion of purification within 48 hours, in contrast to many mAb processes. It is this time-dependent pressure to complete processing that drives the reliance on high flow rate unit operations at the beginning of downstream processes to de-water the culture and allow for operability later in the process. This inherently comes with a number of risks, including the creation of a number of process-related impurities due to shear. This also in part explains the reliance throughout the process on buffers that offer cryoprotectant properties, in an effort to protect the virus from process-related stresses.

The typical unit operations employed for downstream LVV processes are summarized in Figure 3. Some of the greatest stresses are often created during the steps at the beginning and end of the process. This includes filtration during harvest clarification of the bioreactor material, and bioburden reduction of the vector before final fill. For clarification, this is in part due to use of some kind of depth filtration, traditionally used to separate cell mass from the supernatant using a sufficiently specified pore sized membrane to ensure adequate reduction in bioburden. The clarification unit operation may be affected by high levels of DNA which are introduced during transient transfection, and therefore the use of stable PCLs may be advantageous. The levels of DNA often contribute to higher viscosity creating higher back pressure through any form of filtration, but also play a key role in the interaction and binding to virions. While this is something that can be accounted for by adequately sizing of depth or flat filters, stable PCL processes can be somewhat challenging if significantly high cell densities and low cell viabilities at harvest are achieved. LVV production at higher

**FIGURE 3**

**Examples of possible different unit operation orders for DSP.**





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cell densities can lead to lower cell health at harvest and higher host cell protein and DNA content from which purification is initiated. Additionally, increased transmembrane pressures (TMPs) due to the increased load on these filters can result in increased vector shear and cell disruption, which release complex mixtures of DNA associated with histones and other nucleoproteins that can create multicharged complexes displaying a range of positively charged, negatively charged and hydrophobic regions [31]. One approach to mitigate the challenge seen on clarification by filtration is the use of centrifugation. This approach offers an alternative to the pressures placed on the vector in complex cell cultures and allows for wider ranges in harvesting parameters to be used [32]. However, as bioreactor volumes increase, the scalability of this type of method becomes more challenging to execute and may require the implementation of new novel technologies such as continuous solids-discharging centrifugation.

The role of DNA interaction in the efficiency of LVV downstream processing is most commonly addressed by the use of endonucleases to metabolize large amounts of DNA present in the harvest material. This may be employed once or twice in the process dependent on the order of the downstream processing unit operations, and/or the efficiency of the capture step to remove residual endonuclease once used. Indeed, an indicator of the efficiency of an established process for lentivirus purification is the ratio between TU/DNA and also TU/host cell protein (HCP).

In general, the use of chromatography as a capture step to reduce volume and purify the bulk material from clarification is very common. Chromatography offers a greater range and higher selectivity for purifying LVVs based on the mode of action of binding, be it by anion exchange, hydrophobic interaction, or by a non-specific affinity-based interaction such as a heparin-based resin [1]. Irrespective of the ligand used to bind the vector, high cell density cultures offer a significant challenge to these modalities due to the narrow physiological range that maintains LVV stability, and the mixed population size distribution that the virions are expressed as (80–120nm). As a consequence of this, the dynamics of diffusion-based chromatography into the bead becomes more powerful in binding and resolving HCP and DNA rather than lentivirus itself, which predominantly binds by convective mass transfer. This had led to the use of membrane and monolith technologies, which offer highly porous channels and higher flow rates for processing large volumes. Despite this, the challenge in achieving separation/selectivity against contaminants that may co-elute with LVV in harvest material has been largely driven by the choice of ligand and the ligand density. It is for this reason the development of a form of affinity-based chromatography for LVV, and in particular, the processing of stable PCL harvest material, will offer a powerful tool for capture, purification, and concentration in the future. In terms of large-scale processes, this advancement will be wholly dependent on the development of such a resin that uses a robust backbone for suitable flow properties and an eluting agent that is physiologically compatible with LVV. While this mode may clearly be an option for future processes [33], current alternatives such as mixed mode resins are gaining popularity, particularly as a polishing chromatography step. The limitation of scale when using traditional size exclusion is accommodated by the use of flow though technologies employing an exclusion-based outer core and a binding mode in the internal core. Whether LVV material is generated from an upstream process based upon transient transfection-based process or a stable PCL, a form of polishing that significantly lowers HCP and DNA levels while removing aggregates could play an important role in ensuring final product quality. It is often at this stage of the process that aggregation/size distribution profile has one of the greatest impacts on final yield and purity.

While chromatography is often the preferred method for capture in LVV processes, in some cases, tangential flow filtration (TFF) has been used as an alternative. This is mainly due to its flexibility in exchange of buffer components, its ability to remove HCPs and DNA, and its capability to concentrate before further processing. Despite this, the use of either hollow fiber or flat sheet TFF requires sufficient surface area to cope with the turbidity and complexity of the feed, particularly seen with stable PCLs. The required flux rates and continuous recirculation can promote fouling especially at larger process volumes and this requires careful process development. Single-pass TFF is an emerging technology [34] that could offer the advantages of this technique by allowing flow across sufficient membrane area without the potential pitfalls of recirculation and fouling. This method is however still quite new with regards to LVV downstream processes and it remains to be seen if it will replace existing technologies [35]. While TFF is not generally employed early in LVV downstream processes, this highlights a recurring problem in the field – that current technologies for both tangential flow and chromatography unit operations have been developed specifically for mAb processes, and may not be suitable for larger, more complex biomolecules such as LVV.

Typically, the concentration of vector material at this stage and reformulation is achieved using a TFF step, where the formulation is often a complex mixture of proteins, sugars, lipids, and salts [36]. This part of the process is important in determining the final concentration and subsequent dose requirements per patient of the final vector material. As such, the very nature of concentrating and recirculating the product at this stage of the process can place a particular strain on the LVV, which may promote shear and aggregation. For stable PCL processes, this can be especially challenging due to potentially higher cell densities achieved, and may be exacerbated with the introduction of upstream technologies such as perfusion, which may potentially produce more concentrated levels of process-related impurities and LVV. As a consequence of this, it becomes increasingly important to improve selectivity and product quality earlier in the downstream process.

Crucially, the choice of the final formulation and the preceding buffer system in the earlier unit operations greatly influences the degree of purification and, in the later stages, the aggregation profile, which is one of the primary factors affecting sterile filtration. Indeed, the most significant pinch point in any LVV process is the bioburden reduction process. Significant losses across this final filter remain a problem across the industry regardless of whether the vector is generated from a transient transfection or stable PCL-based process. The aggregation profile places a high burden on gaining high step recoveries across a 0.2µm filter. It is for this reason perhaps that stable PCL processes offer a greater ability to scale and produce larger batch sizes with increased reproducibility. It is this reproducibility that helps to establish the correct sizing strategy required to minimize the losses seen during this step.

The ability to develop LVV downstream processes is driven by the available analytical capabilities. The development of rapid (or at-line) in-process analytical methods for LVV processes (transient and stable PCL-based) is ongoing. Currently, infectious titer assays are relied upon heavily and these take place over a number of days. It can mean that a lentivirus downstream process is effectively run blind until the analysis has been completed sometime after the process. Despite this, areas of analysis such as spectroscopy (i.e., DLS, SLS, UV Vis Spectroscopy, mass spectrometry), imaging (TEM), particle analysis (TRPS or NTA, microfluidic flow analyzers) are becoming increasingly important in giving early feedback in process performance. While some of these technologies are still in their infancy, it is clear that they will become key in offering important information as processes advance towards continuous manufacturing in the future.


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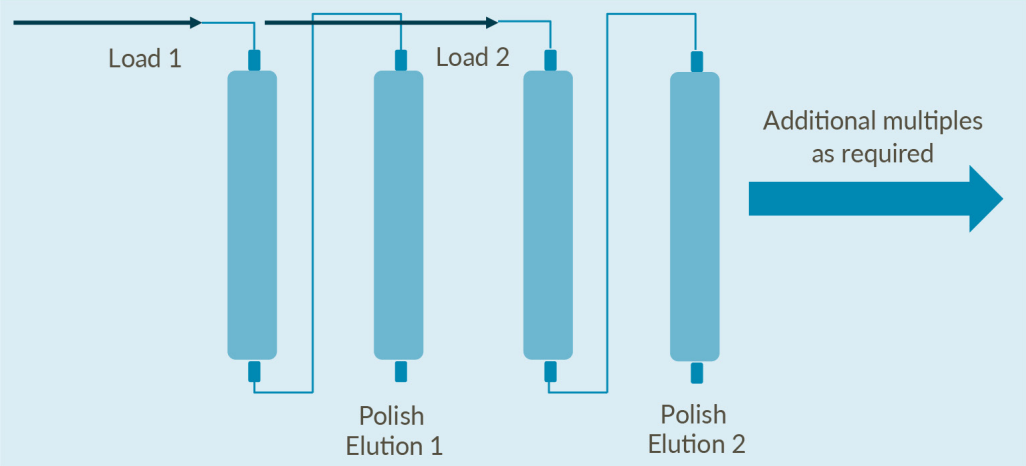
**FIGURE 4**

Continuous chromatography/manufacture.




For LVV manufacture, stable PCLs clearly offer an advantage in the development of a continuous manufacturing process (Figure 4). This would potentially reduce scales and size of equipment, and allow for batch consistency and improvement in stability, due to the reduction of start to finish time. Utilizing this type of process may allow the potential strengths of unit operations such as single-pass TFF, as a form of continuous harvest filtration, and affinity chromatography, for the capture of LVV, to be leveraged. These two unit operation advancements are in their early stages for applicability for vector production, but show the potential for process intensification that can be potentially achieved when using stable PCLs for LVV production.

## CONCLUSION

In this article, we have described the significant advantages that stable PCLs potentially offer, and some of the upstream and downstream processing challenges that are currently faced when utilizing these. Under certain circumstances, transient-transfection-based processes could still be considered as an alternative to stable PCLs, for example, where challenges are faced in the development of stable PCLs for LVVs encoding large or cytotoxic transgenes, or to supply early clinical studies prior to final confirmation of the target sequences. It should be noted, however, that demonstration of comparability when introducing manufacturing process changes should be considered when planning and developing processes for clinical and commercial phases. Recently, the demonstration of comparability has led to delays in the launch of a gene therapy product [37]. Therefore, the early development of commercial-ready LVV manufacturing processes based upon stable PCLs may be advantageous in minimizing process changes through the product lifecycle, and reducing the number of comparability studies required to justify such changes. Eliminating the requirement for these studies would also reduce the time, resource, and risk associated with their execution and submission to regulatory authorities.

In conclusion, it is clear that the introduction of stable PCLs for LVV batch manufacturing holds great potential for increasing process yield, improving batch to batch consistency, assuring supply, and reducing cost of goods, in order to meet the demands for clinical and commercial supply of LVVs for prevalent indications (e.g., oncology). However, further innovation and process characterization are required to develop high producing, robust, commercial-ready processes for LVV production to ensure the supply of these revolutionary medicines for patients.

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# Scale up of a lentiviral production process from the iCELLis® Nano Bioreactor to the iCELLis 500+ Bioreactor

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**Biotechnology companies are interested in scaling up from flatware to commercial scale as quickly as possible, which necessitates using adherent cell culture methods. Here, we describe how Advanced BioScience Laboratories, Inc. scaled up from the iCELLis NAno Bioreactor to the iCELLis 500+ bioreactor.**

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## MATERIALS & METHODS

### Cell expansion

HEK293LTV cells from Cell Biolabs were cultured in DMEM + 6 mM L-glutamine + 10% fetal bovine serum (FBS) in CellSTACK® 10s culture chambers and inoculated into the iCELLis Nano and iCELLis 500+ bioreactors at 8,000 cells/cm$^2$.

### Cell density & metabolite analysis monitoring

Carrier strips from the iCELLis Nano bioreactor were removed from the fixed bed daily to determine cell density using lysis buffer and nuclei counts on the NucleoCounter® NC-200® cell counter. Metabolite concentrations were measured from both the iCELLis Nano and the iCELLis 500+ bioreactors daily using the Nova Biomedical BioProfile FLEX® automated cell culture analyzer. These samples were obtained by removing medium from the aseptic sampling port on the iCELLis Nano and iCELLis 500+ bioreactors.

**Table 1. Parameters used in iCELLis Nano and iCELLis 500+ bioreactors.**

| Process parameter | iCELLis Nano Bioreactor | iCELLis 500+ Bioreactor |
|---|---|---|
| Surface area (m$^2$) | 0.53 | 66 |
| Culture duration (days) | 7 | 7 |
| Seeding density (cells/cm$^2$) | 8,000 | 8,000 |
| Volume per surface area during cell growth (mL/cm$^2$) | 0.13 | 0.13 |
| Media change prior to transfection (day) | 4 | 4 |
| Day of transfection (day) | 4 | 4 |
| Cell density at time of transfection (cells/cm$^2$) | 252,000 | * |
| DNA concentration (µg/cm$^2$) | 0.2 | 0.2 |
| µg DNA/million cells | 0.8 | * |
| µg DNA : µg PEI ratio | 1:2 | 1:2 |
| Transfection complex volume | 10% of working volume | 10% of working volume |
| Perfusion rate post-transfection (mL/cm$^2$/day) | 0.067 | 0.067 |
| pH setpoint | 7.2 ±0.1 | 7.2 ±0.1 |
| DO setpoint | 40% | 40% |
| Linear speed during attachment (cm/s) | 2 | 1.3 |
| Linear speed during cell growth (cm/s) | 1.2 | 0.7 |
| Linear speed during transfection (cm/s) | 2 | 1.3 |
| Linear speed during production phase (cm/s) | 1.2 | 0.5 |

*Cell counts for iCELLis 500+ bioreactor estimated based on counts from the iCELLis Nano bioreactor.

### Transfection

Cells were transfected on day 4 using the parameters used in **Table 1**. 10% of the bioreactor working volume was removed from the bioreactors which was then replaced by the transfection complex via hand-pump (iCELLis Nano bioreactor) or gravity (iCELLis 500+ bioreactor).

### Production

Perfusion was started 4 h post-transfection. A constant flow rate was used for both bioreactors. A bottle or tote with fresh medium was connected to the 'media in' pump of each bioreactor and an empty bottle or tote was connected to the 'media out' pump of each bioreactor. Slowly, new medium was pumped in while virus-containing medium was pumped out.

### Analysis

Samples were collected and stored at -80 °C until ready to be analyzed. Samples were then thawed and clarified by centrifugation before RNA extraction for RT-PCR.

## RESULTS

### Viral titer

The final bulk harvest had a similar titer to DPT-3, suggesting the concentration of virus in the perfusion out drum was the same as inside the iCELLis bioreactor vessel.

The titer (gc/cm$^2$) was 1.02 x 10$^8$ gc/cm$^2$ in the iCELLis Nano bioreactor and 3.47 x 10$^8$ gc/cm$^2$ in the iCELLis 500+ bioreactor.

### Metabolites

Concentrations of both glucose and lactate were similar between the iCELLis Nano and the iCELLis 500+ bioreactors, suggesting similar cell growth and cell density.

## CONCLUSION

A process was scaled up rapidly from the iCELLis Nano bioreactor to the iCELLis 500 + bioreactor with an N = 1 for each bioreactor. The iCELLis 500 + bioreactor produced 3.47 x 10$^8$ gc/cm$^2$ while the iCELLis Nano bioreactor produced 1.02 x 10$^8$ gc/cm$^2$. The concentration of nutrients and metabolites were similar between the iCELLis Nano and the iCELLis 500 + bioreactors throughout the entire run, suggesting similar cell growth between the two scales.

**Figure 1.** Left: Titer obtained via qRT-PCR for the iCELLis Nano and the iCELLis 500+ bioreactors. Right: Total titer yield from each bioreactor, normalized to surface area.

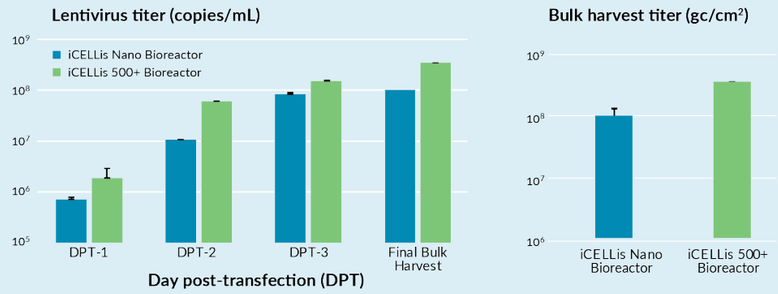
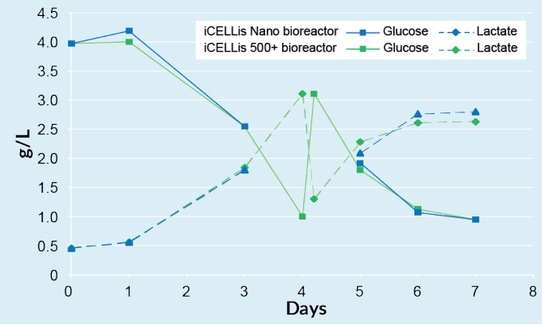



**Figure 2.** Nutrient and metabolite concentrations in the iCELLis Nano and iCELLis 500+ bioreactors in g/L.

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[1]Advanced BioScience Laboratories, Inc; [2]Pall Corporation

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UPSTREAM BIOPROCESSING


INNOVATOR INSIGHT

# Utilizing a media panel rapidly accelerates media optimization for AAV manufacturers

Céline Martin & Jennifer Zatina

Gene therapies offer great promise for the future of medicine, with adeno-associated viral (AAV) vectors emerging as one of the leading delivery vehicles for these innovative therapies. However, the production of AAV-based therapies using HEK293 cells is a complicated process. Regulatory agencies have created expedited approval pathways for these promising, but complex, therapeutics. However, developers face important time constraints in developing robust manufacturing workflows. For these reasons, a successful chemicals, manufacturing, and control (CMC) strategy must be established as soon as possible to prevent delays during clinical trials. To support the implementation of this strategy, AAV manufacturers are looking for solutions to accelerate process and analytical development, notably those that can help with the selection and optimization of a suitable cell culture medium. The Gibco™ Viral Vector HEK Media Panel is the first panel solution for gene therapy manufacturers, and it has the potential to accelerate media optimization. The following article describes how the media panel can be used to quickly identify media candidates for multiple HEK293 cell lineages.

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Gene therapy is a rapidly growing field of medicine that uses viral vectors to deliver genetic material to cells to compensate for abnormal, faulty, or missing genes. Of these viral vectors, recombinant adeno-associated viruses (AAV) are emerging as one of the leading delivery vehicles. This is due to the lack of disease associated with the wild-type virus and its ability to transduce dividing as well as nondividing cells. There are also multiple

serotypes available, including those that can be engineered with differing cell type selectivity and immunogenicity, which developers can exploit for more specific therapies. The availability of these serotypes increases the usability of the vector, advancing the commercial potential of AAV-based gene therapies.

AAV-based gene therapies are seeing rapid growth, with multiple products already approved and over a hundred in clinical trials [1], which has fueled the demand for cGMP production solutions and the expansion of manufacturing capacity for viral vectors. Regulatory agencies such as the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have given gene therapies expedited regulatory approval pathways. These initiatives include Fast Track designation and Breakthrough Therapy status from the FDA [2] and the PRIME scheme from the EMA [3]. For this reason, commercially viable and scalable supply chains need to be established as early as the beginning of a Phase 1 clinical trial, with a robust chemicals, manufacturing, and control (CMC) strategy in place as soon as possible to prevent delays in getting AAV products to late-stage clinal trials.

As a result, manufacturers are focused on developing and improving scale-up and analytical assays to generate highly pure and potent AAV vectors. One step toward this goal is the selection and optimization of a suitable cell culture medium.

## MEDIA OPTIMIZATION CHALLENGES

AAV vectors are typically produced using HEK293 cells due to their reliable growth and the efficiency and success of transfection. The cell culture medium used to grow these cells will impact the final quality of the viral vector products and viral titers but optimizing a single medium for a variety of HEK293 cell lines is difficult. Different HEK293 lineages have varying gene expression profiles [4]. Along with the effect that adaptation to suspension has on metabolism [4], this results in specific cell-dependent requirements that necessitate considerable cell bank–specific medium optimization.

Selecting the optimal medium for an AAV manufacturing workflow has the potential to not only improve titers, but also improve viral particle quality, and it will ultimately simplify the scale-up process. Unfortunately, media formulations are complex and composed of potentially over a hundred components, all of which play a significant role in cell growth and productivity. Optimizing formulations effectively can require in-depth analysis of the key performance drivers in the formulation. This medium optimization process is time-consuming, and gene therapy manufacturers must balance this with the accelerated timelines introduced by regulators to prevent delays.

## OPTIONS AVAILABLE FOR MEDIA OPTIMIZATION

When it comes to media optimization, AAV developers and manufacturers have previously only had two options. The first is the development of a proprietary formulation designed specifically for the cell line and process. However, the development of a proprietary formulation requires lengthy R&D studies and significant investment, followed by extensive sourcing and qualification of raw materials for manufacturing. For this reason, many developers opt for the second option of off-the-shelf catalog formulations, which are readily available. This, however, does not account for the time taken to find and select a medium suitable for optimization. As most catalog formulations aren't designed for specific cell lines or processes, developers need to spend considerable time evaluating media from several suppliers after early proof-of-concept studies. Once a medium is selected, additional time is needed to further optimize it to improve process performance as they scale up and optimization need to be repeated for different iterations of a platform process.

Media panels are a new third option for AAV manufacturers. Media panels contain a diverse set of formulations that offer the greatest amount of nutritional diversity in a small


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library. They are purposely designed to help reduce the time spent screening for an ideal medium. Although media panels have been available for other applications such as CHO-based mAb manufacturing, they have not previously been available for gene therapy applications.

## THE GIBCO VIRAL VECTOR HEK MEDIA PANEL

The Gibco™ Viral Vector HEK Media Panel – with five serum-free and chemically defined media – has been developed to support enhanced performance in HEK293 cell culture. It has the potential to offer increased viral vector titers or quality in some HEK293 cell lines and at least two AAV serotypes. As Table 1 shows, the compositions of the formulations are unique with varying concentrations of key nutritional components. This will allow AAV manufacturers to rapidly screen for improved media performance and recognize the key drivers behind the improved performance.

One of the key advantages of the Viral Vector HEK Media Panel is its versatility, enabling five media to be evaluated for a broad range of HEK293 cell lines quickly. This versatility was demonstrated in the following experiments, where HEK293F and HEK293T cells were used to produce AAV2 and AAV8, respectively.

## HEK293F cell growth & AAV2 productivity

Two suspension HEK293F clones, from the Gibco™ FreeStyle™ 293-F cell lineage, designated 293F1 and 293F2 were evaluated for growth and titer production. The clones were directly adapted from the banked medium to Gibco™ FreeStyle™ F17 Expression Medium as the control medium or one of the five panel media (designated panels 1–5). All cultures were supplemented with Gibco™ GlutaMAX™ Supplement.

Prior to transfection, the population doubling times for the 293F1 and 293F2 cells in all five panel formulations were shown to be comparable to the control (Figure 1). The results suggest that all the Viral Vector HEK Panel media would support sufficient growth for productive AAV2 transfection.

The HEK293F clones were transfected after dilution in fresh media to 3 x $10^6$ cells/mL cells using PEIpro™ transfection reagent (Polyplus). All transfections were performed on cells at passage 3 post-thaw. The AAV2 viral titers were quantified on crude lysate, at harvest 72 h post-transfection, using qPCR. The 293F2 clone produced higher overall average titers across all panel media compared to the 293F1 clone (Figure 2A & B). Interestingly, both clones produced the highest average viral genome titers with Viral Vector HEK panel media 4 and 5 relative to the control. The titers of the 293F1 cells were 10-fold higher in the panel media (Figure 2A), the titers of the 293F2 saw a 2-fold increase (Figure 2B).

## HEK293T cell growth & AAV8 productivity

HEK293T cells derived from adherent serum–banked HEK293T cells were adapted to suspension in a serum-free medium. After

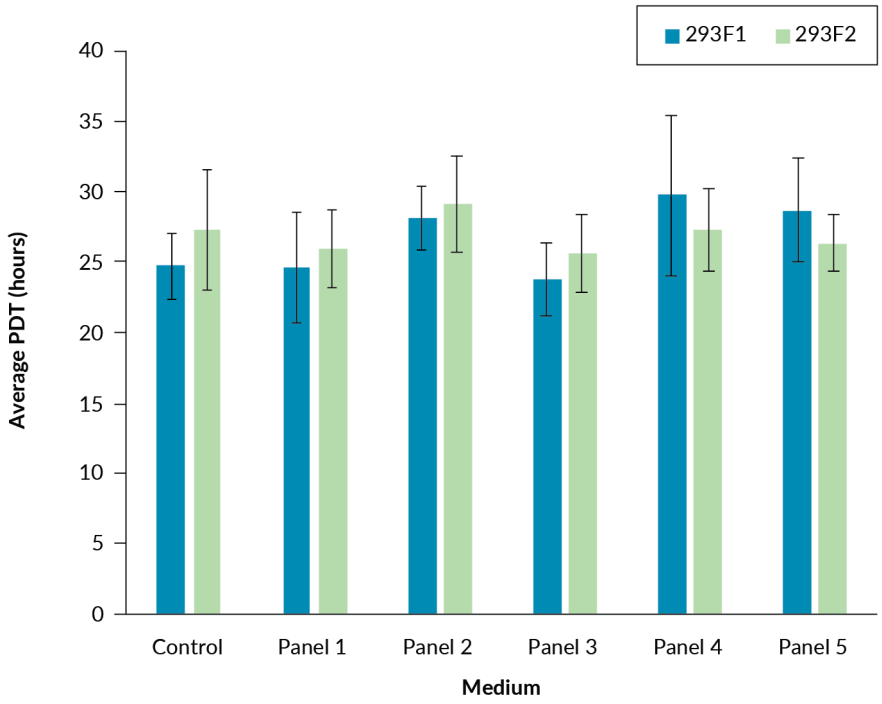
**TABLE 1**

**Component diversity heat map of the Viral Vector HEK Media Panel.**

| Component | Panel medium 1 | Panel medium 2 | Panel medium 3 | Panel medium 4 | Panel medium 5 | |
|---|---|---|---|---|---|---|
| Amino acids | | | | | | |
| Vitamins | | | | | | |
| Lipids | | | | | | |
| Trace metals | | | | | | |
| Polyamines | | | | | | |
| | | | | | | |
| High level | | | | | | Low level |

**FIGURE 1**

HEK293F population doubling time.




The 293F1 and 293F2 cell clones demonstrated comparable average population doubling times (PDTs) over 3 passages after adaptation to the five Viral Vector HEK Media Panel formulations and the control medium from P3 to P5 (biological replicate from different thaw, 293F1 n=4, 293F2 n=3).

recovery, the cells were adapted to the same control medium and Viral Vector HEK media 1 and 5 supplemented with GlutaMAX Supplement, which contain either the lowest (panel 1) or highest levels (panel 5) of key nutrients.

The population doubling times of cells cultured in Viral Vector HEK media 1 and 5 were comparable to the doubling times in the control medium (23 ± 1 hour, at the 3rd passage in 100% of the respective medium), regardless of their very differentiated compositions. This demonstrated the ability of the panel to also support sufficient growth for productive AAV8 transfection (data not shown).

Viral genome titers were comparable in Viral Vector HEK media 1 and 5 and the control medium (Figure 3A), titers were quantified on crude lysate, at harvest 72h post-transfection, using qPCR. The HEK293T cells were transfected after dilution in fresh media to 2 x $10^6$ cells/mL cells using PEIpro™ transfection reagent. Product quality was also assessed by calculating the ratio of full to empty capsids using a capsid ELISA to quantify the total amount of particle. Empty capsids are a manufacturing impurity that can affect the efficacy and safety of AAV vector products [5]. Viral Vector HEK medium 1 facilitated the production of an average of 76% full capsids, compared to 51% and 40% in the control medium and medium 5, respectively (Figure 3B).

## CONCLUSION

AAV manufacturers must be able to rapidly identify media formulations candidates that can be adopted for their HEK293 cell lines, AAV serotypes, and transfection processes. The Viral Vector HEK Media Panel is the first available media panel that allows AAV manufacturers to accelerate and simplify media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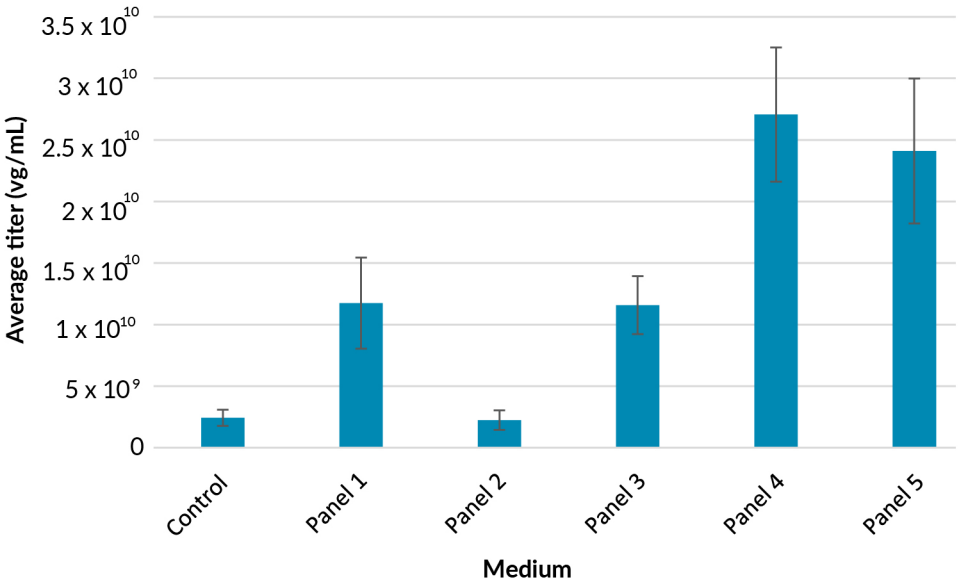
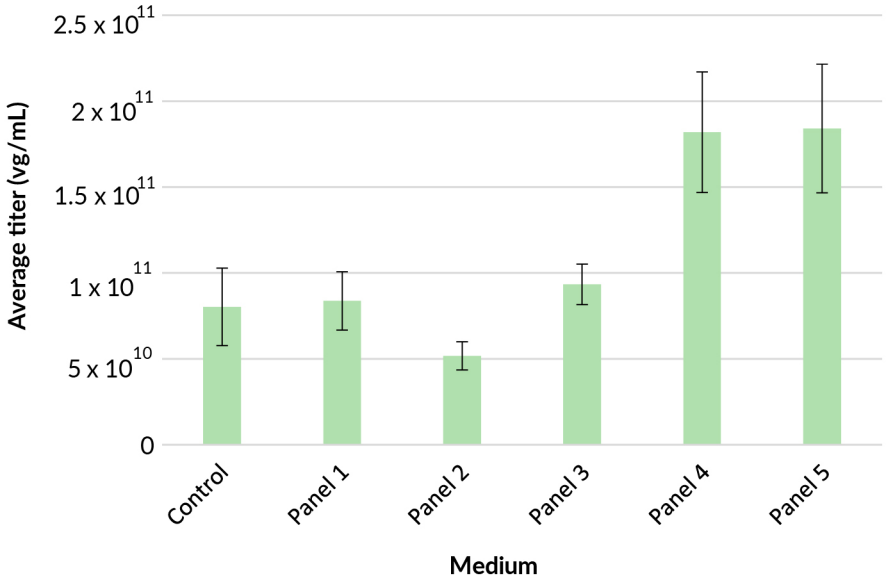
## FIGURE 2

HEK293F AAV2 viral genome titers.

A: AAV2 viral genome titer from 293F1 cells




B: AAV2 viral genome titer from 293F1 cells

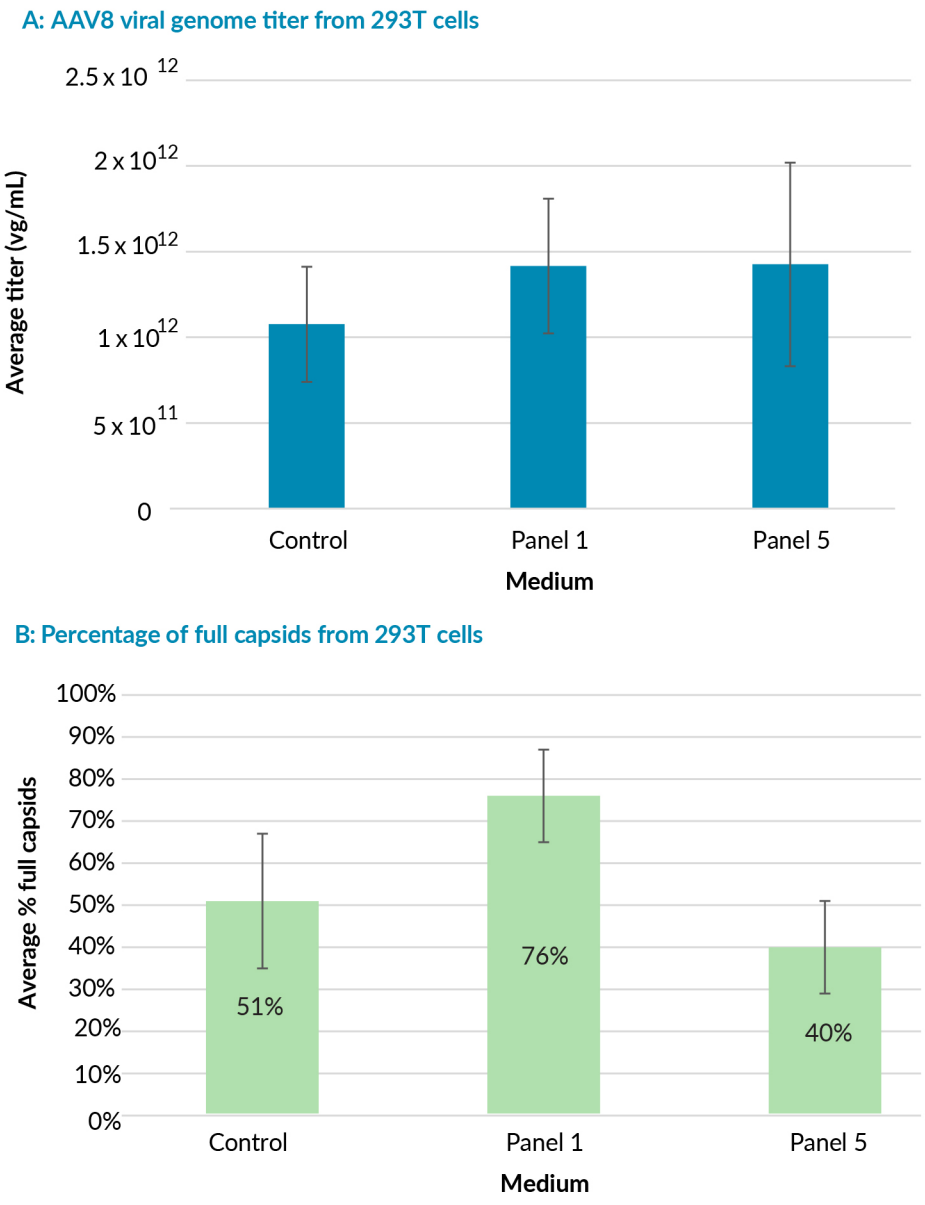



(A) The 293F1 cells produced 10-fold higher average AAV2 titers with Viral Vector HEK media 4 and 5 relative to the control. (B) 293F2 cells demonstrated 2-fold higher average titers with Viral Vector HEK media 4 and 5 relative to the control (n=3).

optimization. The Viral Vector HEK Media Panel enables rapid screening of candidate media that may support higher titers and better quality with diverse HEK293 cell lines while maintaining steady growth. This allows manufacturers to identify promising media formulations faster and establish unique workflows that are in line with the diversity of platforms being developed in viral vector manufacturing.

## FIGURE 3

HEK293T viral genome titers and percentage of full capsids.




(A) Viral Vector HEK media 1 and 5 yielded comparable average titers when compared to the control medium. (B) Viral Vector HEK medium 1 produced 76% full capsids compared to 51% with the control medium and 40% with Viral Vector HEK medium 5 (n=3).

For more information, visit thermofisher.com/hekpanel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## AUTHORSHIP & CONFLICT OF INTEREST

***Contributions:*** *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

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ThermoFisher SCIENTIFIC




# Streamline media optimization for AAV production

Accelerate your AAV manufacturing with a diverse set of ready-to-use formulations. Compatible with a broad range of HEK293 cell types, the Gibco™ Viral Vector HEK Media Panel is not dependent on specific transfection reagents or techniques, enabling efficient media formulation identification for your target cell line and workflow.

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UPSTREAM BIOPROCESSING


INNOVATOR INSIGHT

# Evaluation of a microbioreactor system as a screening tool for optimizing lentiviral vector process development in suspension culture

Nolan Sutherland, Lesley Chan, Kelly Kral & Franziska Bollmann

With an increasing number of lentiviral vector (LVV)-based cell and gene therapy candidates reaching clinical trials, scalable suspension cell culture processes using stirred tank reactors (STRs) are needed to meet future demands. However, to cost-effectively scale LVV production in STRs requires process development which can be expensive and time consuming to perform in bench-top bioreactors. To address these issues, a multi-parametric approach for process development using a micro scale bioreactor system (Ambr® 15 cell culture system, Sartorius) was assessed. Since the medium exchange process step cannot be linearly or methodically scaled-down from a bench-scale STR to a microbioreactor due to system differences, this study focused on adjusting to those differences by developing and testing three different medium exchange protocols. The implementation of one approach (Process 2.0) using an automated cell settling medium exchange protocol produced results which closely aligned with an established LVV bench-scale process in transfection efficiency and productivity, as well as lowered variability between vessels in the cell culture workstation. In summary, this study demonstrates the suitability of the Ambr 15® system as a process screening tool which has the potential to reduce costs and timelines of the development of scalable LVV production systems in suspension culture.

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## LVVS FOR GENE THERAPY

Lentiviral vector (LVV) systems are recombinant viral vectors used for delivering *ex vivo* and *in vivo* gene therapies into primary cells and are commonly used to correct a gene associated with a monogenic disease [1]. In recent years there has been an acceleration in the number of clinical studies utilizing LVVs and by March 2021 according to ClinicalTrials.gov trial registry [2] there were 646 active studies listed. This increase is driven in part by the successful use of LVVs in chimeric antigen receptor (CAR) T-cells as cell therapy to treat blood cancers and includes the FDA approved Kymriah [3] to treat acute lymphoblastic leukemia (ALL). However, with Kymriah for example retailing at a list price of $475,000 US Dollars [4], these types of therapies are currently often prohibitively expensive. One reason for the high price tag is that the manufacturing Cost of Goods (CoGs) is high, with LVV production representing a large proportion of the costs [5]. Therefore, as LVV is becoming more widely used, scalable and efficient processes across the production workflow are critical if LVV is going to be successfully manufactured to deliver a consistent, pure, high-titer product that is safe, efficient, and affordable. Thus, there is now a drive towards reducing LVV manufacturing costs to help ensure the commercial viability of many life-saving gene and cell therapies.

## ADDRESSING SCALABILITY TO REDUCE LVV PRODUCTION COST

Looking to produce LVVs for commercial use, adherent cell-based processes in tissue culture flasks can robustly produce sufficient supply for clinical trials but are limited in their ability to scale to the demand required. Traditionally, these production processes are scaled-out by vessel number, but not scaled-up in size/volume, limiting the batch size to the number of flasks which can be successfully manipulated. For example, during an adherent LVV production process only approximately 40 L can be produced in 40 vessels, whereas production in a single 200 L STR increases the number of LVV units 5-fold volumetrically while the number of vessels to manipulate decreases significantly. This consolidation of vessels and scale-up in volume thereby increases batch consistency while reducing the CoGs per unit of LVV, respectively.

Adherent systems are therefore limited when moving from clinical trials to the commercial environment as they cannot be scaled-up but only scaled-out, increasing manufacturing complexity and processing time. To improve the use of adherent technologies for commercial manufacture, automated systems which use stacking T-flasks or roller bottles have been used, as well as fixed bed bioreactors [7]. However, since adherent cell culture has several disadvantages, including having an additional processing step to detach cells from the surface they are being cultured on and CoGs of LVV production being around 90% more expensive than single-use stirred tank bioreactors (STRs) [5], this has led to the use of suspension cell lines in STRs becoming more common for commercial manufacturing of LVVs.

All steps from a LVV production process in adherent culture systems can be substituted/replaced by corresponding steps in a suspension process. Processes in STRs can be scaled-up from the ~0.25 L lab-scale to a ~200-2000 L commercial-scale through process optimization and development, offering the most flexible and cost-effective platform [5] to produce hundreds of liters of cell culture. Additionally, STRs also provide greater control of the culture environment than flask-based culture. Thus, these types of volumetrically-scalable suspension-based cell culture processes have the potential to meet future demands for LVV based therapies in indications with large patient populations [6].


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## PROCESS DEVELOPMENT OF LVV PRODUCTION

Process optimization for LVV production is performed in bench-scale (2–5 L) bioreactors for eventual scale-up to pilot and manufacturing scale STRs (50–1000 L). This rapidly becomes cost-prohibitive when executing design of experiments or screening studies with numerous replicates to find the optimum process conditions due to the high cost of reagents and consumables in transient transfection-based processes. The work is also operationally intensive and requires substantial lab infrastructure to run multiple systems. With the goal of lowering cost while increasing throughput during development, a microscale bioreactor system was evaluated which could reproducibly perform the LVV production process to screen for changes that significantly affect yield and consistency.

## MICROBIOREACTORS FOR PROCESS DEVELOPMENT

Single-use (SU) microbioreactors for screening mammalian cell culture conditions have been widely adopted in major biopharma companies including AstraZeneca and Merck for process optimization and development since 2010 [8,9]. The drawback with many micro scale bioreactors is that they do not mimic the sparged, stirring action of a STR, and have no control over DO (dissolved oxygen) and pH inside the vessel. Additionally, not all micro scale bioreactors have the capacity for perfusion culture. With these, pH, DO and perfusion capabilities in mind, the Ambr® 15 cell culture high throughput automated microbioreactor system (Sartorius), an established technology for mimicking benchtop STRs [10], was selected as the LVV screening platform. The microbioreactor system mimics the characteristics of classical STRs at the miniature scale (10–15 mL) with each microbioreactor having its own agitation impeller and gases supplied by sparging or overlay. The system uses cost-effective, SU microbioreactors that are controlled by an automated cell culture workstation. Twenty-four vessels (12 vessels across two cell culture stations) can be operated simultaneously with the benefit of independent gassing for DO/pH control and built-in liquid handling.

High throughput tools with parallel processing capability, such as the Ambr® 15 cell culture system, help to address a major manufacturing bottleneck. The system can be used as a screening tool for process development, clone selection and effective media optimization in less time with reduced reagent use and labor saving [11]. Furthermore, the system has been shown to be an excellent tool for mimicking perfusion processes in small scale to increase the viable cell density of Chinese Hamster Ovary cells used for monoclonal antibody production. The studies [12,13] used two approaches, a centrifugation method and a cell settling method for medium exchange, and these were used as the basis for methodology in this LVV study.

## PROOF-OF-CONCEPT STUDY: ADAPTING A MICROBIOREACTOR FOR LVV PRODUCTION

To determine if the micro bioreactor system could be used as a predictive screening tool for LVV production with a suspension cell line, a series of medium exchange protocols were assessed. Exchanging the culture medium post-transfection is essential due to cytotoxicity issues caused by transfection reagents, which can adversely affect titer [14]. Several factors are easily scalable with the Ambr® 15 cell culture system using existing protocols including medium loading and conditioning, inoculation, gassing control strategy, agitation, and sampling. However, two operations: medium exchange and transfection liquid handling are less well defined for suspension cells and must be further developed for a robust, reproducible LVV production process.

The aim of this study was to implement and optimize an automated microbioreactor LVV production process for screening.

The study focused on optimizing the medium exchange process step, a unit operation which can have a substantial impact on process performance and LVV titer. Additionally, the medium exchange process step cannot be linearly or methodically scaled-down from a bench-scale STR due to system differences, mainly the lack of analogous mini/micro scale medium exchange technologies, liquid handling mechanisms, and ability to consistently perform across 24 vessels in one process run.

Therefore, to adjust to system differences, an established proprietary LVV production process developed in a benchtop bioreactor was assessed in the SU microbioreactors and was redesigned through three iterations until it achieved culture characteristics and productivities comparable to bench-scale. These included achieving similar culture growth rates and viability, transfection efficiencies and infectious titer.

## MEDIUM EXCHANGE PROTOCOLS

A proprietary HEK293T cell line was inoculated at a proprietary low cell density and cultured for a set duration until the proprietary target cell density for transfection was reached. The vessels were controlled at the targets of 37˚C, pH 7.0 ±0.2, and 50% DO by the Ambr® 15 system and with an impeller tip speed of 0.4 m/s. The viable cell density (VCD) and percentage cell viability were measured using an automated cell counter-(Vi-CELL™ XR, Beckman Coulter) throughout the culture duration. When cells achieved a target VCD, they were transfected using a proprietary transfection reagent with a proprietary four-plasmid system encoding the core proteins and transgene for LVV assembly. A 5th reporter plasmid was included encoding the Green Fluorescent Protein (GFP) gene. After a set duration post transfection (proprietary), the medium was exchanged to ensure robust transfection and production of functional LVV. Vector supernatants were harvested 48 hours post transfection and clarified by centrifugation (500 RPM for 5 minutes) to remove cells. LVV titer data for each production run was determined by qPCR of DNA extracted from a target cell line transduced with the LVV containing supernatants, reported as Transducing Units per milliliter (TU/mL). Productivity was also determined by quantitation of HIV-1 p24 antigen concentration (ng/mL) using a proprietary ELISA assay [15]. Transfection efficiency (percentage GFP expression) was also measured by flow cytometry analysis on the production culture for each run at defined timepoints. These data were compared to the mean historic LVV titer data from 22 process runs in a benchtop bioreactor (2 L single-use bioreactors) generated using the same proprietary cell culture parameters.

Three medium exchange methods were evaluated in this study (See Figure 1 for an overview of the processes evaluated.) Each of the processes for medium exchange varied in complexity and number of manual handling steps.

- **Process 1.0**: Microbioreactor vessels were manually removed from the cell culture station, the contents were transferred to sterile centrifuge tubes, these were centrifuged at 500 RPM for 5 minutes to spin down the cells into a pellet. The supernatant was then removed, fresh media added at an equivalent volume and cells were resuspended manually using a serological pipette. The culture volume was then transferred back to the microbioreactors by pipette, to be loaded back onto the automated bioreactor system.

- **Process 1.5:** The manual handling step of transferring the vessel contents into a centrifuge tube was omitted. Here, the microbioreactor vessels were centrifuged directly in specifically designed Ambr® 15 centrifuge adapters and were centrifuged at 500 RPM for 5 minutes to pellet the cells in one corner of the vessel. Then


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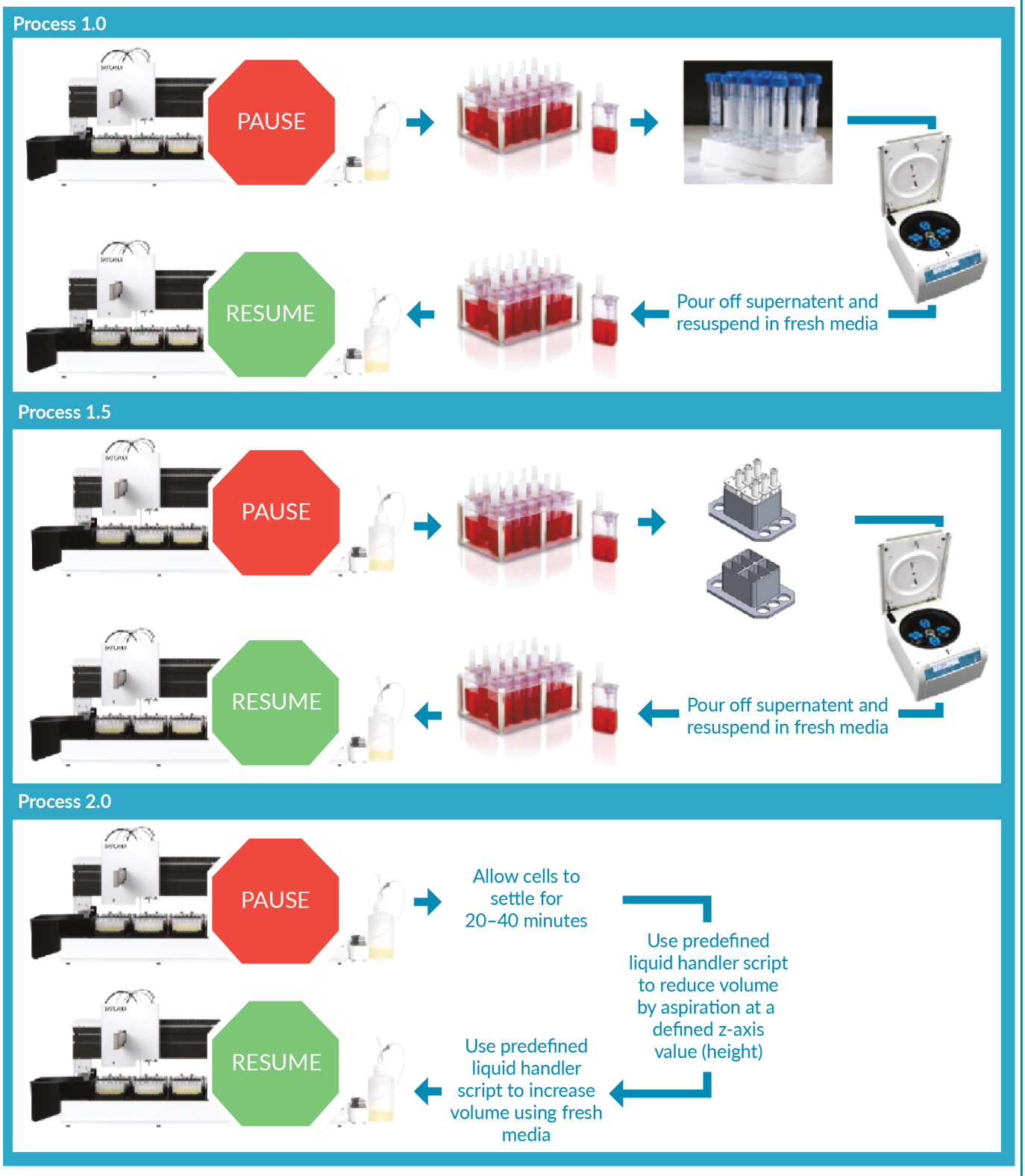
## FIGURE 1

**Different workflows for medium exchange with ambr® 15 cell culture system**




the supernatant was removed by pouring or aspiration, fresh media added, and cells were resuspended manually using a serological pipette. The microbioreactors were then loaded back onto the automated bioreactor system.

- **Process 2.0:** Since both Process 1.0 and Process 1.5 required manual handling which was time-consuming and posed a contamination risk, an automated cell settling process (Process 2.0) was developed. This involves pausing the agitation of the microbioreactors and allowing the cells to settle before exchanging a portion of the supernatant with fresh media. This is achieved by first drawing down the volume of each vessel in sequence using a 1-mL tip and then adding back an equivalent volume of fresh media once all the vessels of a cell culture station have been reduced in volume. A settling time of 20 minutes prior to spent medium removal of the first vessel was sufficient for the majority of the cells to settle to the bottom, limiting a decrease in cell density due to the removed volume. The Ambr® 15 system can only perform one drawn down at a time, which lead to the final vessel of a cell culture station being accessed after ~40 minutes of settling.

This process eliminated manual handling steps by using predefined liquid handling scripts in the Ambr® 15 software to facilitate the medium exchange by the liquid handler.

To determine if VCD or viability were compromised by the cell settling method for the medium exchange used in Process 2.0, VCD and viability data were measured before and directly after cell settling in three different experimental runs (designated experiment 1, experiment 2 and experiment 3) using the ViCELL XR automated cell counter (Figure 2).

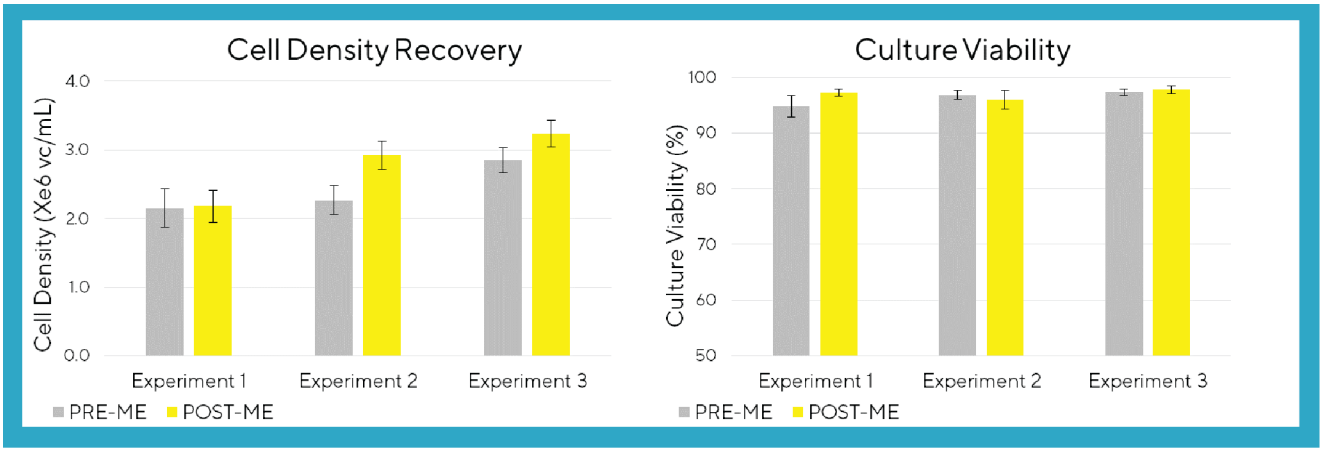
## EFFICIENCY OF LVV PRODUCTION WITH MEDIUM EXCHANGE PROCESS 1.0 AND PROCESS 1.5

Titer and transfection efficiency data from Process 1.0 and Process 1.5 (Figure 3) showed that eliminating one pipetting step and using specifically designed centrifuge inserts enables more consistent results between vessels with the Process 1.5 medium exchange protocol than using the Process 1.0 protocol.

When titer data from replicates are averaged within a study where titers vary across conditions, Process 1.5 demonstrates well defined and reproducible trends for this output (data not shown). However, there were still instances of variability between replicate vessels observed, albeit at low frequency. This allowed for the identification of trends in productivity during development exercises but required the use of replicates or triplicates for each condition during the runs.

## FIGURE 2

**VCD and culture viability produced by Process 2.0 medium exchange protocol over 3 experimental runs.**




Each bar represents the mean of 24 vessels with error bars of standard deviation (SD) showing data reproducibility. Grey bars = pre-medium exchange and yellow = post-medium exchange.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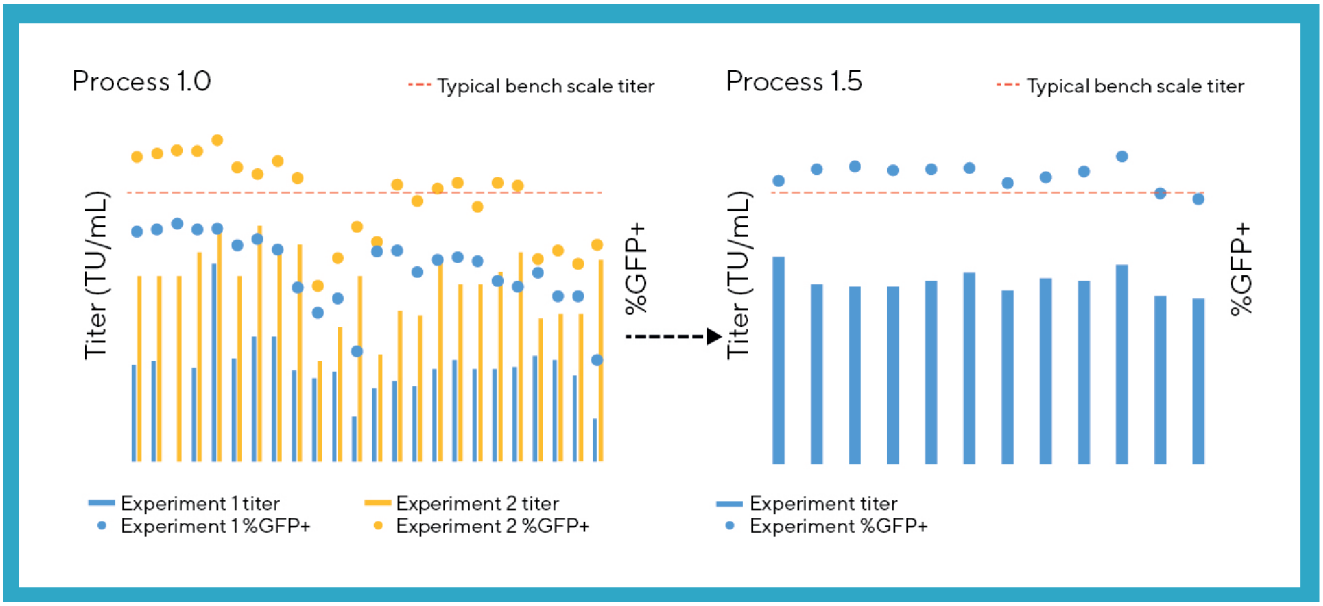
## FIGURE 3

**Comparison of LVV infectious titer (TU/mL) and transfection efficiency (%GFP+) from Process 1.0 and Process 1.5 medium exchange protocols (each data point represents data from 1 microbioreactor).**




Note initial titer and %GFP+ values are proprietary so are not stated on the graph's axes, but the graph's axes are the same for direct visual comparison of the two plots.

## EFFICIENCY OF LVV PRODUCTION WITH MEDIUM EXCHANGE PROCESS 2.0

A VCD of 2-3 x$10^6$cells/mL and cell viability of 95-98 % is consistently achieved across all microbioreactor replicates, as shown by Figure 3. These results indicate that VCD and cell viability are not compromised by the automated cell settling method used in Process 2.0 for medium exchange.

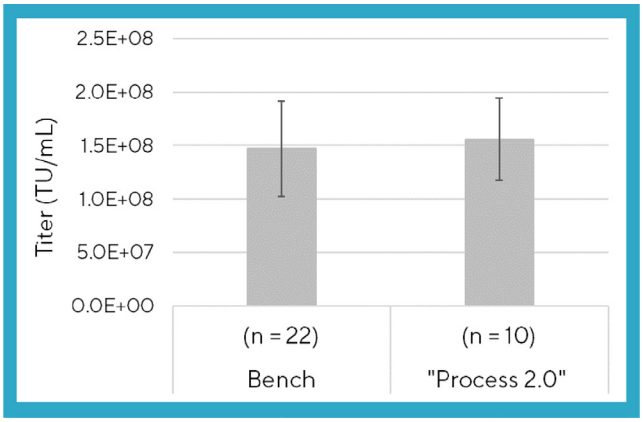
Furthermore, the average LVV titer achieved when using the Process 2.0 cell settling method for medium exchange in 10 Ambr® 15 microbioreactors was comparable to the average titer achieved using an established proprietary cell culture and medium exchange process run at bluebird bio in 22 bench scale bioreactors (Figure 4).

## COMPARATIVE ANALYSIS OF LVV PRODUCTION USING DIFFERENT MEDIUM EXCHANGE PROTOCOLS

To determine whether the Process 2.0 medium exchange protocol is more robust than Processes 1.0/1.5 in terms of LVV yield predictability, the viral particle titer (p24 amount in ng/mL) and the transfection efficiency (%GFP+) from all three processes were plotted and analyzed statistically using an ordinary least squares (OLS) regression analysis. The results from the two methods of measuring process performance, which both correlate to infectious titer, have a positive correlation with Process 2.0 with a $R^2$ value of 0.64 versus little

## FIGURE 4

**Averaged titer results (TU/ml) from bench-scale runs and microbioreactor vessels using the established processes with SD error bars.**



correlation ($R^2$ value of 0.0021) with Processes 1.0/1.5 (Figure 5). Therefore, this indicates that using the Process 2.0 cell settling method for medium exchange using the Ambr® 15 cell culture system offers an increase in process robustness. Process 2.0 also has a more consistent yield of LVV produced compared to the more manual Processes 1.0 and 1.5. This indicates that using an automated microbioreactor system offers the benefits of less variable results which are often caused by different operators manually performing the same protocol in a different way. It also demonstrates that reduced operator input could lead to more objective and reliable process development data.

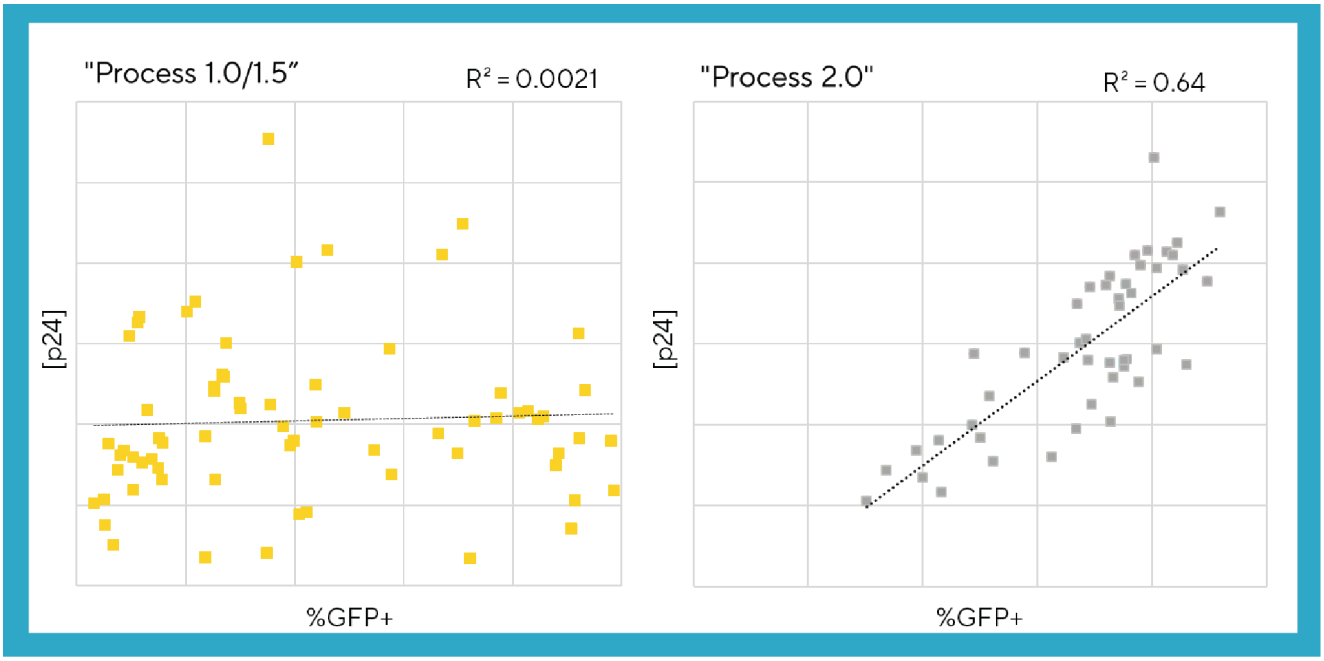
## CONCLUSION

In this article, the development of a medium exchange protocol for a LVV production process using an Ambr® 15 cell culture system has been described. The process developed focused on the medium exchange step, as this process step cannot be scaled-down from a bench-scale STR due to system differences. However, by adjusting to system differences and developing novel process steps and techniques through trialing three different medium exchange protocols, LVV titer and transfection data showed that the Ambr® 15 system can meet the productivity of bench top bioreactors in terms of infectious titer. As the Ambr® 15 bioreactors utilize smaller volumes of media (10–15 mL) than benchtop bioreactors they also have the potential to increase screening capacity, while making costs savings when performing this type of screening.

From the three medium exchange processes assessed, the automated cell settling step, followed by spent media removal and replenishment used in Process 2.0, produced the lowest variability between microbioreactor vessels and improved overall LVV production. The implementation of Process 2.0 also produced results which closely aligned with LVV yield from an established bench-scale process. In summary this study, demonstrates the automation power of the Ambr® 15 cell culture system and its suitability as a process screening tool which can significantly reduce operator variability

**FIGURE 5**

**Plotted results of p24 production (ng/mL) versus transfection efficiency (%GFP+) under "Process 1.0/1.5" or "Process 2.0".**




Linear regression was performed on all the data points, with each plot consisting of 3 separate runs..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and handling time with process development of LVV production in suspension culture.

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FASTFACTS

# Scalable cell culture and transient transfection for viral vector manufacturing

**Ann Rossi Bilodeau, PhD**

**The acceleration of clinical cell and gene therapy programs is creating a growing worldwide demand for lower-cost and higher-yield viral vector manufacturing, especially for adeno-associated virus (AAV) and lentivirus. Increasing capacity by improving cell densities and cell numbers per batch could address this need, whether by a scaled up suspension process, or a scaled out adherent process. However, it's not enough to simply grow more cells; the production process, including transfection methods, must also be optimized to maintain product quality while minimizing operating costs. This poster will explore two key factors affecting scalability and yield: choice of cell culture platform, and optimizing transient transfection.**

## CHOICE OF CELL CULTURE PLATFORM

The cell culture platform used is a key determining factor in the capacity of viral vector manufacturing. Suspension culture is more easily scaled; however, transfection of adherent cultures remains a tried-and-true method with proven protocols and an extensive knowledge base.

**Table 1** compares the number of vessels needed and the total surface area achieved for the major Corning bioprocess vessels. The best system for a given process depends on many factors: there is no one-size-fits-all solution. For example, if footprint is a key consideration, the Corning® CellCube® system is an obvious choice. However, the CellCube requires a bioreactor, whereas vessels such as the Corning CellSTACK® culture chambers, require only incubators.

**Table 1. Adherent culture platform comparison.**

| Platform | No. of Vessels | Total Surface Area ($cm^2$) | Media Volume (L) | Required Equipment |
|---|---|---|---|---|
| Polystyrene Microcarrier 40L Bioreactor[1] | 1 | 403,000 | 40 | Bioreactor, controlle |
| Dissolvable Microcarrier 40L Bioreactor[2] | 2 | 400,000 | 40 | Bioreactor, controller |
| Corning® CellCube® 100-layer module warm room | 4 | 340,000 | 32 | Controller, oxygenator, |
| Corning HYPERStack® 36-layer vessel | 20 | 360,000 | 78 | Incubators |
| Corning HYPERFlask® vessel | 209 | 359,480 | 117 | Incubators |
| Corning CellSTACK® 40-layer vessel manipulator | 14 | 356,160 | 38 to 45 | Incubators/ warm room |
| Corning CellSTACK 10-layer vessel | 56 | 356,160 | 38 to 45 | Incubators/ warm room |
| Roller bottle | 400 | 340,000 | 51 | Racks, warm room |

[1]Assumes 28 g/L of Polystyrene microcarrier, 1400 g total Polystyrene microcarriers used
[2]Assumes 2 g/L of Dissolvable microcarrier, 100 g total Dissolvable microcarriers used

## OPTIMIZING TRANSFECTION

Another critical aspect for successful viral vector production is transfection, with transient transfection the most efficient means of producing viral vectors. Best practices for transient transfection include:

- Spend sufficient time optimizing your culture, ensuring you have healthy, actively dividing cultures.
- Choose transfection reagents that have been optimized for your processes and are scalable, such as PEIpro® from Polyplus.
- Develop a robust protocol with the transition from pilot-scale to production-scale in mind, whether the final production scale is intermediate or large, adherent or suspension.
- Don't assume viral titer will correlate with transfection efficiency.

## PROOF-OF-CONCEPT: SCALABLE TRANSFECTIONS IN AN ADHERENT PLATFORM

We carried out two proof-of-concept studies of green fluorescent protein (GFP) transfections, to act as a starting point for customer optimization. **Figure 1** shows transfection in a Corning CellSTACK 10- chamber vessel. HEK293 cells were seeded at 5,000 cells per $cm^2$ with PEIpro and allowed to grow to roughly 50% confluence or transfection. The transfection mix was added directly to the CellSTACK 10-chamber vessel, mixed, and incubated until harvest, with no medium exchange before or after transfection. Two days after transfection, the cells were harvested and a GFP transfection efficiency assay was performed. There were 83% GFP-positive viable cells, typical for this protocol.

We achieved very similar results with the CellCube 25-chamber system **(Figure 2)**, with 70% to 80% GFP-positive viable cells. The only difference in protocol is that the CellCube must be rotated to ensure transfection of both sides.

**Figure 1. Corning CellSTACK culture chamber PEIpro transfection.** Left: Top chamber of CellSTACK vessel, showing cell confluence on day of transfection. Middle: CellSTACK 10-chamber vessel transfected with GFP (top) and negative control (bottom). Right: GFP-positive cells in the CellSTACK vessel.

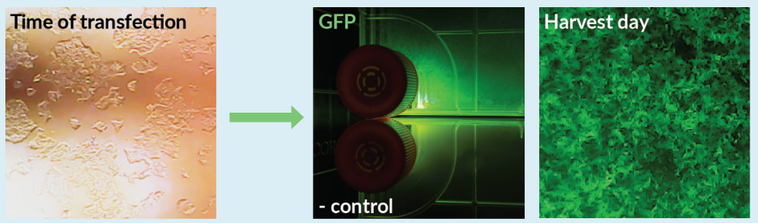
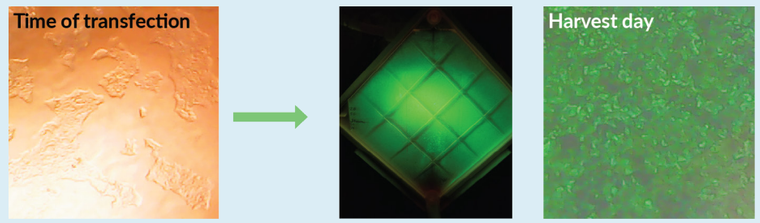



**Figure 2. Corning CellCube system PEIpro transfection.** Left: Cell confluence on day of transfection. Middle: CellCube 25-chamber vessel transfected with GFP. Right: GFP-positive cells in the CellCube vessel.




## CONCLUSIONS

Many cell and gene therapy applications are dependent upon viral vectors for recombinant gene delivery. Efficient transfection of a large biomass of cells represents a significant challenge for transient viral vector production systems. However, identifying a scalable adherent cell culture platform for high-density cell culture helps to streamline the transfection process. Further, utilizing transfection reagents that are optimized for transfection at scale ensures robust and reproducible transactions from process development through to large-scale manufacturing.

CELL & GENE THERAPY INSIGHTS

*Cell & Gene Therapy Insights* 2021; 7(9), 1035 • DOI: 10.18609/cgti.2021.135



In partnership with: CORNING

# FASTFACTS

# Addressing HEK293 cell lineage and AAV diversity with media panel

**Jennifer Schieber,** Senior R&D Scientist, Thermo Fisher Scientific

**Adeno-associated virus (AAV) has become an attractive vector for gene therapy; however, low titer yield often limits its viability as a therapeutic. Here, we highlight how a media panel supports AAV production by helper-free triple transfection using HEK293 cells with increased viral titers agnostic of manufacturing processes or cell lineage.**

*Cell & Gene Therapy Insights* 2021; 7(9), 933 • DOI: 10.18609/cgti.2021.124

When it comes to HEK293 cells, each cell line is unique. Since the original HEK293 cells were derived in 1973, the cell line has been repeatedly modified by transfection and adapted to grow in a variety of conditions. Therefore, it's not surprising that HEK293 cell lines demonstrate a significant diversity in genomic and proteomic profiles and nutritional needs. To address this, Thermo Fisher Scientific has developed an approach for basal media screening that increases the chance of finding a great match for a specific HEK293 line and improving performance.

Using principal component analysis, over 60 potential candidates were narrowed down to five formulations, which provide a broad range of different nutrient concentrations and have very specific raw materials **(Figure 1)**.

We tested the compatibility of the media panel with different transfection methods and found that the five panel formulations perform very differently depending on the HEK293 cell line **(Figure 2)**. Response to base media and titers are highly dependent on the HEK293 cell line/lineage/population used. Depending on your cell line, a screen may yield higher results or be a starting point for optimization.

**Figure 1. Media panel heat map.**

| Components | HEK panel medium 1 | HEK panel medium 2 | HEK panel medium 3 | HEK panel medium 4 | HEK panel medium 5 |
|---|---|---|---|---|---|
| Amino acids | | | | | |
| Vitamins | | | | | |
| Lipids | | | | | |
| Trace metals | | | | | |
| Polyamines | | | | | |

High level — Low level

## CASE STUDY 1: EVALUATION OF DIFFERENT SEROTYPES

The viral vector HEK media panel was evaluated in a single cell line and multiple AAV serotypes. For this experiment, only the top three formulations were considered, and f17 was used as the control media.

The panel formulations yielded similar genomic titer, cell growth, and transfection efficiency compared to F17. However, when harvested fractions were analyzed separately for intracellular and secreted AAVs, some differences did emerge. Looking at serotypes in which AAV was mostly secreted (AAV8 and AAV9), there was an apparent correlation between using media panel 4 and an increased amount of AAV in the extracellular fraction **(Figure 3)** – a finding that warrants further investigation.

**Figure 2. Potential to increase titer with Viral Vector HEK Media Panel.** All titers normalized to FreeStyle F17 Expression Medium. Titers range from 109 to 1011 vg/mL depending on the cell line and process.

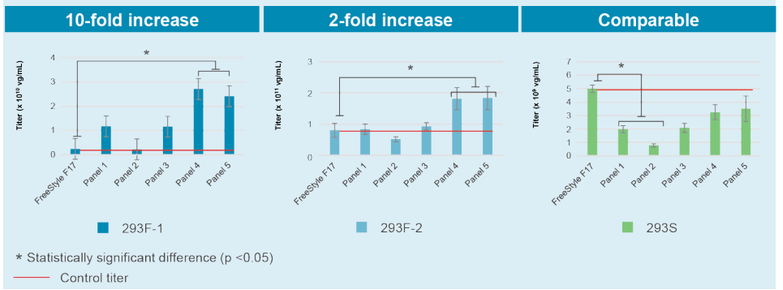



**Figure 3. Increased yield for secreted serotypes.** Apparent increased extracellular vg/mL in Panel 4 on AAV8 and AAV9. At scale, the AAV8 process has been qualified without a lysis step at harvest to limit contaminants. Panel 4 was selected to be validated in future bioreactor runs for AAV8 production.

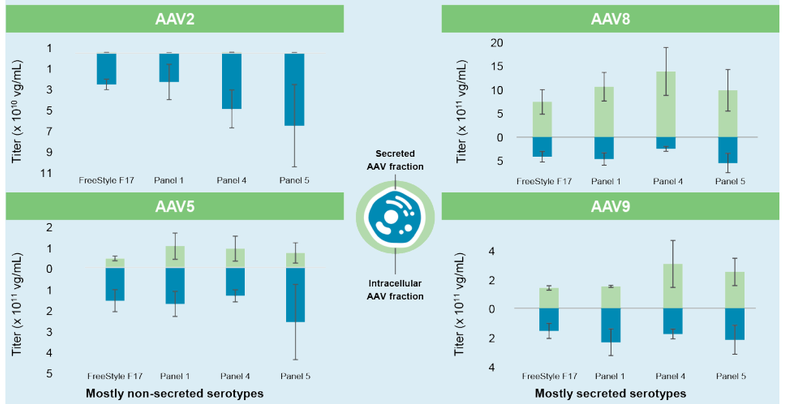
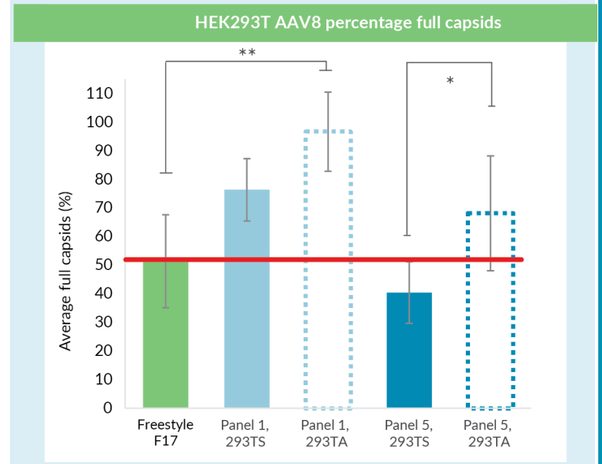



**Figure 4. HEK293T AAV8 percentage full capsids.** Percentage of full capsids determined by qPCR and ELISA analysis. All experiments were performed on AAV8 with comparable total yield (vg/mL).




## CASE STUDY 2: IMPACT OF ADAPTATION STRATEGY

The Viral vector HEK media panel was also evaluated for use in adapting cells from adherence to suspension. Only panels 1 and 5 were tested as they have the most differentiated level of nutrients.

Once cells were adapted, triplicate biological replicates were run to assess if the adaptation strategy had an impact on titers or particle quality after performing AAV8 triple transfections. No significant difference was seen in average genomic titer between F17, panel media 1, or panel media 5.

However, looking at the empty:full ratio, we saw an apparent increase in % full capsids when cells were adapted directly into the panel media (293TA) compared to having been first adapted to the control medium (293TS) **(Figure 4)**.

CELL & GENE THERAPY INSIGHTS

UPSTREAM BIOPROCESSING


INNOVATOR INSIGHT

# Key considerations for maximizing LV and AAV production in transient transfection workflows

Leisha Kopp, Beth Larimore, Nolan Sutherland & Anindya Dasgupta

Recombinant adeno-associated virus (AAV) and lentivirus (LV) are essential components of gene and cell therapies, which show incredible promise for the treatment of genetic and acquired diseases. Accordingly, the need for large-scale manufacture of safe and effective viral vectors has never been greater. In this article, critical parameters for optimizing viral vector production are discussed, along with how *Trans*IT-VirusGEN® Transfection Reagent and accompanying enhancer components can support manufacturers from research and development through commercial manufacturing.

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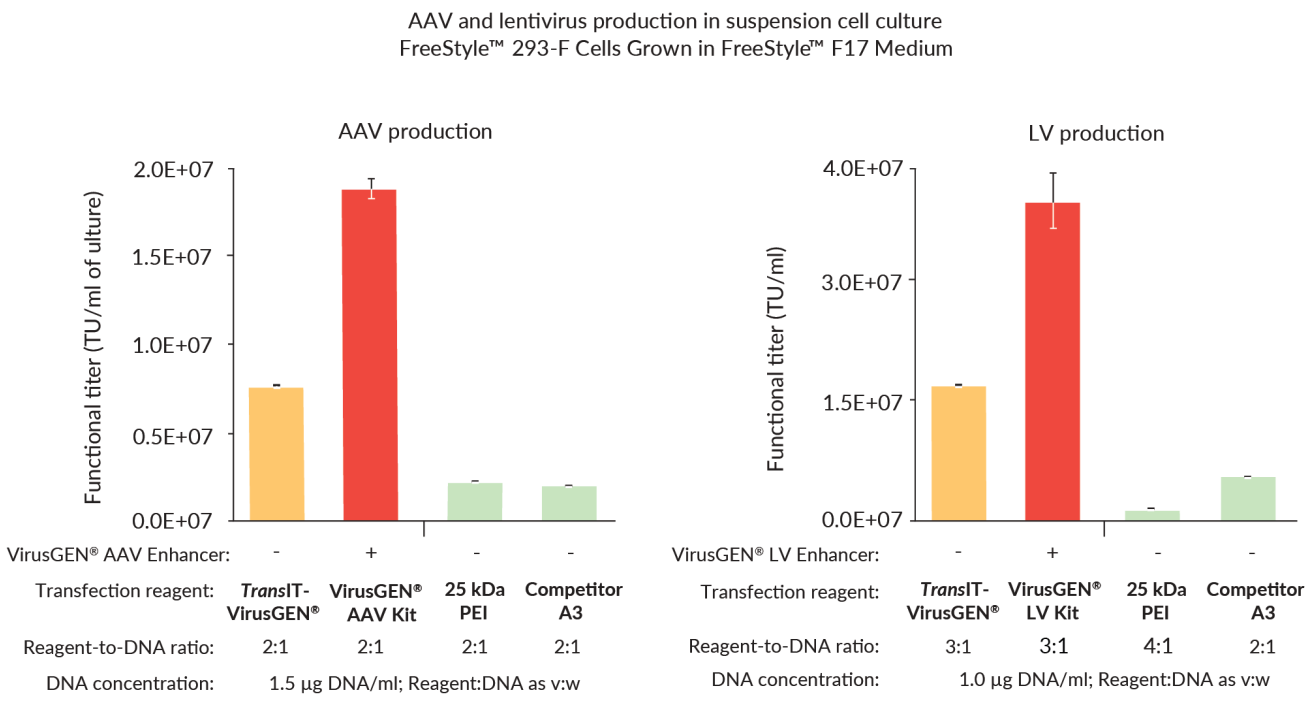
## THE POTENTIAL OF CELL & GENE THERAPIES REQUIRES IMPROVED VECTOR PRODUCTION

Cell and gene therapies hold the potential to address diseases and indications that were previously considered challenging or even hopeless. These therapies commonly utilize AAV and LV vectors, and a critical consideration is the virus quantity required for creating effective therapeutics.

Gene therapies typically utilize AAV to deliver a corrected genetic sequence, and the average delivered dose is between 1.0E+11 to 1.0E+16 viral particles per patient. The treatment is administered *in vivo* and must impact the entire organ or system.

## FIGURE 1

***Trans*IT-VirusGEN Reagent (yellow bars) and the VirusGEN AAV and LV Kits (red bars) generate higher functional AAV and LV titers than PEI-based reagents (green bars).**




In addition, the treatment has to contend with the patient's immune response. For cell therapies, where cells are modified outside of the body before re-administration to the patient, the required dosage is lower, i.e., between 1.0E+8 to 1.0E+10 viral particles per patient. Lentivirus is often used in cell therapies to transduce cells *ex vivo* which means that the virus does not have to contend with the patient's immune response. However, a large amount of virus is still needed for a single patient dose.

Transient transfection is the critical first step in AAV and LV production and involves delivering a series of plasmid DNAs to HEK293 cells in culture. Transfection must be extremely efficient to enable cells to produce virus at the highest possible levels.

Within life science research, transfection is often considered a fundamental scientific tool. However, maximizing transfection efficiency for therapeutic viral vector production requires a deep understanding of all critical parameters – including cell density at the time of transfection, reagent-to-DNA ratio, plasmid ratios, total DNA amount, complexation dynamics and timing of transfection – as well as how these parameters interact in this intricate, complex process.

Many different components go into enabling successful transfection and subsequent gene expression. One of the most critical components is the transfection reagent used since transfection efficiency leads to higher virus titers and superior virus quality. However, with so many options available on the market, how can cell and gene therapy developers choose the best option for them?

## *Trans*IT-VirusGEN® TRANSFECTION REAGENT FOR HIGH TITER VIRUS PRODUCTION IN MULTIPLE FORMATS

The *Trans*IT-VirusGEN Transfection Reagent is specifically designed by scientists at Mirus Bio for improved LV and AAV production

over existing reagent formulations. During the development process, libraries of lipids and polymers were screened to find a formulation that effectively delivered multiple different plasmids to HEK 293 cells in a manner than maintained good cell health, so that the cells could produce high quantities of functional virus.

In addition to high transfection efficiency, VirusGEN also offers flexibility. Both LV and AAV can be produced using *Trans*IT-VirusGEN in a variety of different adherent and suspension HEK 293 cell lines and cell culture formats, including T-flasks, HYPERStack® vessels, shake flasks and stirred-tank bioreactors.

Batch-to-batch reliability is also required with any commercial transfection reagent, and Mirus has demonstrated that similar performance can be expected with every VirusGEN lot and across reagent grades – from research grade to SELECT to the newly-released *Trans*IT-VirusGEN GMP Transfection Reagent. No commercial license is required to use this product for research or further manufacturing, and it is chemically defined and animal-origin free.

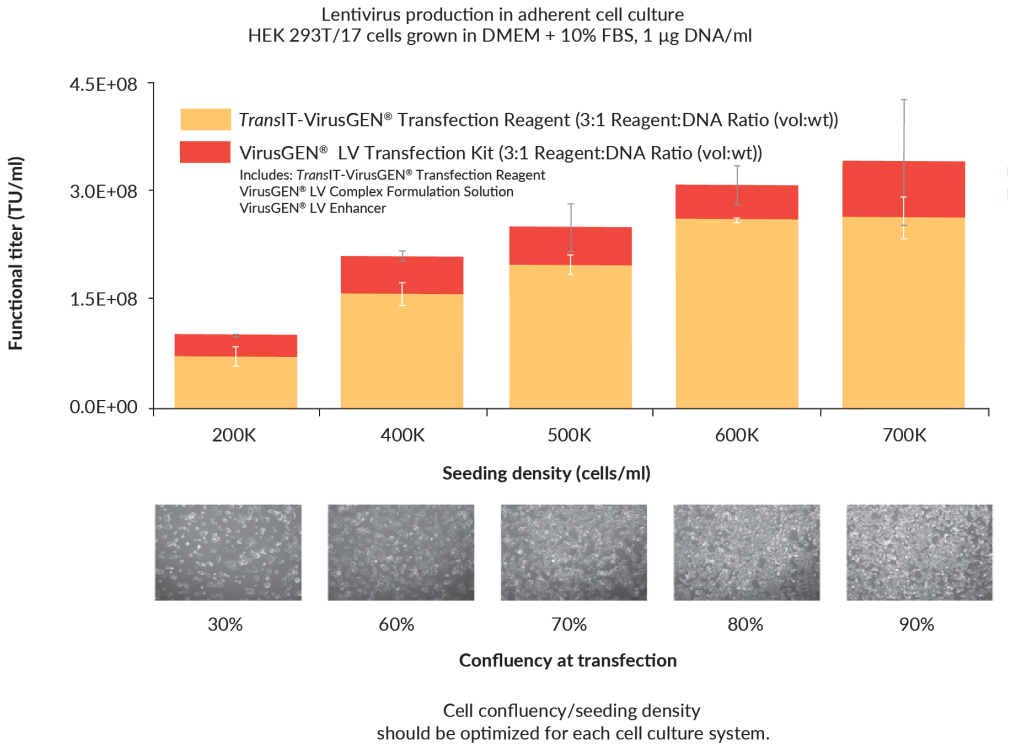
Virus specific enhancer and complex formation solution components have also been developed to pair with *Trans*IT-VirusGEN Transfection Reagent which further increase functional AAV and LV production over reagent-alone transfections.

## TRANSFECTION OPTIMIZATION WITH THE VIRUSGEN AAV TRANSFECTION KIT

The following studies demonstrate the significant benefits *Trans*IT-VirusGEN Transfection Reagent, with or without enhancer components, can bring to the transient transfection process.

**FIGURE 2**

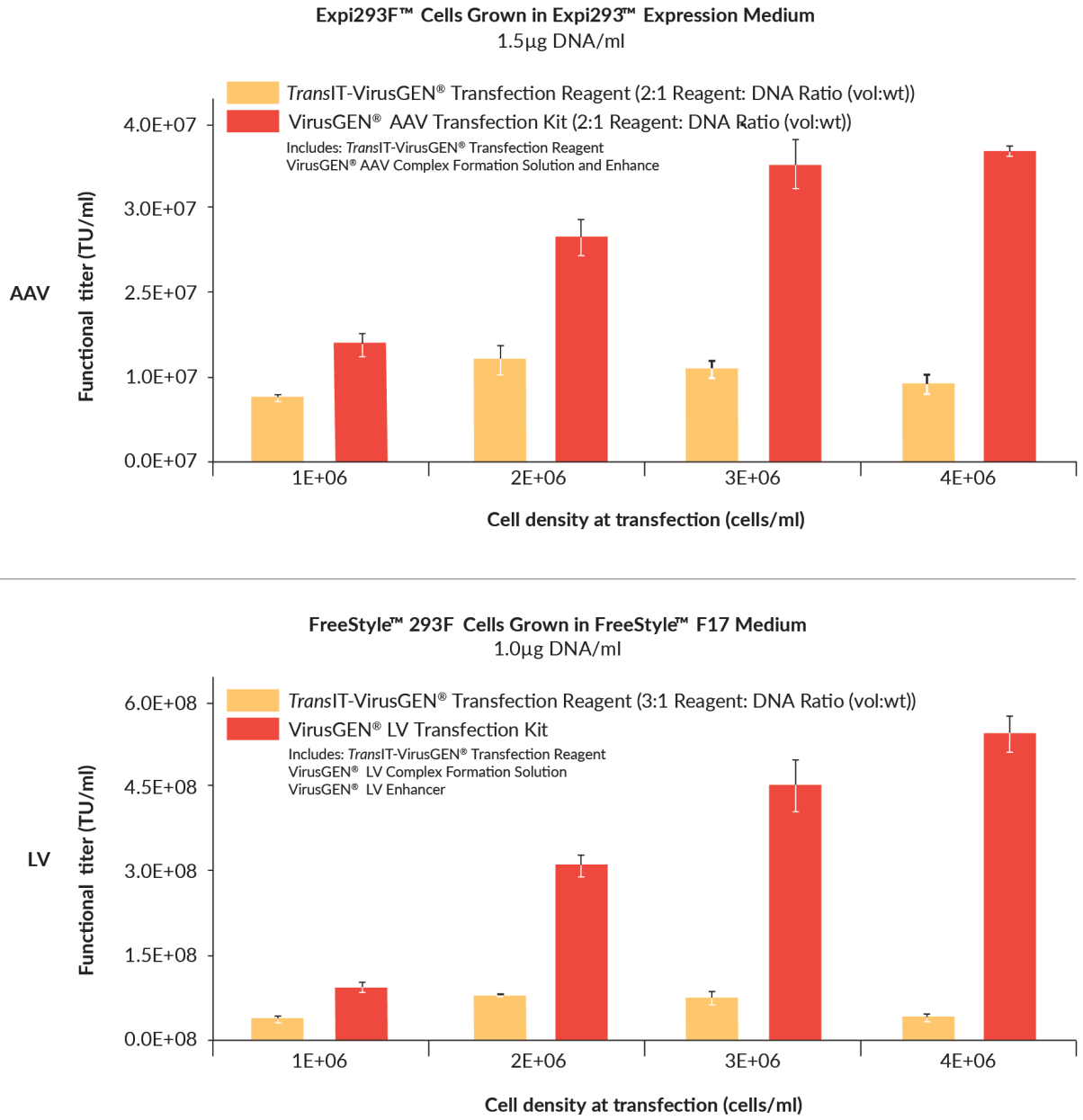
**Effect of cell density on transfection optimization.**




VirusGEN reagent (yellow), and VirusGen plus LV enhancer (yellow plus red) were compared. Cell density is also shown.

## FIGURE 3

**Effect of cell density on AAV and LV production, with 1, 2, 3, or 4 million cells per ml at the time of transfection, using VirusGEN alone (yellow), or VirusGEN plus enhancer (red).**




## Key consideration 1: choosing a reagent

As discussed above, one of the first variables to address when optimizing a transfection process is the reagent used. Here, AAV and LV was produced in suspension cell culture using *Trans*IT-VirusGEN Transfection Reagent, with and without AAV enhancer, compared against two commonly used PEI-based formulations (Figure 1). *Trans*IT-VirusGEN Transfection Reagent clearly outperforms PEI-based reagents in head-to-head comparisons for both recombinant AAV and LV manufacture.

## Key consideration 2: cell density

Cell maintenance schedules and cell density at the time of transfection are additional

critical parameters to consider for LV and AAV production, as robust cell health and division rate are paramount to a successful transient transfection.

This study involved lentivirus production in adherent HEK293T/17 cells. Cells were grown in DMEM + 10% FBS and transfected at densities ranging from 30–90% confluence at the time of transfection. Lentivirus production levels were measured 48 hours post-transfection for each condition. (Figure 2).

As the data indicates, there is a significant difference in virus production levels when transfecting cells that are too sparse, i.e., 30% confluent, versus cells that are 80–90% confluent at transfection. It is important to note that cells can be too confluent for transfection as well. If cells are 100% confluent, this may result in contact inhibition and cause cells to become senescent. Therefore, 80–90% confluency is a desirable target for transfection to maximize virus production levels.

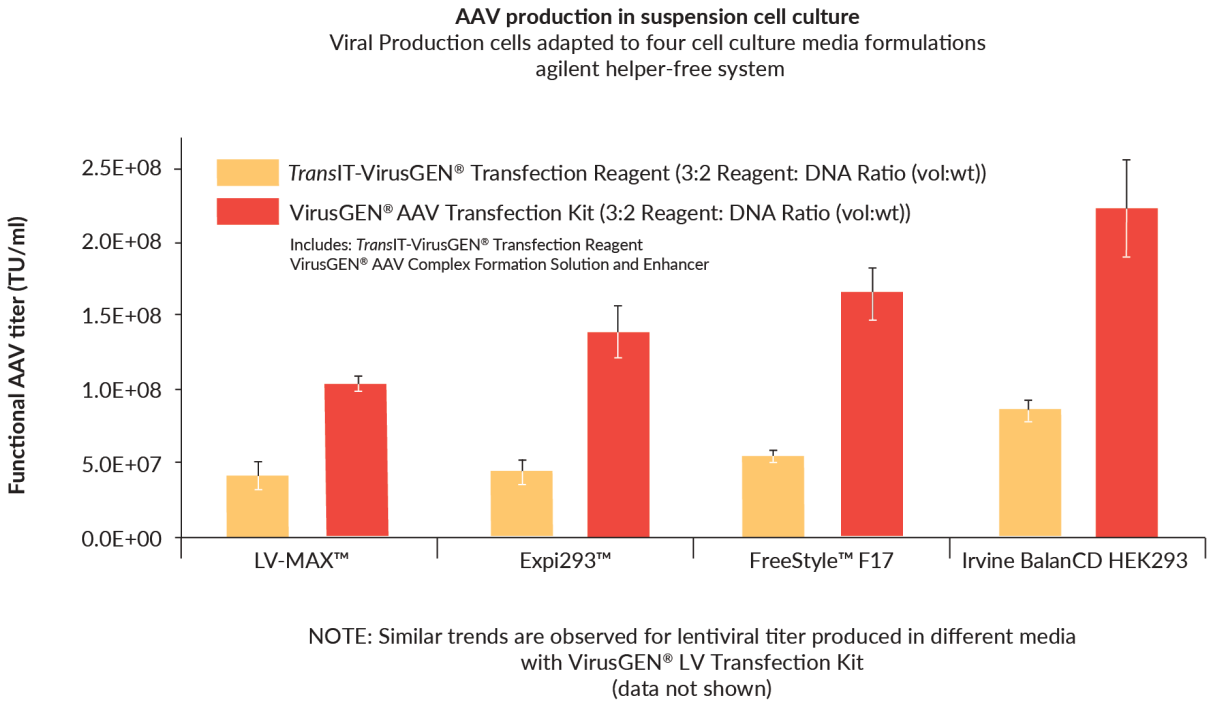
Cell density is also an important parameter for virus production in suspension cells. In Figure 3, either AAV (top) or LV (bottom) was produced by transfecting cells at densities ranging from 1E6–4E6 cells/ml at the time of transfection. For AAV and LV produced using *Trans*IT-VirusGEN® Reagent alone (i.e., no enhancers) highest titers are observed at 2 million cells per ml. However, when incorporating the enhancer components, maximum AAV and LV production levels were observed at 2 million cells per ml, and above.

It should be noted that the suspension cell type and the suspension culture media will also have an impact on growth rate and transfectability. Therefore it is important to evaluate for optimal density with each new cell type and media composition tested.

When working with suspension cells for AAV production, it is also important to passage the cells the day before transfection to ensure they are high viability and actively dividing at the time of transfection. Similar to adherent cells, suspension cells that have grown to a high density are less amenable to transfection and will underperform for virus

## FIGURE 4

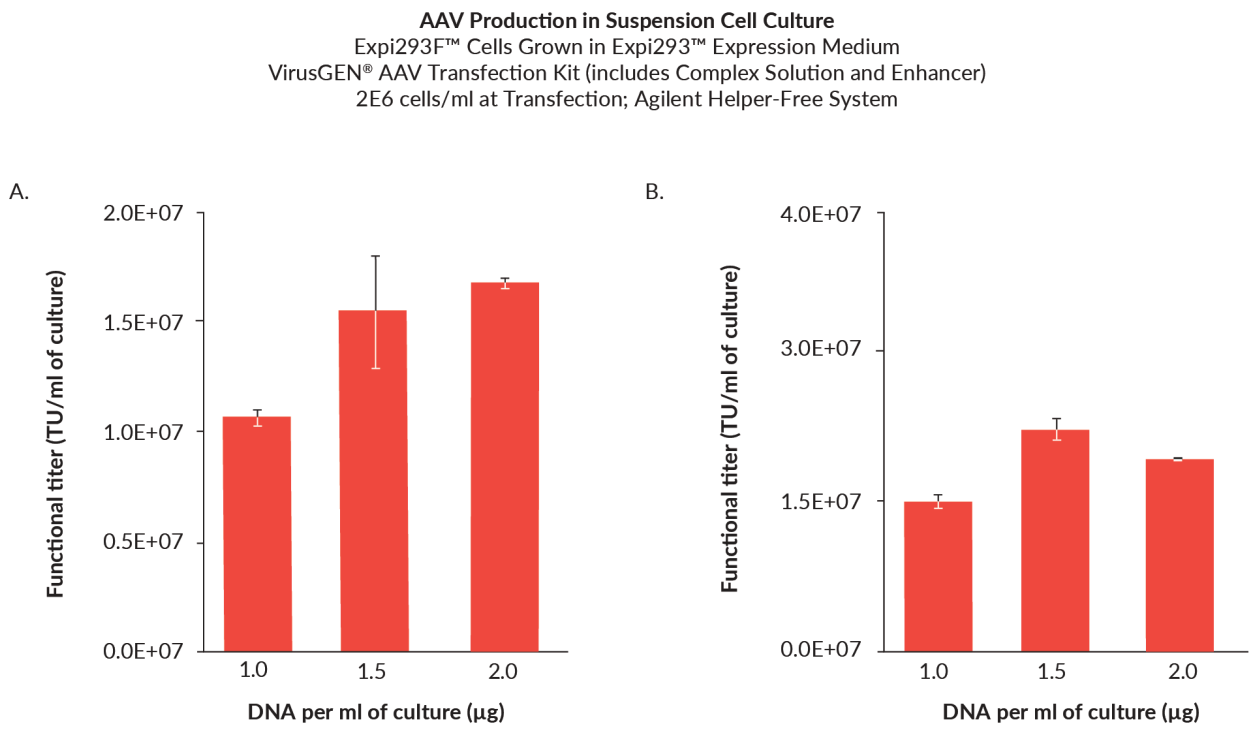
**Outcomes of different culture media on AAV production in suspension cell culture using Viral Production (VP) cells transfected with VirusGEN alone (yellow), or VirusGEN plus enhancer (red).**




NOTE: Similar trends are observed for lentiviral titer produced in different media with VirusGEN® LV Transfection Kit (data not shown)

## FIGURE 5

**Comparison of performance on AAV production when transfecting with VirusGEN plus enhancer using different reagent-to-DNA ratios.**




**Reagent:DNA ratio (ul:ug)** and **total DNA**
should be optimized for each recombinant vector and cell culture system.

production until they return to log-phase growth.

### Key consideration 3: cell culture media

Mirus tested a number of different serum-free suspension cell culture medias. Figure 4 compares Viral Production cells from Thermo Fisher Scientific adapted to four different media formulations: LV-MAX™ Production Medium, Expi293™ Expression Medium, and Freestyle™ F17 Expression Medium from ThermoFisher, and BalanCD HEK293 from Irvine Scientific. Higher overall functional titers were observed with the Irvine media, but it is important to note that the *Trans*IT-VirusGEN Transfection Reagent performed well in all the media used, and the VirusGEN Complex Formation Solution and Enhancer increased titers over reagent alone in all four media. For lentivirus, similar results were seen again with a higher expression level using BalanCD media from Irvine.

### Key consideration 4: reagent-to-DNA ratio & total DNA

Reagent-to-DNA ratio and total DNA per milliliter of culture are two additional parameters to consider, and both should be optimized for each unique recombinant virus and cell culture system. Figure 5 shows a comparison of AAV functional titers when virus was produced using 2:1 and 3:1 reagent-to-DNA ratios. Comparing both graphs, it is clear that the 3:1 ratio is inferior to the 2:1 ratio for AAV production.

For the 2:1 reagent-to-DNA ratio (Figure 5A), maximum performance is seen when delivering 2 micrograms of total DNA per ml of culture. For the 3:1 reagent-to-DNA ratio (Figure 5B), 1.5 micrograms total DNA appears to be the best choice.

Overall, it is key to find the conditions that provide a high virus titers. Fine tuning and testing different ratios with a specific vector set, cell type, and cell culture media is crucial in order to discover the truly optimal parameters for a specific viral vector expression process.

## Considerations for harvest & analysis

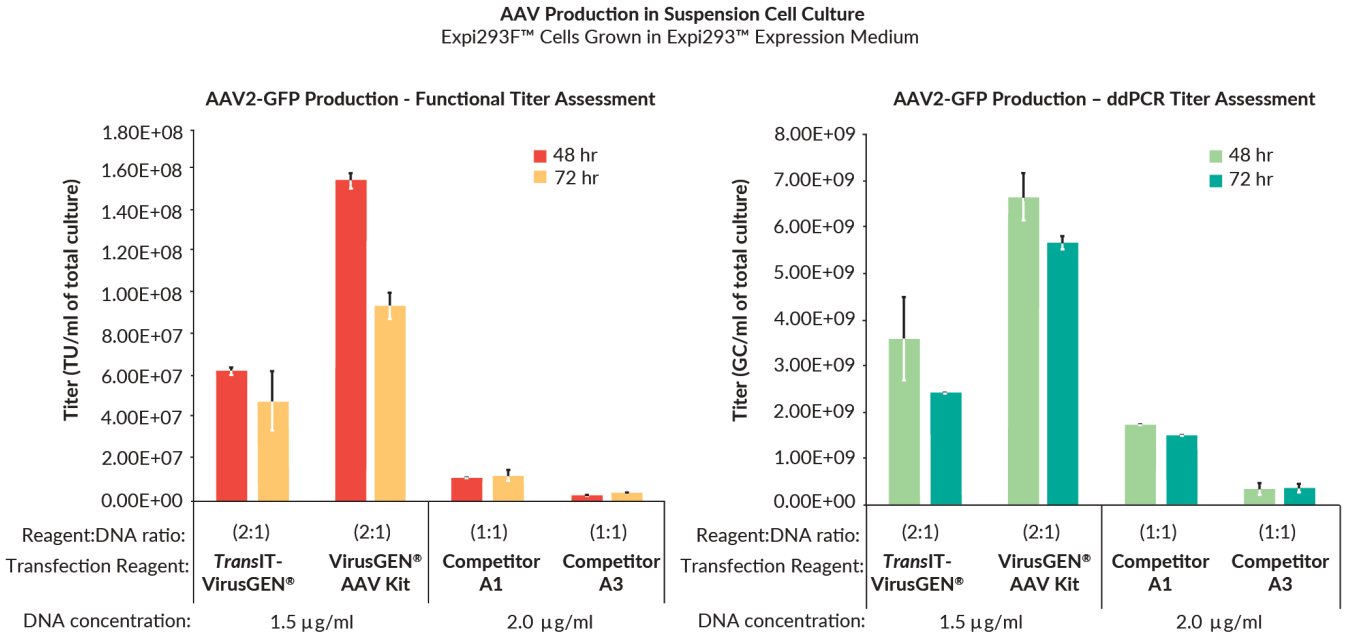
When assessing viral vector titers, and especially when working with AAV, researchers will utilize either qPCR or ddPCR to measure the genome copies per ml of total culture (GC per ml). This is commonly referred to as the physical titer. In addition to measuring GC per ml, Mirus, routinely performs infectivity assays to determine the functional titer (TU per ml) of a virus prep since this measures the viral particles capable of transducing gene expression in target cells.

In Figure 6, physical and functional titers are assessed for four different transfection conditions and two different harvest points. Overall, similar trends are observed between the two titering methods (i.e., VirusGEN Reagent plus AAV Enhancer produces highest titers whereas competitor products produce lower titers); however, physical titer differences are less dramatic, as the genome copies measured via ddPCR are not necessarily capable of transducing target cells. For this reason, functional titer could be considered a more reliable measurement for the transduction potential of a virus population.

It is also important to consider harvest time points. With Mirus Bio's lipid and polymer-based transfection reagent formulations, expression can occur sooner than what is typically observed with PEI-based reagents. Accordingly, we recommend testing several harvest timepoints (e.g., 48- and 72-hours post-transfection) to determine what is optimal for each culture situation.

## FIGURE 6

**Comparison of functional and genome AAV2 titers when transfecting with VirusGEN and VirusGEN plus enhancer.**




Expi293 cells were transfected with *Trans*IT-VirusGEN Reagent and *Trans*IT-VirusGEN AAV Transfection Kit yield higher functional and physical titers than competitor reagents A1 and A3 (reagent for AAV production and PEI reagent, respectively) at 48 (red and green bars) and 72 hours (yellow and turquoise bars).

# Q&A

**Leisha Kopp**
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**Beth Larimore**
Associate Director of Viral Vector Process Development, Bristol Myers Squibb

**Nolan Sutherland**
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## TAKING A HOLISTIC APPROACH TO THE VIRAL VECTOR WORKFLOW

Mirus Bio supports customers throughout their viral vector manufacturing process. An optimized transfection workflow is not solely reliant on high performing reagents – it is important to understand how all the different aspects of the workflow perform together. Process development for each platform and/or target will empower manufacturers to truly get the most from their recombinant virus production which will in turn, lead to achieving higher viral vector titers results in fewer runs, cost and time savings, and ultimately, better and faster therapeutic development. In the following panel discussion, a group of industry experts listed above delves deeper into the challenges facing viral vector manufacture today, along with the potential solutions.

**Q** Beth, throughout your career you have held viral vector process development roles of increasing responsibility at several prominent companies. What are some of the biggest challenges you faced early in your career, in terms of viral vector engineering and expression?

**BL:** I started my career in downstream purification and monoclonal antibodies. That experience gave me a good foundation on the principles of process development, and allowed me to see a very mature industry and platform.

When I transitioned into working on lentiviral process development, what stood out to me was how the industry was still developing. The clues to that were the viral process vendors, who didn't have much, if any, experience to share with me. All of the tools were really designed for

protein purification applications. There were no GMP transfection reagents available at the time. We had tremendous challenges with the quality and supply chain for our large-scale adherent vessels. Lentivirus production in suspension was quite rare at the time. I would say the CMO landscape and the talent pool was pretty sparse. It was hard to find people with relevant experience.

Now, the industry is in an exponential growth phase, and I think most if not all of these bottlenecks have started loosening up. It is a really exciting time.

**Q** Nolan, maintaining high viral vector titers when moving from small-scale studies in shake flasks to large-scale production in bioreactors is notoriously difficult. In your experience, what steps can you take to ensure scalability, and what is the smallest scale and format you trust will translate to success at larger bioreactor scale?

**NS:** When moving from shake flasks to stirred tank bioreactors we definitely ran into a lot of challenges. The academic process we inherited out of the development groups just didn't translate. What we had going for us at the time was a rapidly maturing adherent process, which informed a lot of the bioreactor unit development.

Our main goals when developing a suspension process included increasing yield per batch compared to adherent, and an animal component free process. In addition, we wanted to adapt the unit operations of the adherent as closely as possible, based on the robustness of that process and any comparability required from regulators during a possible post-commercial change to suspension.

To assure a lot of our sustainability, we focused on breaking up the upstream process by those unit ops, and then evaluating and developing technologies which had scalability built in. We chose perfusion filters and other technologies from vendors who already had scaled products marketed. We also focused on off-the-shelf medias and reagents that would be easy to source. The transfection step was one of the most difficult to scale, and it required more creativity than leveraging available technologies.

When choosing a scale-down model, it is really a matter of where you are in your development cycle. We moved very quickly from scale up and tech transfer right into process characterization. As most people will know, process characterization has to be completed using a qualified scale-down model which is shown to be able to mimic the at-scale process at every unit op, either by an exact comparison or with a known offset in their outputs.

For LV processes, there are unit ops which can't be scaled down below the bench top stirred tank reactor systems, so that was the scaling cut-off for our model. Therefore, our qualified scale-down model is at the benchtop scale.

Finally, other development exercises that are maybe earlier in stage or more exploratory in nature could benefit from a higher throughput and lower cost system which could be a screening platform of some sort. There are a lot of these out there now. A lot of companies are coming out with scaled down systems, such as the Ambr® system from Sartorius, which are also stirred tank reactors.

For screening, a system can be chosen which doesn't exactly mimic every unit op of the qualified scale-down model, or the commercial scale, but if you can patch together those lapses and still produce vector with similar supernatant titers and other characteristics, then the model can be really informative for your process. Correlation to the bench scale is also key for your screening platform, where positive results from that screening model can be optimized in the qualified scale-down model.

I actually have an innovator insight in peer review on this topic, so stay tuned.

**Q** Anindya, in your recent role at Cincinnati Children's Hospital you led a program to develop high titer lentiviral vectors. In your experience, what modifications to expression platforms tend to lead to the most significant improvements in expression levels?

**AD:** To echo Beth and Nolan's points, you need to have a system that you can scale up. It is also advisable, although not always possible, to have a system that you can take to GMP manufacturing.

At Cincinnati Children's, I was involved in GMP manufacturing, and I faced the situations and challenges that come in a clean room. In that process, we decided to first test a system that we never tested before – an electroporation-based device that has a GMP compliant version, so that you can integrate it into your manufacturing. You can scale it up from a mid-scale 20 billion up to 200 billion cells that you can electroporate in 30 minutes.

We optimized a lot of transfection protocols, and we got 95% or more efficiency. The most important modification that we did was the transfection method, which was electroporation. We found several factors that affect the optimization process. One is the type of vector producing cells you are using, whether it is adherent or suspension. Our legacy system is an adherent system, and you can go to an iCELLis® and other fixed bed bioreactors to do that. But there is a limit up to which you can scale up, so then the suspension system comes into being.

We tested both cell types in this transfection system. We found that cell density is very critical, and we had about 1 x $10^8$ cells per ml. The ratio of the expression plasmid to the packaging construct is also critical, and if people are interested, there is a paper in molecular therapy [1] that talks about how that affects packaging of your lentiviral particles. It also talks about gag-pol mRNA as a surrogate marker that you can use to somewhat approximate your packaging efficiency, which is an interesting thought.

We found that the amount of DNA that you electroporate is crucial. Electroporation creates harsher conditions, so you are going to end up with more dead cells than you do in chemical-based transfection. Therefore, you need to have the right balance of the amount of DNA and of viability, and we found that about 200–400 micrograms per ml DNA is good.

We also found that the amount of DNA per amount of cells, meaning the number of micrograms of DNA, is also critical in the transfection process. Post-transfection, we found that the addition of DNAase right after electroporation is extremely critical, especially in the survival of suspended cells. We found the effect to be minimal in adherent cell types.

We also tested transfection enhancers, particularly sodium butyrate, and we found it gives you about 1.5–2-fold more expression. But again, you need to consider the time of sodium butyrate addition, and the amount that you need. There is some optimization that needs to happen. Interestingly, we found that the enhancer had less enhancing effect on adherent cells, and it is more pronounced in suspension cells.

The way we cultured the cells was also critical. In adherent cells we stuck to our GMP media, which is basically your regular DNA with serum. But the situation changes when you start using suspension cells. We used a platform with serum-free media, and we found that some media was better than others. How you cultured those cells in suspension, and the type of vessel you use for cultures, is also important.

You can also think about changing your temperature. It has been suggested that 37° C is optimal, but if you go down to 32 °C you can have more pronounced expression of your LV.

The time of harvest was also critical in this system, and we found that 24–48 hours for transfection is ideal. 32 hours is also not bad.

So, we found that this electroporation-based transfection system, along with several modifications that we used with that, gave us a titer, and unconcentrated titer, from 5 x $10^7$ to 5 x $10^8$ infection units per ml. And again, the whole point was to have a scaled down version that we can use in a GMP process.

**Q** How would a two to 10-fold increase in viral vector titer change your production workflow? What aspect of the overall expression process would higher titers improve the most?

**BL:** The major impact would be that we can do fewer runs, and a lot of benefits would flow from that regarding cost of goods and supply availability. It would reduce the quality control tax on the lot and reduce the full-time equivalent hours per released vial. For those of us who are reliant on CMOs, we are really at the mercy of their calendars, so fewer lots would have lots of benefits.

**NS:** If you are looking more towards the oncology landscape, increases in productivity are absolutely required to meet the needs of that area. Comparatively, the severe genetic disorder landscape does not have the same scalability requirement, and is more focused on supply.

There are also considerations from an upstream perspective. We are constantly trying to increase our titer, but there are also downstream impacts to that. You need to be working very closely with your purification group to make sure that impurity profiles and capture steps are scaling as you are increasing those yields.

**AD:** Even a two-fold increase is a tremendous advantage. It also depends on your disease indication. Some indications may not need that number of particles, and some may. When you are developing your process, you need to be prepared to test or optimize two or three processes, and adapt that to your manufacturing needs.

**Q** Where do you anticipate seeing the greatest cost savings from significantly improved LV or AAV titers?

**NS:** In my opinion, the greatest savings will be reduced cost of overall manufacturing – both the vector process and the cell therapy process – due to higher yields producing more vials per patient.

Increasing those yields in a single batch will lower the demand of vials for your cell therapy process, and you will be able to treat more patients with a single batch of vector.

We have a lot of room to significantly impact those calculations, purely based on yield. Unfortunately, the consumables around the transfection process will probably remain high in cost, so there is a huge balance to be focused on when looking at cost of improvements versus impact per yield.

A lot of our focus is on developing a next-generation process which reduces deviations, is more robust, and overall increases yield from previous generations, whether through increases in productivity or through more reliably being able to consistently produce across batches. LV processes can be more variable in yield than other biologics, so increasing process robustness is also just as important as increasing your process yield.

Specifically to the vector process, we may actually be creating an overall more expensive process in terms of operation through extending it to run longer, but this again will be balanced with cost per titer unit, which we are really trying to decrease. Dollar per titer is really what we are trying to decrease at this point.

**LK:** Our focus has always been on optimization, creating higher titers and finding different solutions – whether that's cells, media, or vector sets – that enable those higher titers. What we hope to see is a reduction in the number of runs required, and ultimately, enabling customers to produce more molecules faster, because this field moves at a lightning speed.

**Q** What methods or assays do you employ to determine upstream viral titers and virus quality?

**AD:** We are based on technology that was developed for particular use over the years. It is a functional titer, and at the moment there is no substitution for that. You can still use the physical titer, of course, that is p24, or you can do a genomic titer. You can also use something like product enhanced reverse transcription to measure the reverse transcriptive assay, but those are the most physical properties, physical ways, and functional categorization is through a functional titer infectious-based method.

I strongly believe that there should be more in-line processes to calculate your amount of genome particles. You will have empty particles, partially filled particles with spliced host DNA, and you will have full particles. There are things that are coming up now, and the FDA would probably be interested in having this data as well, as part of in-line processing that can be done quickly to see how your optimization is taking place.

**BL:** I agree with Anindya. One thing to add is that as our assays become more sophisticated, it is really clear that your process optimization is only as good as your assay. So if you have a new, more sophisticated assay, and you go back and look at your previous optimization, you may not have been optimizing the right things.

**NS:** For upstream titers, we typically have either a GFP titer, which is cell-based, or genomic titers that are qPCR-based. Like the other panelists, we also calculate our particle to infectivity (P to I) ratio. We do particle based on an ELISA against the P24 protein, and the LVV particle, which is a capsid protein, and we are looking to equate how many particles are actually infective in our prep.

On the upstream side that is something we are looking to optimize. We are looking to make a higher number of infectious particles; lowering that P to I ratio.

Something else we are looking into is trying to get away from these cell-based assays, and doing more instrument-based determination. What is coming up in the field now is a lot of nanoparticle flow cytometry. With this we can not only probe for envelope proteins, but probe for genomes. This means you can start to equate and determine empty versus full LVV particles as well as total nanoparticles, and the psuedotype-positive particles that have infectivity based on something like VSVG, versus a non-infective, non-psuedotype particle.

**Q** Leisha, have you characterized empty/full content with the Mirus transfection reagent, and how does that compare to empty/full content with competitor agents?

**LK:** This is something that everyone producing AAV is struggling with: how to generate more full particles versus empties, and how to characterize them.

Infectivity assays, which we often do at Mirus, are great because they will only measure functional viral particles – those that are completely full capsids. But it's worth noting that there are caveats to that. It is a cell-based assay with some inherent variability. There is also serotype and cell type compatibility to consider. At Mirus, we often functionally titer by transducing HT-1080 cells with AAV2 and this works very well. AAV2 transduces a lot of different cell types well. However, if you switch to something more challenging like AAV5, which doesn't transduce many cells well at all, that functional titering assay becomes much more of a challenge and perhaps less reliable, because not all AAVs that work well *in vivo* will work equally well *in vitro*.

This is why companies heavily rely on those GC/ml measurements, ELISAs, and the ratio of the two to determine percent empty/full capsids. We have all acknowledged on the panel here that this assessment method is not ideal. It is definitely not 100% clear what you are really reading there. We have started looking at analytical ultracentrifugation to try to get a better idea of how that all teases out in terms of percent full, or percent empty. The problem with AUC is that it is not necessarily a high throughput method, but I know there are companies working on that to address the need in cell and gene therapies.

The methods that everyone spoke to are also really valid. It is clear that we need to improve analysis methodologies. There are a lot of different teams working on that now that we are talking with.

But back to functional titering – the TU/ml measurement you obtain will be from full and functional capsids so if using as a relative measurement where you are comparing different expression systems, the data is likely still valid. This is also where VirusGEN tends to shine – the ability to produce fully functional viral particles compared to competitor reagents.

**Q** How would you approach optimization of viral vector expression at small scale, and does that change when shifting to larger expression formats such as the iCELLis bioreactor for adherent, or stirred tank bioreactors?

**BL:** Overall, I would say we are very cautious with scale up and scale down. We systematically check and double-check the reproducibility of our results at small-scale, pilot-scale, and full-scale. If we do an optimization experiment at a given scale, we are cautious to make only internal comparisons among those samples. But we also retain samples so that later we can make direct comparisons across scales in a single assay

**NS:** As I mentioned before, we have utilized an Ambr® 15 system for a lot of small-scale studies, and that is a great system. A lot of the limitations are around the perfusion steps at a 15 ml bioreactor scale – with any experimentation that is looking at perfusion rates, you know they aren't that translatable to the at-scale or the bench scale process. But for other aspects you are looking to screen in terms of titer, transfection enhancers, or media components and things like that, it is pretty indicative.

If you can match your cell growth kinetics, and you can match your transfection kinetics in these systems, they are typically quite indicative outside of that perfusion step.

**Q** Leisha, is the Mirus AAV enhancer available to purchase?

**LK:** Right now it is not available to purchase. We are beta testing the AAV Enhancer, which will be a component of a the VirusGEN AAV Transfection Kit launching in either July or August of this year. If someone is interested in evaluating the VirusGEN AAV Kit, we can certainly provide them with research grade material, so that they can test. We will also work together with them on optimization.

**Note:** The VirusGEN® AAV Transfection Kit and VirusGEN® GMP LV Transfection Kit are now commercially available.

**Q** … and do you have any data regarding retroviral production?

**LK:** We have focused mostly on lentivirus production, and of course those are very similar. We have several customers that are using VirusGEN for retrovirus production, and it is definitely very compatible, but we don't have that data internally.

**Q** Finally, what advice would you offer to a startup biotech or pharma company entering into the cell and gene therapy space?

**BL:** What I often see is a startup company that has a great idea, something that is super promising, and they then want to scale up. My advice is to get an experienced CMC person in early.

A lot of decisions that are made early can continue to have ripple effects. For example, if you choose the wrong kind of serum or wrong raw materials. Going fast and trying to fix things later can often cause lots more headaches. I always advise to try and do things right the first time.

**AD:** I believe that how you prepare for your pre-IND application package will drive you towards fulfilment. As Beth alluded to, there are all these factors to scale up or out in later stages of production. If possible, use the scale-down version in your initial developmental studies as soon as you can, so that you can integrate that as you scale up, because often things don't scale up as you desire.

**NS:** Bluebird can probably be seen as a case study for going from startup to commercialization, so anyone can look into our history and what works and what doesn't work.

We have such an immediate impact to patients with these therapies, and it is very easy to be patient-focused, and it is easy to lose sight of your people. A huge focus should also be on the people. It is a very competitive landscape, and it can be a very stressful landscape in doing some of this work.

Foster a culture at the company that is open, honest, and transparent with the employees. Because there are just so many challenges, and also all these products are going at lightning speed. There is a lot to learn, there are a lot of inherent challenges, and at this point in time it is really hard to retain people if the culture is waning.

**LK:** Don't skimp on the optimization steps. I know everyone is trying to move at a really fast pace, but it is definitely worth pausing and looking at all parameters to ensure your stealth isn't tripped up by a lack of attention to details.

Also, don't be afraid to reach out to others that have experiences or expertise that you may lack. Scientists are generally pretty collaborative. If you find teams at pharma companies or in medical research organizations that are able to talk and share their experiences, utilize and learn from that.

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## BIOGRAPHIES

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**Applications Scientist, Mirus Bio LLC**

Leisha Kopp is an Applications Scientist at Mirus Bio LLC, a biotech company providing innovative transfection products to cell culture researchers worldwide. Leisha has over 15 years of molecular biology and mammalian cell culture experience in industrial labs, and her combined bench and business knowledge enables support of scientists in all stages of the drug discovery process – from R&D to commercial manufacturing. Leisha is a graduate of the University of Wisconsin-Madison, with key interests in biotherapeutic antibody discovery and gene therapy.

### Beth Larimore

**Associate Director of Viral Vector Process Development, Bristol Myers Squibb**

Dr. Beth Larimore is Associate Director of Viral Vector Process Development at Bristol Myers Squibb and is based in Seattle. A Bay Area native, Beth earned a BA in Biological Sciences at Cornell University and a PhD in Molecular and Cellular Biology at the University of Washington, where her doctoral work focused on the basic biochemistry of a tumor suppressor protein frequently mutated in human cancers. Beth started her industry career in purification of antibody therapeutics, but has worked on viral vector process development since 2014. She has held process development roles of increasing responsibility at Amgen, Immune Design, Nohla Therapeutics, and Juno Therapeutics/BMS. She is motivated by challenging scientific and engineering problems and feels privileged to contribute to novel, life-changing therapies.

### Nolan Sutherland

**Scientist, Molecular and Vector Biology, bluebird bio**

Nolan Sutherland graduated from the University of Massachusetts, Amherst with a BS in Biochemistry and Molecular Biology where his research areas of focus were on molecular cloning and RNAi. Over the past 10 years he has worked in both Upstream and Downstream Process Development groups, getting his start in the area of enzyme replacement therapies before entering the gene therapy field at bluebird bio. Since that time, the majority of Nolan's work has been focused on developing suspension-based lentiviral vector production systems for commercial manufacturing. His areas of expertise include process establishment and improvement at the miniature and bench-scales, emerging technology evaluations, transient transfection optimization, nanoparticle analysis and characterization, as well as scale-up and technology transfer. With a new role in viral vector R&D, he aims to support the development of novel vectors for *in vivo* use.

### Anindya Dasgupta

**Director Vector Development, Expression Therapeutics**

Anindya is the director of vector development at Expression Therapeutics. He obtained his PhD from University of South Carolina, USA. His post-doctoral training and research associateship at the school of medicine, Emory University, Atlanta, USA, were focussed on the evaluation of novel anti-cancer therapies and the development of strategies for expansion and lentivirus based bioengineering of $\gamma\delta$ T cells in serum free media. Anindya is a co-inventor of a patent on anti-cancer strategy. At his recent role at Cincinnati Children's Hospital Medical Centre he led vector development to manufacture high titer lentiviral vectors.

## AUTHORSHIP & CONFLICT OF INTEREST

***Contributions:*** *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

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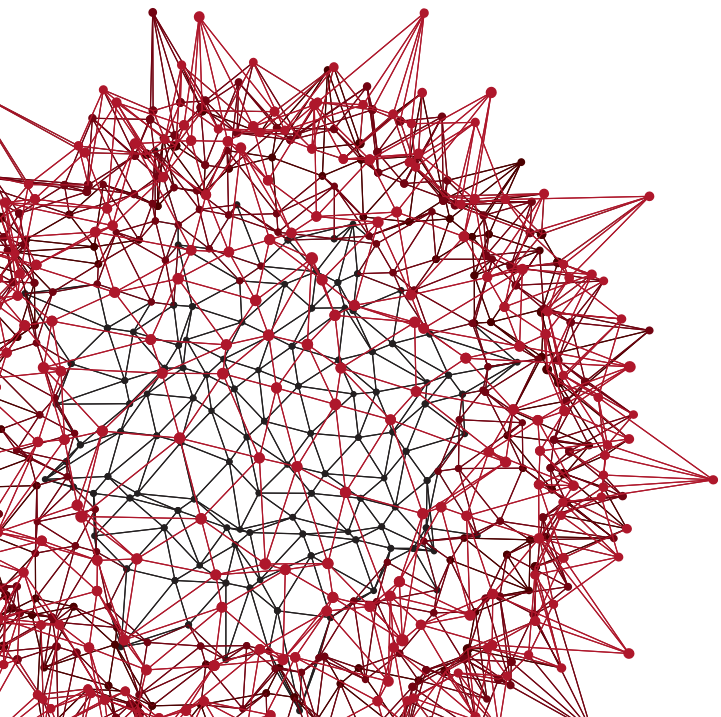

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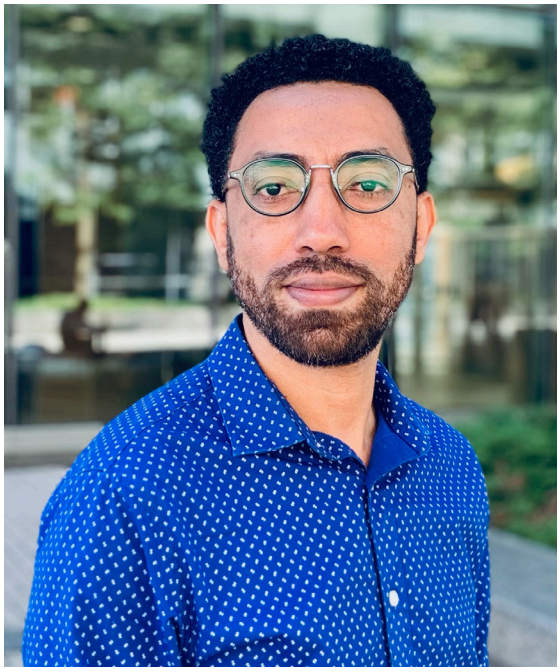
VIEWPOINT

# Overview of major AAV production platforms



> "The current AAV-based viral vector production methods cannot meet demand for conducting large-scale clinical trials and commercial applications. Therefore, there is an urgent need to develop innovative AAV production schemes to turn the promise of gene therapy treatments into a reality."

**JOTE BULCHA,**
**University of Massachusetts Medical School**

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Gene therapy holds great promise for curing genetic disorders that are not currently addressed by conventional medicine, including many rare diseases. At the center of gene therapy products are the viral vectors that have been instrumental in paving the way for preclinical and clinical advances. Among the most widely used viral vectors are adeno-associated virus (AAV) vectors, which have been widely used to deliver a genetic payload to a target tissue. Significant progress has been made in delineating AAV vectors of high therapeutic efficacy to target a specific tissue. To this end, AAV vectors have been used in a significant number of clinical trials as well as commercialized gene therapy products [1]. On the flip side, AAV-based gene therapy products are very expensive mainly due to the complexity of AAV vector production and purification.

Currently, the widely adopted AAV vector manufacturing scheme is transient triple transfection. In a triple transfection production method, HEK293 cells are co-transfected with plasmids containing AAV *cis* construct, which carries a transgene expression cassette flanked by ITRs, a plasmid that contains Rep and Cap expression cassettes, and a plasmid that carries adenoviral helper genes (E2a/b, E4, and VARNA genes). The HEK293 producer cells have integrated copies of adenoviral E1A and E1B genes. Generally, the crude lysate is harvested 72 hours post-transfection which is then subject to subsequent purification processes. The transient triple transfection method confers an advantage over other methods as it can be flexible and set up fairly quickly for early clinical developments. To this end, straightforward transfection protocol, low cost of developing the plasmids, and the agility of the method to produce different serotypes are the pros of this technique. However, this method still suffers from scalability and cost-effectiveness compared to a stable producer cell line method for AAV production. One of the major challenges in AAV production is the low full/empty viral particle ratio, which could range less than 30% [2]. It has been suggested that uncoordinated timing of Rep and Cap expression could be one of the culprits for low viral yield [3]. To circumvent this issue, there are ongoing efforts to optimize the Rep and Cap expression either by manipulating their promoter, developing a split Rep and Cap system or through optimizing the transfection ratios of the plasmids.

Stable cell lines are another alternative to the transient triple transfection method for producing AAV. Typically, stable packaging cell lines have a copy of Rep and Cap integrated into their genome. In addition, producer cell lines may contain other components such as the AAV *cis* construct that carries transgene of interest and other adenoviral helper genes. Depending on the engineered cell line design, AAV production can be achieved by transfection of the *cis* vector construct and infection with Adenovirus to confer helper function, or alternatively, the *cis* construct and the helper function can be provided by AAV-Adenovirus hybrid and Adenovirus coinfection respectively. To date, most of such packaging cell lines are based on HeLa cells. However, there is a safety concern related to the use of cancerous cell lines, such as HeLa cells, for commercial AAV production [4]. Developing a HEK293-based stable Rep and Cap expressing cell line remains a challenge due to the toxicity related to Rep expression. Compared to the transient triple transfection method, stable cell line-based AAV production offers a significant advantage in terms of production scalability. However, developing such cell lines is not an easy undertaking for the desired vector-serotype combination. The stability and the associated production titer of producer and packaging cell lines have to be well characterized for longer passage generations, which makes the development of a stable producer cell line cumbersome.

The baculovirus-based AAV production approach is yet another alternative to the mammalian cell-based AAV manufacturing scheme. In this modality, a baculovirus expression vector is employed to deliver Rep/Cap genes and AAV *cis* construct, carrying transgene of interest, via coinfection of *Spodoptera frugiperda* insect cells (Sf9). As a


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variation to the coinfection scheme, a single infection of Sf9 cells with a recombinant baculovirus vector carrying all the components can be used [5], making the baculovirus-based system more versatile and flexible [6]. In addition to this, the baculovirus-based system has an edge over the transient triple transfection method based on scalability and cost-efficacy measures [6]. Furthermore, the baculovirus-based system has been shown to yield high AAV titers with minimal encapsidation of contaminating DNA, which in turn makes it a good alternative for large-scale AAV manufacturing [7]. However, the impact of post-translational modification of the AAV particles produced in the insect cells needs to be closely investigated.

The current AAV-based viral vector production methods cannot meet demand for conducting large-scale clinical trials and commercial applications. Therefore, there is an urgent need to develop innovative AAV production schemes to turn the promise of gene therapy treatments into a reality.

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## BIOGRAPHY

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## AUTHORSHIP & CONFLICT OF INTEREST

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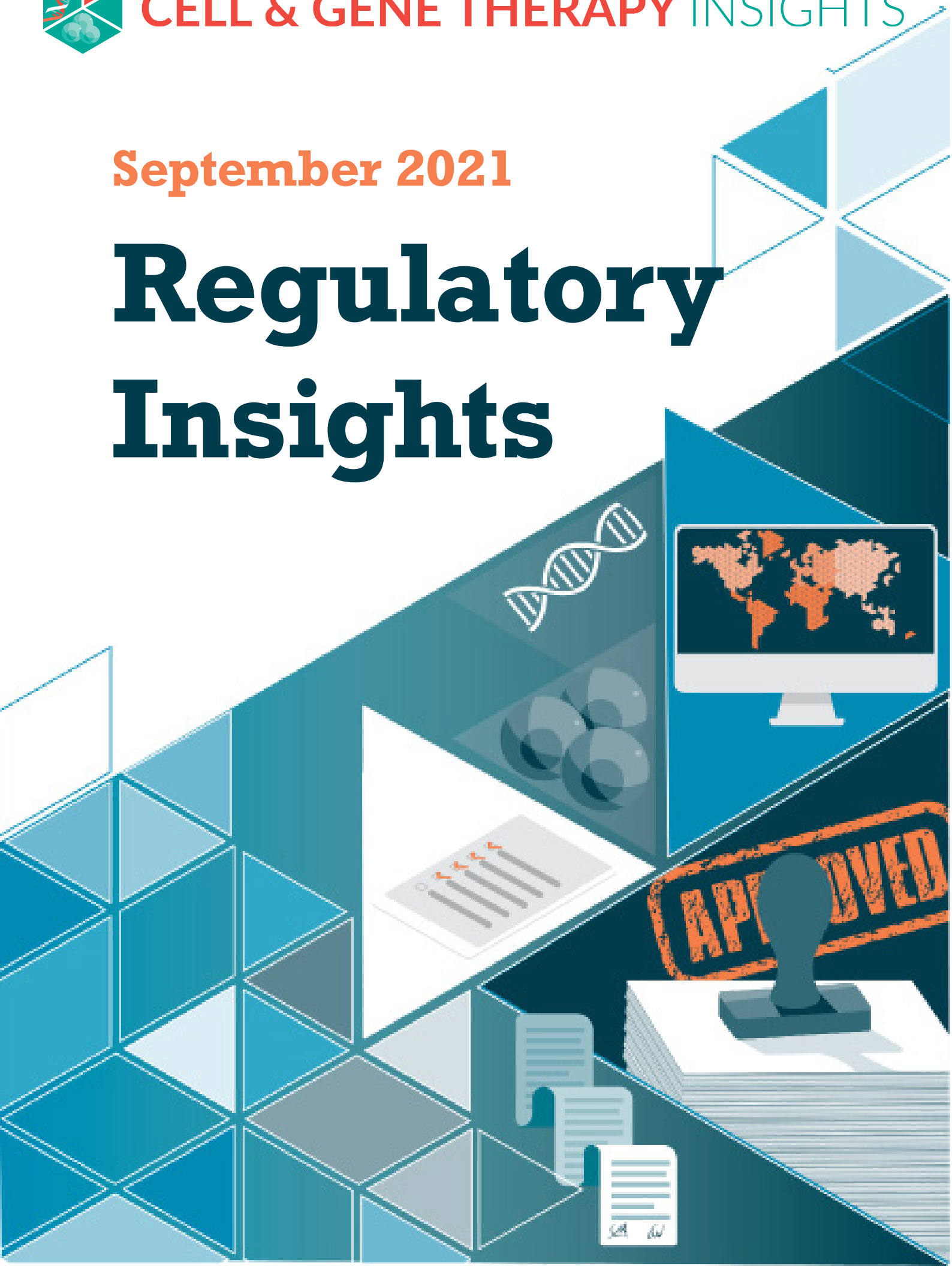
CELL & GENE THERAPY INSIGHTS

September 2021

# Regulatory Insights



CELL & GENE THERAPY INSIGHTS

# Regulatory Trends

-

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## REGULATORY PERSPECTIVE

Clinical trials with investigational medicinal products consisting of or containing genetically modified organisms: implementation of Clinical Trials Regulation EU 536/2014

**Nathalie Lambot, Jacquelyn Awigena-Cook, Tatiana Reimer, Annelie Persson, Julien Romanetto, Beatrix Friedeberg, Virginia Acha, Shera Dandapat, Thorsten Ruppert, Caroline Correas, Keith Wonnacott, Tobias Fleischmann, Christa Holzhauser, Ariane Galaup, Fátima Montes, Silvia Garcia, Pär Tellner, Stuart G Beattie; European Federation of Pharmaceutical Industries, Brussels, Belgium**

1093–1106

## COMMENTARY

The UK's emerging regulatory framework for point-of-care manufacture: insights from a workshop on advanced therapies

**Edison Bicudo, Irina Brass, Penny Carmichael & Suzanne Farid**

1005–1015

CELL & GENE THERAPY INSIGHTS

# Regulatory Insights

REGULATORY PERSPECTIVE

# Clinical trials with investigational medicinal products consisting of or containing genetically modified organisms: implementation of Clinical Trials Regulation EU 536/2014

Nathalie Lambot, Jacquelyn Awigena-Cook, Tatiana Reimer, Annelie Persson, Julien Romanetto, Beatrix Friedeberg, Virginia Acha, Shera Dandapat, Thorsten Ruppert, Caroline Correas, Keith Wonnacott, Tobias Fleischmann, Christa Holzhauser, Ariane Galaup, Fátima Montes, Silvia Garcia, Pär Tellner, Stuart G Beattie; European Federation of Pharmaceutical Industries, Brussels, Belgium

Although originally applicable to genetically modified crops, advanced therapy medicinal products (ATMPs), such as gene therapies, that consist of or contain genetically modified organisms (GMOs) and also viral-based vaccines, need to comply with the European Union (EU) GMO legislation, as implemented in each EU Member State before a clinical trial can commence. Under the European Clinical Trials Regulation 536/2014 (CTR) due to *go live* on 31st January 2022, a single electronic clinical trial application dossier will need to be submitted to all the Member States involved in the trial, via the European submission portal

(Clinical Trials Information System, CTIS). However, national documents, such as informed consent forms, will still need to be submitted, as part II of the dossier, to each *Concerned* Member State, also via CTIS. There will be a single coordinated and harmonized assessment of the clinical trial application between the involved Member States, with one country leading the coordination of the assessment (the *Reporting* Member State). The CTR has as yet not addressed GMO documentation (ERA, common application form, etc) required for an IMP with a genetic component. There is no defined interplay between the CTR and the current GMO legislative framework. There is no specified procedure, nor structure for submission of a GMO application via CTIS, as part of the new single submission and coordinated evaluation procedure for clinical trials defined by the CTR. Upcoming challenges to clinical trial sponsors under the CTR across Member States are identified in this article. Further, national GMO competent authorities are called upon to adopt a pragmatic and simple approach as a first step to facilitate the GMO assessment and its coordination with approval of a clinical trial application.

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## INTRODUCTION

In recent years, across the European Union (EU) we have witnessed an increasing number of clinical trials with ATMPs and vaccines consisting of or containing genetically modified organisms (GMOs) [1]. As of 2021, approximately 15 ATMPs have received marketing authorization within the EU (with four subsequent withdrawals). Authorized COVID-19 vaccines in the EU include those that are genetically engineered and regulated as GMOs.

There are a variety of investigational medicinal products (IMPs) consisting of or containing GMOs (GMO-IMPs) [2]. Such GMO-IMPs include the following: human somatic cells modified *ex vivo* (for example, CAR-Ts); vaccines; recombinant virus-based vectors, including those containing genome editing nucleic acid sequences (which may also be delivered non-virally) and bacterial vectors.

Clinical Trials and GMOs are both regulated by European Commission (EC) directives across the EU, which allows for interpretation and implementation at national level. Due to the resulting complexity of the European Clinical Trials framework and the lack of harmonized requirements for GMO-IMPs across European Member States, the initiation of clinical trials remains a time- and resource-intensive process. This can lead to delays to patient access to innovative and promising advanced therapy technologies. A pragmatic, simplified approach for the GMO assessment and its' coordination with the clinical trial authorization should be considered by each EU Member State.

In the EC communication about the Pharmaceutical Strategy for Europe (dated 25th November 2020) [3] the EC recognized that regulatory requirements for GMO products in the EU *"should be fit for purpose"* but are *"currently hindered by the fragmentation of national requirements"*.

The European Federation of Pharmaceutical Industries and Associations (EFPIA), the European Association for Bioindustries (EuropaBio) and the Alliance for Regenerative Medicine (ARM) have recently called upon the European Commission and the National Competent Authorities to exempt ATMPs containing or consisting of GMO, from the GMO legislation [4].

In July 2020, a temporary derogation from some provisions of the GMO requirements was granted for potential COVID-19 treatments and vaccines [5]. The exemption was made on the basis of a clear recognition of such complexities and resulting delays to

clinical development. This regulation was adopted with the intent to *"accelerate the authorization and availability of successful vaccines against COVID-19"*. The EU strategy for COVID-19 vaccines acknowledges that *"There is considerable variety across Member States in the national requirements and procedures implementing the GMO Directives used to assess environmental risks of clinical trials of medicinal products that contain or consist of GMOs. This is likely to cause significant delay, particularly for multi-center clinical trials in several Member States."* [6]. Further recited extracts from the regulation show how the EC recognized that the national requirements and procedures for the *"environmental risk assessment and consent by the competent authority of a Member State is complex and can take a significant amount of time"*. The Commission further acknowledged how their *"Attempts to streamline the process through informal coordination between Member States' competent authorities have been unsuccessful"*.

The EC Pharmaceutical Strategy stated how *"Solutions will be explored during the evaluation of the pharmaceutical legislation"*. However, there is no timeframe towards such solutions, or ideally an exemption. The authors (EFPIA) hereby reiterate their support for a permanent exemption from GMO requirements for ATMPs containing or consisting of GMOs, as well as for vaccines.

The recent EC study on New Genomic techniques (NGTs) [7] was conducted to determine the need for regulating the use of new genetic tools differently to the currently applicable EU GMO framework. Reports, including the EC staff working document [8], were published recently and included important messages with regard to the burden of the GMO framework for ATMPs in Europe. The study states how *"there are strong indications that"* the GMO legislation is *"not fit for purpose for some NGTs and their products, and that it needs adaptation to scientific and technological progress"*. Per the feedback from trade associations, including that from EFPIA, the report acknowledges how stakeholders *"consider that the GMO legislation is not specifically designed for medicinal products and hinders the conduct of clinical trials."* The report also noted how stakeholders *"ask for reconsideration of the application of the GMO legislation to medicinal products consisting of or containing GMOs. More specifically, they believe that there are no environmental and biosafety risks for non-replicating viral vectors or GM human cells, as these do not duplicate and cannot survive in the environment."* While the report does not specifically suggest that a legislative change to exempt ATMPs is the way forward, it does anticipate the need for policy instruments to *"future-proof"* legislation (as stated by the authors of the EC reports on NGTs).

The Clinical Trial Regulation (EU 536/2014) will be implemented and the Clinical Trial Information System will *go live* on the 31st of January 2022 [9–11] with a 3-year transition period. The Pharmaceutical Strategy proposes to find a solution to the fragmentation of GMO requirements amongst EU Member states, ideally through an exemption for ATMPs (and vaccines) by 2022. It is critical for the timely initiation of clinical trials with GMO-IMPs that in the meantime, a pragmatic and simplified approach for the GMO assessment and its' coordination with the clinical trial authorization should be considered at the national level by EU Member States, ideally with solutions towards greater harmonization of the GMO procedures.

## CONTEXT & BACKGROUND

Current regulatory frameworks in Europe for clinical trials with investigational medicinal products with a GMO component:

1. Presently, two European legislations define the requirements to be followed prior to commencing a clinical trial with an IMP consisting of or containing a GMO across the EU:

    - The current **European Clinical Trials Directive 2001/20/EC**, as implemented in the different Member States, applies

to the evaluation of the clinical trial protocol, the information provided to the investigator (investigator brochure), the information provided to the participants (informed consent form), as well as the non-clinical, clinical, and quality module information about the IMP (IMP dossier, IMPD). The current European Clinical Trials Directive 2001/20/EC requires the submission of the IMPD to the national health competent authorities of the involved Member States by the sponsor. Additionally, a common module clinical trial application is submitted to the relevant ethics committees, by the investigators.

- The current European GMO Directives 2001/18/EC (Deliberate Release) and 2009/41/EC (Contained Use) are interpreted differently across different Member States and apply to the Environmental Risk Assessment (ERA) of the GMO component of the IMP and its use.

  - EC Directive 2001/18/EC for Deliberate Release was enacted primarily to protect food consumers and the environment with regard to large scale agricultural use of GMO plants. Despite no negative environmental impact being reported after several decades culture of GMO crops, the subject remains controversial [12]. However, GMO medicinal products, such as gene therapies, are not designed to propagate in nature and cannot survive outside of controlled storage conditions. GMO medicinal products are utilized at several orders of magnitudes lower than GMO crops and after many decades of clinical development of GMO medicinal products, there have been no reports of any impact to the environment.

    The application of the Deliberate Release or Contained Use Directives differ across Member States, sometimes even for the same GMO-IMP. When Deliberate Release has been identified by the authorities, this requires the submission of a dossier consisting of the GMO application, including the ERA and common application form (for an ATMP) by the sponsor to the corresponding national GMO competent authority of the Member State. In addition, information specific to the clinical trial site is in most cases also required. In case of an identified Contained Use, the procedure can also be complex and variable across the Member States.

2. Various data requirements and regulatory procedures exist in the different Member States for the ERA of the GMO component of the IMP:

The national GMO competent authorities differ (for nearly, but not, every Member State) from the national health authorities that evaluate the clinical trial evaluation application. In the majority of Member States, a single submission of both clinical trial and GMO applications is not possible, where the respective authorities do not necessarily interact with each other. Data requirements for a GMO application differ depending on whether it falls under either the Contained Use or Deliberate Release Directive and vary considerably in format and content across countries.

A first step to harmonization across Europe was taken in 2017 with the publication by the EC of a Common Application Form [13] to be used as part of the submission of the ERA dossier for Deliberate Release. Common application forms and good practice documents were introduced for a variety of ATMPs and vaccines, including for human cells genetically modified by retroviral or lentiviral vectors; and also, for adeno-associated viral vectors (AAV) and for other viral vectors. However,


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additional forms and data are often requested by the national GMO competent authorities as recently shown by a survey (Clinical Trial implementation (CTi) Monitor 2020) [14] conducted by EFPIA and by the ARM 2020 GMO survey [4].

The European Commission hosts a website that provides overviews of the national regulatory requirements for GMO applications in the 27 EU Member States [15]. Since having been drafted in 2017, the overviews have not been kept up-to-date and many links to national websites no longer work.

In 2018, the European Commission published a Q&A document related to the interplay between the medicinal products framework and the GMO framework (regarding authorization procedures). The document acknowledged how *"The EU legislation governing the authorization of clinical trials does not specifically address environmental aspects."* [16].

Despite these initiatives, the evaluation procedures (pathway and timelines) are very different across Member States. The EC acknowledged that there is not a common approach for assessment of GMO aspects of clinical trials with IMPs for human use in the EU when issuing the regulation specific to the temporary derogation from some provisions of the GMO requirements was granted for potential COVID-19 treatments and vaccines.

Four groups of Members States can be defined:

- Member States where a single application to the national health authorities including the GMO application takes place.
- Member States where separate GMO applications and clinical trial applications are conducted in parallel.
- Member States where the GMO approval is needed before the clinical trial application can be submitted.
- Member States where there is no defined process.

Those differences are illustrated for EU Member States that host the majority of clinical trials with GMO-IMPs in Table 1.

## 3. The European Clinical Trials Regulation 536/2014 (CTR) aims to harmonize the clinical trial application framework across Europe but does not address GMO applications (ERAs)

With the European Clinical Trials Regulation 536/2014 to become applicable on 31st January 2022 [10,11] a single electronic clinical trial application dossier will need to be submitted to all the Member States involved in the trial via a unique European portal (Clinical Trials Information System, CTIS). However, national documents, such as informed consent forms, will still need to be submitted, as part II of the dossier, to each *Concerned* Member State, also via the European platform (CTIS). There will be a single coordinated and harmonized assessment of the clinical trial application between the involved Member States, with one country leading the coordination of the assessment (the *Reporting* Member State).

The CTR has as yet not addressed the GMO documentation (ERA, common application form, etc) required for a GMO-IMP. There is no defined interplay between the CTR and the current GMO legislative framework. There is no specified procedure, nor, even a structure for a GMO application submission via CTIS, as part of the new single submission and coordinated evaluation procedure for clinical trials defined by the CTR.

If the national GMO procedures and their respective timelines (which vary across Europe) are not adapted to the CTIS and the CTR, the different GMO procedures and data requirements across Europe will continue to be required. It will be imperative to ensure GMO procedural timelines are synchronized with CTA timelines, in order to ensure

## TABLE 1

**Regulatory GMO requirements in selected EU Member States.**

| EU member state | GMO competent authority | Submission | Contained use*/deliberate release** | Document and data requirements |
|---|---|---|---|---|
| Austria | BMSGPK (Federal Ministry of Social Affairs, Health, Nursing and Consumer Protection). | Parallel, or in advance of CTA | CU | Application in German, where technical documents in English accepted. GMO-IMPs not defined as somatic gene therapies (such as GMO vaccines) not required to submit request for authorization separate to the CTA. |
| Belgium | National Biosafety advisory Council (BAC) for DR. Service Biosafety and Biotechnology (SBB) and regional competent authorities for CU. Classification via Federal Agency of Medicines and Health products (FAMHP)/ National BAC. | Parallel, or in advance of CTA | CU or DR | Classification via FAMHP Scientific Technical Advice procedure. For DR 90-day assessment with 30-day public consultation required in local language (Dutch or French). For CU, typically shorter review time (30–70 days). |
| Bulgaria | Ministry of Environment and Water (MoEW). | Prior GMO authorisation required | DR, where clinical sites should comply with CU requirements | Application in Bulgarian. Technical documents in English acceptable, except for information related to safety of clinical personnel. No public consultation. Plan for clinical site training and CV of PI required. Technical questions expected. |
| Finland | Board for Gene Technology. | Parallel, or in advance of CTA | CU or DR, depending on replicative capacity and containment measures | Applications in English may be acceptable in some cases. Public consultation when DR (60 days). |
| France | Ministry of Research (MESRI) for CU: GMO classification / CU agreement per clinical site. Ministry of Environment (MTES) for DR. High Council of Biotechnology (HCB) for opinion and assessment involved. | Parallel, or in advance of CTA | CU or DR (DR upon determination by HCB) | Class 1 is notification only. Via HCB for DR determination, If DR applies, an additional submission is required to MTES. Public consultation of 15-30 days may apply, in French. Electronic submission via DUO portal. In French#. |
| Germany | Paul-Ehrlich-Institut (PEI) with consultation of Federal Office of Consumer Protection and Food Safety (BVL), plus local GMO/Federal office of consumer protection and food safety. | Single submission via PEI in parallel, or separately in advance of CTA | DR | Single application includes CTA and GMO data. DR limits the GMO storage time to 6 months at site: any longer requires application for CU. |
| Ireland | Environmental Protection Agency. | Parallel to CTA | DR | 28-day public notice. Agency decision 14 days after notice period#. |
| Italy | Ministry of Health (ISS) for CU Ministry of Environment for DR. | Parallel, or in advance of CTA | CU or DR | CU: form specific by class (1–4) Complex public consultation in case of DR (30 days), in Italian. Application in Italian#. |

Table modified from Westra-de Vlieger *et al.* 2019 [17]. For recent industry timelines, please see Supplemental materials to 2021 *Human Gene Therapy* article [4].
*CU often required clinical site-specific notifications and/or submission to authorities.
**ERA requirements plus a dossier in the summary notification information format (SNIF) for publication on the EU register.
#Technical documents in English are acceptable.


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**TABLE 1**

**Regulatory GMO requirements in selected EU Member States.**

| EU member state | GMO competent authority | Submission | Contained use*/deliberate release** | Document and data requirements |
|---|---|---|---|---|
| Netherlands | Gene Therapy Office as single point of contact. Ministry of infrastructure, Environment and Water management (IenW) for permit application. | Parallel, or in advance of CTA | DR | Pre-submission meeting possible IenW require 120-day assessment for environmental risk for a GMO of unknown risk. For known risk, 56-day assessment. 28 days for "copy permits" across multiple clinical sites for a previously approved GMO. |
| Poland | Ministry of the Environment – GMO unit. | GMO authorisation required prior to CTA | CU | Public consolation required (30 days via GMO register); laymen summary of technical information required in dossier. Application forms in Polish#. |
| Spain | Consejo Interministerial de OMG (CIOMG) and Comision Nacional de Bioseguridad (CNB). | Parallel, or in advance of CTA | DR | Questions focus on local site operations. GMO review and approval times many be extended if public consultation is sought. Application forms in Spanish#. |
| Sweden | Medical Products Agency (MPA). | Single submission, via MPA in parallel or separately in advance of CTA | DR | Single application to MPA for CTA and GMO aspects. SNIF and ERA required. Short, non-confidential, summary posted for public consultation via MPA website (30-day within 90-day assessment period). |

Table modified from Westra-de Vlieger *et al*. 2019 [17]. For recent industry timelines, please see Supplemental materials to 2021 *Human Gene Therapy* article [4].
*CU often required clinical site-specific notifications and/or submission to authorities.
**ERA requirements plus a dossier in the summary notification information format (SNIF) for publication on the EU register.
#Technical documents in English are acceptable.

GMO assessment does not cause any undue delay.

## TRANSITION PERIOD

As detailed in the European Commission draft Clinical Trials Regulation Q&A document (version 4 from July 2021) [18] upon the implementation of the Clinical Trials Regulation, there will be a 1-year transition period, during which sponsors will be able to selectively use the procedure that currently exists under the Clinical Trials Directive, submitting each clinical trial and GMO application to each national authority, without being required to the use the CTIS portal. During the two subsequent years (leading to the end of January 2025 per the current timeline) it will be possible for ongoing trials to remain under the Clinical Trials Directive framework, but all new clinical trial applications will have to be submitted under the harmonized framework of the CTR, via CTIS.

As a consequence, it will be difficult for a sponsor to leverage the advantage of the single dossier submission under the CTR, to initiate a clinical trial in Europe for an IMP with a GMO component. Moreover, the question of how these procedures would fit with the procedure and timeframe of the CTR is raised. That is, if the timings of both CTA and GMO assessments are not aligned, and a single simultaneous opinion cannot be provided, this can lead to a delay in the commencement of the clinical trial.

Implementation of a GMO exemption scheme before the end of the first year of the transition period for the CTR (the end

of January 2023) is important to avoid new CTA submissions for ATMPs under the CTR having to conduct the whole GMO assessment process in parallel. The same consideration also applies to vaccines.

## IT IS IMPORTANT FOR EACH MEMBER STATE TO BE PREPARED FOR SUBMISSION OF NATIONAL GMO APPLICATIONS WHEN THE CTR IS IMPLEMENTED

It is important that each Member State prepares for the CTR implementation and to consider how the GMO application (that may include an ERA) will fit into the coordinated review of the clinical trial and its timelines.

Some countries have already adapted, or are in the process of adapting, their legislation to have a coordinated submission procedure to obtain an authorization under both GMO and clinical trial legislations in a timely manner, according to the latest intelligence, as reported for instance for Germany (see below for national case studies).

## 1. Understanding national readiness in view of CTR implementation

Each year since 2014, EFPIA has conducted a survey, the Clinical Trials Implementation (CTi) Monitor, to assess the national readiness for the implementation of the EU CTR. The latest survey was undertaken in November 2020 and was completed by the Pharmaceutical Industry National Trade Associations of 23 countries.

The executive summary, as published on the 6th of May 2021 [14] reads as follows:

*"The European Clinical Trials framework will undergo a major change when the Clinical Trials Regulation 536/2014 [19] comes into application towards the end of January 2022. The legislation becomes a regulation, rather than a directive, which will ensure key aspects have identical rules throughout the EU.*

*EFPIA consider the implementation of the Clinical Trials Regulation to be an opportunity to demonstrate Europe's commitment to clinical innovation, scientific collaboration, and transparency of clinical trials information.*

*In order to meet the essential elements for successful implementation of the Regulation and reaching its objectives, EFPIA has identified three key and distinct requirements as follows:*

- *To deliver flexible, efficient, and streamlined execution of the authorization procedure to avoid administrative delays.*
- *To enable the required collaboration between concerned Member States, in addition to sponsors.*
- *To appropriately manage the transparency of data beyond the active phase of the clinical trial.*

*In collaboration with EFPIA's national trade association members, EFPIA are monitoring the preparation of the Regulation on the national level through our comprehensive National Trade Association Clinical Trials Implementation Monitor survey (CTi Monitor survey)."*

In the 2020 edition of the CTi Monitor Survey, five specific questions were added to better understand the potential adaptation of the national legislation, as needed in context of GMO procedure requirements and the timeline for implementation of the CTR.

While the survey showed that four countries have already adapted their legislation with regard to the GMO procedure (17% of the 23 responding national trade associations) it also underlines that the majority of national trade associations had indicated that they *"don't know"* if their country is planning to change its GMO legislation. In addition, no clear picture emerged from the response about the use of the Common Application forms for GMO applications. The use of the relevant common application forms would have indicated further harmonization of the


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data requirements in the Member State. It is unclear whether these forms would be used and be part of the clinical trial application, or whether they would be submitted separately in addition to the clinical trial application; or, whether local GMO application forms would still be requested by some GMO competent authorities (often duplicating the content provided in the common application form).

As part of the 2020 CTi Monitor Survey, Pharmaceutical Industry National Trade Associations were asked the question: *"Will the European Commission Common Application Forms for GMOs be part of the submission package (part II) for clinical trials under CTR?"*. Figure 1 illustrates the intended use of the relevant common application form, as part of the national GMO application as provided by the national trade associations.

Under the CTR, national health and GMO competent authorities need to be aware of the upcoming challenges related to the interplay between the clinical trial and the GMO legislative framework, with regard to procedures and data requirements for IMPs with a GMO component. Each Member State needs to ensure that a pragmatic and simple approach is in place to facilitate the GMO assessment and its coordination with the clinical trial authorization.

## 2. Member States' case studies (representative of similar challenges encountered across the European Member States)

### Austria

Currently under the Clinical Trial Directive, where there are two separate, parallel processes.

Clinical trials with an IMP with a GMO component currently have to be submitted to both the Competent Authorities for medicines and the Ministry of Health preferably simultaneously. The Common Application Form for Clinical research shall be used. Plans are foreseen to evaluate potential adaptation of the system towards a simplified process. For example, a combined approval of the clinical trial and GMO release authorization. As part of the GMO release authorization process the consultation (review) period could be reduced or, may no longer need to be performed. This outcome from the legislative process is still pending.

### Belgium

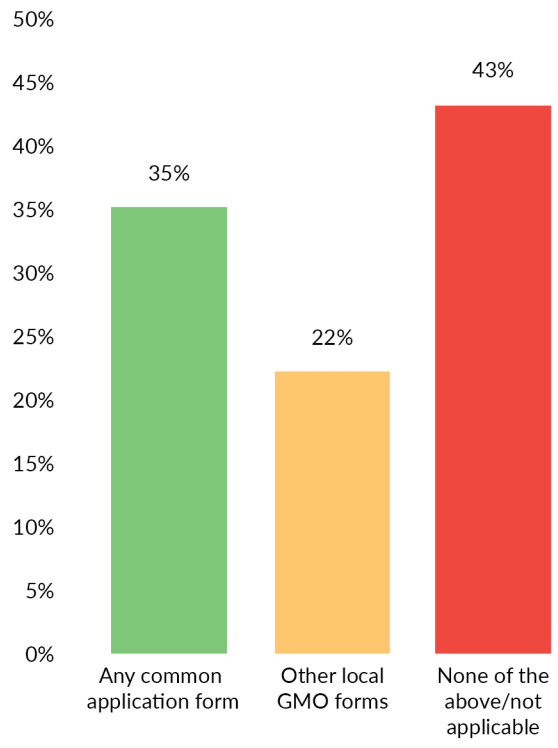
An adaptation of the current regulatory framework for clinical trials with GMOs should be foreseen to streamline and simplify the process under the CTR.

In Belgium, no adaptation of the existing legislation has been undertaken as yet, but the issue has been raised by the pharma industry trade association pharma.be.

Currently, under the Clinical Trial Directive, clinical trials with GMOs can fall under the Contained Use procedure and/or the Deliberate Release procedure.

**FIGURE 1**

**EFPIA CTi Monitor Survey: use of EU Common GMO Application Forms.**



The Directive 2001/18/EC (transposed into Belgian law by the Royal Decree of 21st February 2005) applies to the Deliberate Release of GMOs and requires that an environmental risk assessment (ERA) should be carried out before release. The GMO dossier together with the clinical trial application is submitted to the national competent authority, the Federal Agency for Medicines and Health Products (FAMHP), which transfers the GMO dossier to the relevant advisory body, the Biosafety Advisory Council (BAC). This GMO dossier is then evaluated by the BAC, who thereby transmits their opinion to the FAHMP for a final decision.

The Contained Use procedure is implemented at regional level across Belgium (Brussels, Flanders, Wallonia). Each region has their own independent procedure, timeline and documentation in their regional language. A notification/authorization for the Contained Use (of the GMO-IMP) needs to be sent/obtained for each new study.

Most clinical trials under the Deliberate Release procedure will also necessitate the submission of a biosafety dossier according to the Contained Use procedure and the submission of a biosafety dossier according to the Deliberate Release procedure in addition to the submission of a CTA dossier to the ethics committee and the FAMHP **(Figure 2) [20]**.

The EC common application forms are all currently implemented by the GMO competent authority in Belgium.

The procedure for the evaluation of these clinical trials with GMOs is complex and lengthy. Moreover, the environmental assessment embedded in the clinical trial evaluation procedure in case of a deliberated use of GMOs will, as such, not fit in the evaluation procedure foreseen under the CTR. For instance, it is unclear whether the documentation will be part of the CTA, as part I or part II, or how the different timeframes for the clinical trial and the GMO evaluation will be aligned.

Therefore, it is necessary to streamline and simplify this regulatory framework for clinical trials with GMOs and to foresee its integration in the evaluation process according to the EU CTR and the Belgian *Law of 7 May 2017* implementing the CTR with adequate reduced timelines.

## France

There is upcoming reform of GMO supervising authorities that include the Contained Use assessment of GMO medicinal products. Common Application Forms are currently implemented by the GMO competent authority in France.

The Haut Conseil des Biotechnologies (HCB) is the current independent authority in charge of reviewing applications for use of all types of GMOs (GMO plants and seeds, GMO animals and GMO medicinal products) that are submitted to public authorities by research institutions or sponsors. The HCB can also be consulted on ethical, economic and social aspects relating to GMOs.

Presently, under the Clinical Trial Directive, in case of a clinical trial application for a GMO-IMP in France, a submission for a Contained Use assessment is required in parallel of submissions to the French health authority (Agence Nationale de Securité du Médicament, ANSM) and the ethics committee. The Contained Use application for the GMO-IMP is sent to the Ministry of Research via an online portal by the clinical site(s). Either the EC common application form(s) or national forms are currently accepted for such applications. The Ministry of Research then refers to the HCB for GMO classification and for a permit for the clinical site(s). During its Contained Use assessment, the HCB mentions in its decision if there is a potential risk for the deliberate release of the GMO-IMP.

A reform of GMO supervising authorities including GMO-IMP Contained Use assessment is currently under discussion for implementation in early 2022.

## Germany

A future joint process for the clinical trial and GMO under the CTR; with an additional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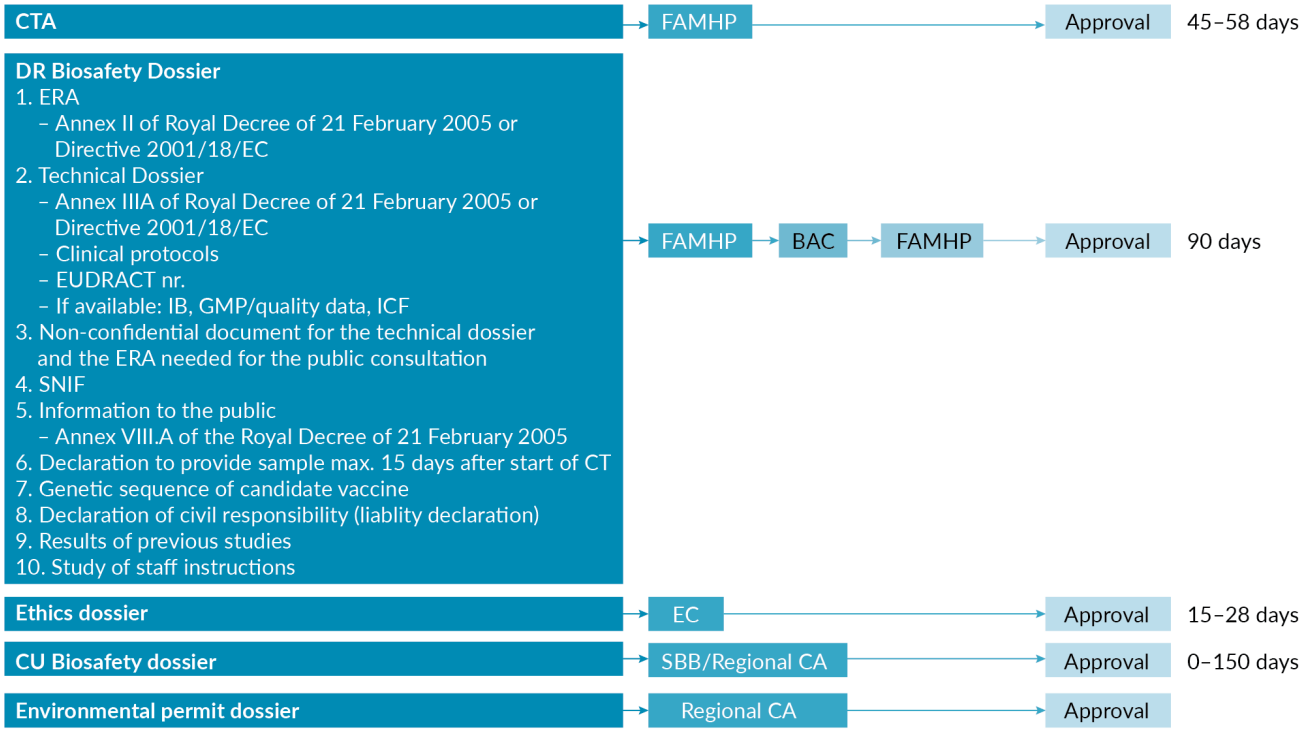
## FIGURE 2

**Overview of the regulatory procedure in Belgium for a clinical trial with a GMO classified as deliberate release.**




step to provide information specific to the GMO component.

The upcoming version of the German Drug Law (AMG) that will apply after implementation of the CTR, stipulates that the clinical trial authorization will also include GMO release authorization in the future. A release authorization, as determined by an assessment of the ERA, will also to be placed in the authorization via the CTIS to the sponsor in the future. Therefore, in Germany it is intended to be one process: the CTA via the CTIS, with authorization to include that for release of the GMO-IMP. An authorization from local federal state authorities will not be needed in the future.

The national trade association in Germany, the Verband Forschender Arzneimittelhersteller (VFA) underlines that there is a problem with the submission of the GMO data via the CTIS. The EC common application forms for GMOs will not be part of the submission package (neither Part I nor Part II) for the CTA, via the CTIS. For Germany, an inclusion in Part II of the CTA dossier would result in regulatory problems, since the upcoming version of the AMG foresees that Part II is solely under the responsibility of the ethics committee. To resolve this CTIS issue, when a sponsor comes to apply for a clinical trial for a GMO-IMP, via the CTIS, the applicant will need to send a parallel application regarding the GMO aspects (based on the EC common application forms) via the Common European Submission Portal (CESP) to the GMO national competent authority. The deadlines are in parallel and the assessment by the national competent authorities will be undertaken jointly/in parallel. Objections and responses to GMO submissions must, again, be communicated separately, via the CESP, since these aspects are not included in the CTIS.

In Germany, there will be a clear procedure under the CTR to approve both CTA and GMO applications. An additional step will be needed to provide the information on the GMO aspects of the IMP. There could be

some challenges with regard to timelines for both assessments. When Germany is acting as the *Reporting* member state under the CTR, coordinating the procedure for the evaluation of the clinical trial, it can more easily control the timeline for the evaluation process, to alignment with their own national GMO procedures and requirements. When Germany is acting as a *Concerned* Member State, there will be the challenge to also include the full German GMO process within the overall EU assessment, since the timelines for the *Concerned* Member State are much shorter, in accordance with the CTR. It will be critical that the applicant ensures that the application is submitted in parallel (via the CTIS and GMO-related via the CESP) and that responses to GMO questions can be provided expediently.

It should be noted that the inclusion of the EC common application form(s) for GMOs in the CTIS was suggested by different stakeholders. During the consultations on CTR EU 536/2014, as well as during the "programming" of the CTIS, different EU Member States and national competent authorities, including the Paul-Ehrlich-Institute for Germany, had repeatedly proposed that that the CESP should be opened to allow for submission of GMO applications to allow for the possibility of a synchronized approval of the CTA with authorized release of the GMO-IMP. These discussions are ongoing.

### Spain

Currently under the Clinical Trial Directive, there are two separate and independent procedures: one for the CTA and another for evaluation of the GMO-IMP.

The Spanish law on GMOs (Law 9/2003 of 25th April, establishing the legal framework for the Contained Use, voluntary release and commercialization of GMOs and Royal Decree 178/2004, of 30 January, approving the General Regulations for the Development and Implementation of Law 9/2004, of 25 April, which establishes the legal basis for the Contained Use, voluntary release, and commercialization of GMOs) implements the EU Directives on GMOs. No further adaptation is foreseen.

The GMO topic is addressed by a different competent authority (Ministerio para la Transición Ecológica y el Reto Demográfico) than the Spanish Pharmaceutical Authority (AEMPS) which assesses clinical trial applications, although AEMPS is represented in GMO assessment discussions. In Spain, since the CTA and GMO assessment procedures, including decision for approval, are separate and independent from each other, they can be performed in parallel, without issue in terms of timeline compatibility.

Common Application Forms are currently used by the GMO competent authority in Spain, as are the corresponding Good Practice documents on the assessment of GMO-related aspects in the context of clinical trials with for AAV clinical vectors and human cells genetically modified by means of viral vectors [21].

## CONCLUSION

The initiation of a clinical trial with a GMO-containing IMP is currently a lengthy and complex process in Europe due to the fragmentation of the GMO requirements at the national level. Moreover, the different risk classifications and the different national procedures and requirements for the GMO assessment pose a challenge to align with the new coordinated evaluation of a CTA under the CTR. It will therefore be difficult for the sponsor of a clinical trial to leverage the advantage of the single dossier submission under the CTR to initiate a clinical trial in Europe in case of an IMP with a GMO component. Due to challenges described in this document and to the lack of clarity to sponsors, Europe appears less attractive to host clinical trials with GMO-containing IMPs than the United States, where ordinarily a *"categorical exclusion"* exists for gene therapies, vectored vaccines, and related recombinant viral or microbial products.

EFPIA, EuropaBio and ARM have recently called upon the European Commission and the national competent authorities to exempt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ATMPs and vaccines containing or consisting of GMOs from the GMO legislation [4]. EFPIA (and Vaccines Europe) reiterate their support to this *best-case scenario*, for both ATMPs and vaccines. An exemption from GMO requirements could make Europe a more attractive region for clinical development of, for instance, gene therapies and could accelerate European patients' access to these potentially life-saving medicines and vaccines.

In the meantime, concerns remain how all these different procedures for the GMO assessment will fit with new procedures and timeframes established under the CTR. There is a need to ensure that for each Member State a practical and efficient system is in place to allow for the GMO assessment of the IMP and its coordination with the clinical trial authorization at the time of the implementation of the CTR.

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*With regard to this advocacy, the author is sitting astride joint trade association efforts (with frequent meetings) and welcomes input from the readers of CGTI to the issues highlighted in the article.*

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CELL & GENE THERAPY INSIGHTS

## Regulatory Insights

COMMENTARY/OPINION

# The UK's emerging regulatory framework for point-of-care manufacture: insights from a workshop on advanced therapies

Edison Bicudo, Irina Brass, Penny Carmichael & Suzanne Farid

Point-of-care (POC) manufacture can be defined as the production of therapies in clinical settings or units close to hospitals and patients. This approach is becoming increasingly viable due to the emergence of flexible manufacturing technologies. Expecting an increase in this kind of production, the UK's regulatory agency, the Medicines and Healthcare products Regulatory Agency (MHRA) is proposing a regulatory framework specifically designed for POC manufacture. To discuss the challenges of POC manufacture and the MHRA's proposal, the EPSRC Future Targeted Healthcare Manufacturing Hub (FTHMH) organized a workshop drawing insights from specialists in cell and gene therapy manufacture. Through presentations and discussion roundtables, the workshop highlighted the challenges for the UK and other countries implementing POC manufacture. The workshop attendees stressed four main issues: quality control; standardization and equipment use; availability of qualified personnel; and the challenges to be met by hospitals participating in POC manufacture systems. This commentary provides a summary of the points discussed in this workshop.

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## CONTEXT: MANUFACTURING ADVANCED BIOTHERAPEUTICS

Academic institutions and companies have for some years developed personalized cell and gene therapies [1,2], also called Advanced Therapy Medicinal Products (ATMPs), such as CAR-T cell therapies. At the same time, new manufacturing equipment has been developed to optimize ATMP production [3–5]. It is sometimes claimed that these trends enhance the prospect of bringing manufacture close to the patient, but it is also recognized that several operational, regulatory, public policy, and healthcare challenges remain for this to become routinely used.

Traditionally, drugs and therapies have been produced in a centralized manner, with a small number of large manufacturing units strategically positioned across the globe [6]. With newer, flexible, and automated equipment, it becomes increasingly possible to achieve decentralization that could lead to a larger number of manufacturing units located close to hospitals [4]. In some cases, it might be possible to perform manufacturing activities within the hospital, constituting so-called point-of-care (POC) manufacture. In some countries, ATMPs have been manufactured at POC, which has involved, for the most part, production of therapies to be tested in clinical trials. However, there is already a small group of hospital-based clinicians/researchers producing biotherapeutics for routine clinical procedures in dedicated Good Manufacturing Practice (GMP) manufacturing facilities. For example, mesenchymal stem cells have been manufactured at the Mater Hospital Brisbane (Australia) for the treatment of acute myeloid leukemia [7]; and red blood cells have been produced by NHS Blood and Transplant (UK) for treating cardiac illnesses [8]. In some cases, such production involves the use of miniaturized manufacturing systems (so-called GMP-in-a-box) which can sometimes be transferred from hospital to hospital.

ATMPs have short shelf lives when delivered fresh, that is, they have to be administered to the patient promptly after their manufacturing process has been completed. This is a major reason why hospitals, health services, and some companies are becoming interested in bedside manufacture.

Since freshly delivered ATMPs have these particular characteristics, it has been challenging to formulate and implement regulations to frame their development and production [2,9]. The decentralization of production, the emergence of new manufacturing platforms, and particularly the conduct of POC manufacture in a large number of hospitals, bring about additional regulatory challenges, as regulatory agencies need to learn to ensure the continued safety, efficacy, and quality of medicinal products manufactured outside centralized facilities.

These regulatory demands have been felt in many countries, particularly those willing to offer a dynamic landscape for the development of ATMPs, like the UK [10]. The Medicines and Healthcare products Regulatory Agency (MHRA), the UK's regulatory agency, has paid much attention to these trends. For example, in a recent partnership with the National Institute for Health and Care Excellence (NICE) and the Scottish Medicines Consortium (SMC), the MHRA launched the Innovative Licensing and Access Pathway (ILAP) [11], a new regulatory route aimed to speed up the marketing authorization of chemical entities, biological medicines, and repurposed medicines.

POC manufacture has also been on the MHRA's radar. In a recent application submitted to the agency, the applicant plans to manufacture a blood product at POC, in a system that will involve over 200 hospitals. To deal with this application, and similar ones, a new approach has to be created for managing risks, ensuring quality control, and tracking liabilities. For this reason, the MHRA is now proposing a new regulatory framework specifically designed for POC manufacture. This is done in the framework of the 2021 Medical Devices Act, which allows for regulatory divergence between the European Union and the UK after the UK departure from the Union.

To develop the POC regulatory proposal, the MHRA initially consulted specialists in medicines manufacture, by means of three


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workshops held in 2020–2021. It was then possible to identify the main issues to be dealt with in POC manufacture systems [12]:

- Frequent production of autologous therapies, which are custom-made therapies whose starting material is constituted by samples taken directly from the patient;
- Production of small batches; frequently, a batch is formed of only one product for a particular patient;
- The shelf life of products (that is, the period for which products remain active and safe) are either short, amounting to seconds in some cases; in this way, it is not viable to have the manufacturing units located far away from the patient;
- The manufacturing system can involve several units, hospitals or even mobile units such as adapted vehicles; this circumstance poses considerable challenges in terms of inspection of manufacturing sites; and
- Depending on the skills and the infrastructure that is available, different units of the same manufacturing system might need to employ different techniques, devices, or software packages.

It was noted, therefore, that current regulatory schemes that might be used in POC manufacture (such as the Specials scheme [13], which is applicable to unlicensed therapies produced for special clinical needs) are not completely suitable for large POC manufacturing systems. The MHRA has then designed a new regulatory framework which was initially presented in an online workshop in March 2021. After receiving feedback from the specialists who attended this event, the MHRA released, in August 2021, a public consultation.

This Commentary aims to provide an overview of the MHRA's framework regulatory proposal and, subsequently, summarize the discussions that took place in an online workshop organized by the Future Targeted Healthcare Manufacture Hub (FTHMH) [14] hosted at University College London. Held on 29 June 2021, with talks by Ian Rees (MHRA), Dr Qasim Rafiq (FTHMH), and Laura Sands (Lonza), as well as several breakout sessions, our workshop provided a space for the expression of insights and concerns about POC manufacture and the MHRA's regulatory framework proposal from the viewpoint of ATMP manufacture, delivery, and administration.

## THE MHRA'S REGULATORY FRAMEWORK PROPOSAL

Given the issues described above, the MHRA aims "[…] to establish a proportionate regulatory framework that supports the safe development of medicines which need to be manufactured and supplied in close proximity to patients or new supply chains that enhance patient access" [12].

It is important to note that the emerging regulatory framework does not focus on any particular class of products and can therefore impact on "[…] a wide range of POC product types including blood products, medical gas products, Advanced Therapy Medicinal Products (ATMPs) and small molecule products" [12]. In order to enable the formation of manufacturing systems that may comprise a large number of sites while making sure that quality control is always in place, the MHRA proposes the concept of Control Site. This will be the institution responsible for establishing and overseeing the POC manufacturing process, with responsibilities that will include: staff training, quality control, provision of manufacturing equipment, adverse event reporting, auditing of manufacturing sites, and others. The Control Site will take the product from the development phase to the market, which will comprise securing a clinical trial authorization, conducting the clinical trial, and obtaining a marketing authorization.

The Control Site will oversee the Manufacturing Sites, which will be, for the most part, NHS hospitals and, in some cases, manufacturing units located close to a hospital or a mobile manufacturing unit installed, for example, in an adapted vehicle. As Manufacturing Sites may be in large numbers, one of the main challenges is to ensure consistent quality across the whole production system. Precise and timely communication will then need to be in place between the Control Site and Manufacturing Sites, including for the notification of adverse events, by means of a reporting system to be created by the Control Site. When the product involves high risks, such communication may need to happen in real time.

All the information pertaining to the manufacturing system will be stored in a POC Manufacture Master File. This kind of document has been used in other fields, such as for plasma and vaccine production. In POC manufacture, the Master File will be more flexible, as its contents and the frequency of its update will depend on the product's nature and associated risks. The POC Master File will contain information about: GMP inspections; staff; adverse events; batches; patients receiving the product; participating sites, among other items.

In this manner, the regulatory proposal introduces a layered system where the Control Site figures as an entity mediating between the MHRA and the sites, as illustrated in Figure 1 [15].

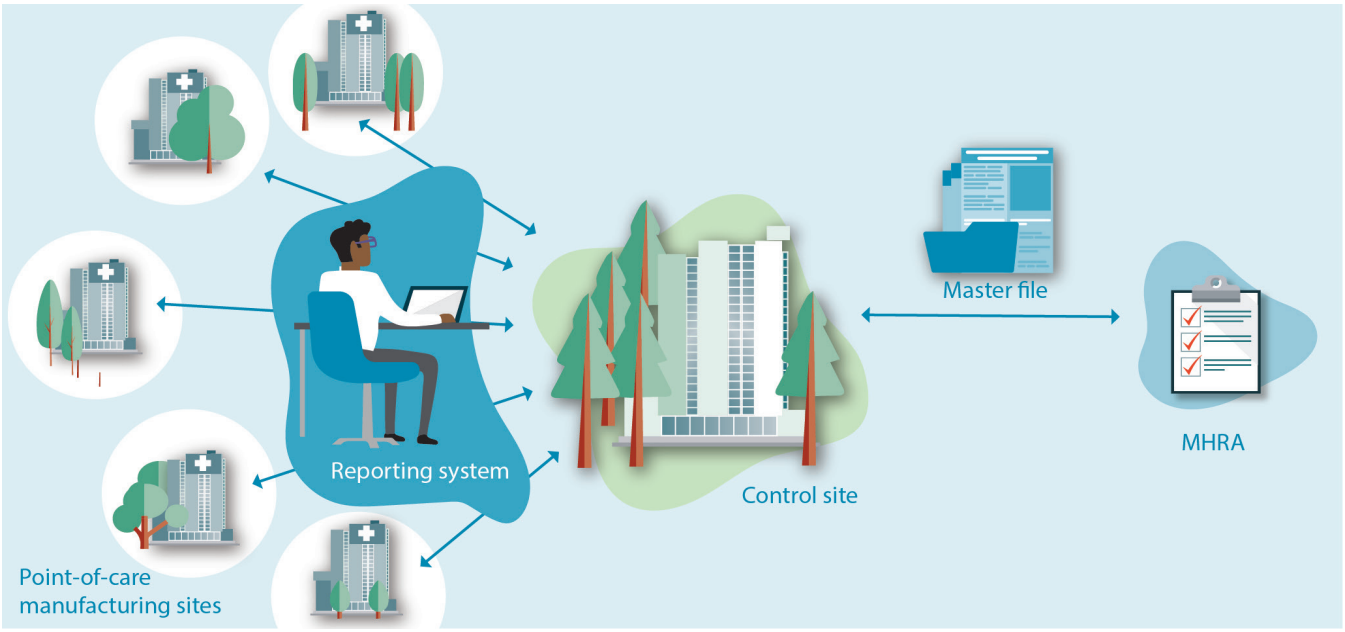
Due to the variety of products to be covered by the emerging regulation, as well as the diversity of players that may be willing to manufacture products at POC, it is crucial that the framework be proportionate, flexible, and able to accommodate various manufacturing systems, with either a small or large number of Manufacturing Sites. Thus the MHRA will be open to adjust some aspects, such as the contents of the POC Master File and the frequency of Manufacturing Sites inspections.

## THE WORKSHOP ON POC MANUFACTURE

The Future Targeted Healthcare Manufacture Hub (FTHMH) held an online workshop

**FIGURE 1**

**A schematization of the MHRA's regulatory proposal.**




Created with BioRender. Source: [15].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on 29 June 2021 with two goals: to discuss the current scientific, technical, institutional, and regulatory challenges of POC Manufacture; and to collect feedback on the MHRA's regulatory framework proposal, based on the example of ATMP manufacture, delivery, and administration. The workshop convened 32 specialists in the field of biotherapeutics, manufacturing technologies, and regulation, as summarized in Figure 2.

As an initial activity of the workshop, participants were polled on what they considered to be the most challenging aspect of POC manufacture to be tackled in the next years. The responses we received are summarized in Figure 3.

According to these responses, the main challenge to be dealt with is "Equipment and infrastructure" (40% of participants), followed by "Qualified personnel" (35%). This is reflected in the comments that participants made about the MHRA's proposal, as explained below. It is interesting to note that 20% of participants selected "Regulation," in line with the MHRA's diagnostic that a new regulatory framework is needed to foster POC manufacture at this moment.

Three keynote presentations were given in the workshop. Ian Rees (MHRA) presented the agency's POC regulatory framework proposal, its concepts, guiding principles, and implementation structure. Dr Qasim Rafiq (FTHMH) addressed technical and scientific issues in the decentralization of advanced biotherapeutics manufacture, including POC manufacture more specifically, and emphasizing the growing relevance of highly automated production systems. Subsequently, Laura Sands (Lonza) described the company's approach to technology development for manufacture decentralization, highlighting the potentialities of the Cocoon© system and its possible use for cell therapy manufacture in clinical settings. Presentations were followed by discussion roundtables. The sections below provide a summary of the main points and discussions made in our workshop, focusing mainly on participants' views about the MHRA's POC regulatory framework proposal.

## SUMMARY OF WORKSHOP DISCUSSIONS

Generally, workshop attendees expressed a positive view about the MHRA's POC regulatory proposal. The concept of Control Site and the flexibility of the framework (that is, the willingness to adjust the regulatory oversight to different kinds of products and manufacturing systems) were particularly welcome by participants. However, they also raised several issues to be further considered by the regulator. Below we highlight the issues that emerged the most frequently in the

**FIGURE 2**

**Affiliation of workshop attendees.**

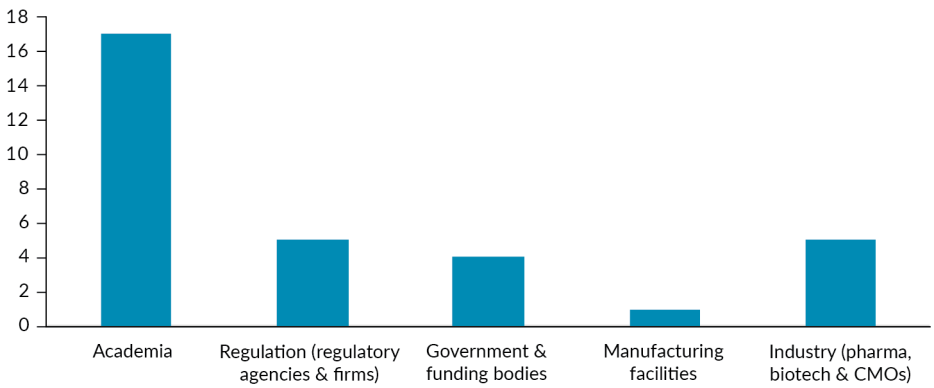
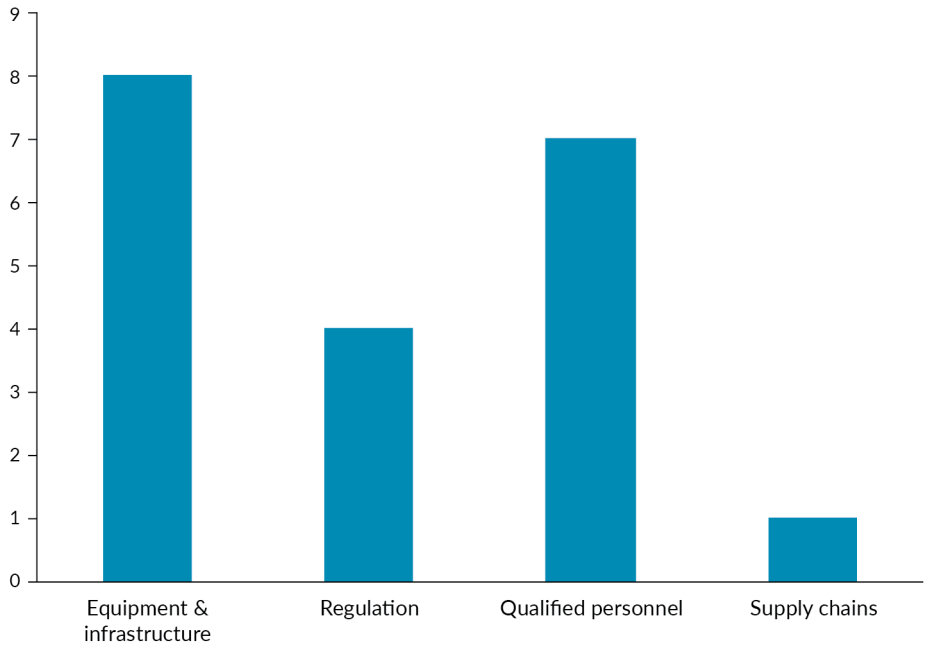


**FIGURE 3**

Survey responses on hardest challenges of POC manufacture.




breakout sessions, pointing to some aspects that deserve further refinement and consideration. In order to outline all the discussions in a systematic way, this section focuses on four main themes as they were addressed in the workshop: quality control; equipment and standardization; human resources and training; and implementation in hospital sites.

## Quality control

When production is centralized and involves few manufacturing units, it is relatively simple to perform equivalent control measures. However, this practice may be compromised in a more decentralized system, and even more so if large numbers of manufacturing sites are employed. The main challenge is that small procedural changes may be implemented at each site, either consciously or unconsciously.

Workshop participants stressed, for example, the issue of software updates. If different Manufacturing Sites implement updates independently, minimal variations will be introduced in manufacturing systems that are likely to rely on software support for a growing range of tasks. To minimize the chance of discrepancies, it will be crucial to have data integrity approaches, automated software updates, and continuous monitoring.

Another challenge in terms of quality control is the broad range of materials to be handled and processed in POC manufacture. This makes it difficult to decentralize all aspects of quality control. Some workshop attendees pointed out that some Control Sites may prefer to test and approve some materials in a centralized fashion, subsequently releasing them for use in clinical settings.

Participants also expressed doubts about the role to be played by Qualified Persons (QPs), who are professionals responsible for assuring the quality of medicines and certifying batches. MHRA representatives confirmed that the framework also covers offsite qualified person (QP) release, whereby the QP monitors the manufacturing process without having to be physically present at the site. This type of monitoring will only be viable with highly automated manufacturing systems and, ideally, real-time monitoring,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but it will be necessary to wait until such technologies are available.

Lastly, accurate quality control will depend on the features of the Master File. Several workshop participants noted that it is important to have a clearer idea of the nature and organization of this document. The MHRA explains that the File's information can be used along the path leading to product registration and marketing authorization. It will then be important to have timely guidance about such process.

## Equipment & standardization

In the UK, some initiatives have supported the development and implementation of ATMP manufacturing technologies, as is the case of the Advanced Therapy Treatment Centres network [16]. It is not certain, however, that such technologies will be evenly diffused across regions and hospitals. Variability between Manufacturing Sites can then become a key challenge of POC manufacture. Indeed, hospitals have different infrastructures and staff with varied experience in ATMP production. and quality control. It is known that some NHS Trusts have devoted themselves to therapy manufacture, including some with experience in the production of cell therapies. At the same time, there are Trusts whose pharmacies have much more modest manufacturing skills and capabilities. This variability can be solved, or at least minimized, by means of closed, automated systems, in such a way that mobile manufacturing units (GMP-in-a-box) are taken to hospitals whenever certain products are necessary. If the newest technologies are mobilized, it can be possible to manufacture therapies at POC with little need for manipulation of materials and products and, consequently, little need for having highly trained staff.

According to workshop participants, then, some risks of POC manufacture can be reduced with technological solutions. In our event, examples were provided of such solutions. According to Laura Sands (Lonza), the Cocoon© system for cell manufacture is flexible enough to be used in various settings, including clinical ones. And Dr Qasim Rafiq (FTHMH) presented Autostem, a project led by the National University of Ireland Galway and aimed to develop an automated system for stem cell manufacture. Even if such a system is used in regional manufacturing hubs, and not exactly at POC, it can enable a precise production with less occurrences of human errors.

Another key issue is the level of investment needed for setting up those manufacturing systems. With devices incorporating cutting-edge technologies, and with small numbers of providers on the market, automated manufacturing platforms can be costly. In addition, technologies will be necessary for implementing data integrity systems and monitoring site variability, as mentioned above. The financial schemes that will enable the deployment of such technologies to hospitals are not yet clear. Furthermore, some POC manufacture systems may involve a large number of sites, compounding these financial challenges.

Therefore, the workshop discussions are in line with considerations that had been voiced before [17], namely: the feasibility of POC manufacture will depend on the number of sites and the reimbursement models agreed upon with hospitals.

## Qualified personnel

Even with highly automated systems in place, it will be necessary to rely on a range of professionals. For example, QPs will be crucial players for assuring quality control, as explained above. In addition, other professionals must be mobilized for tasks such as: materials handling; pre-process checks; manufacturing device operation; documentation and regulatory compliance; and coordination of the different players and departments involved.

Training of such personnel was stressed as a key enabler for POC manufacture by

workshop participants. The MHRA's regulatory framework proposal includes training as one of the responsibilities of the Control Site. It might be relatively easy to devise training schemes for staff related to the Control Site. However, if hospital staff are to participate in manufacturing activities, then those individuals will need to undergo training as well. To be sure, the Control Site can design training programs to be rolled out across various Manufacturing Sites. However, this brings about the challenge that was pointed out above for quality control: how to make sure that such training is implemented in a standardized way, with no significant variations between different sites. These issues are particularly relevant when one considers that manufacturing technologies are likely to evolve constantly and rapidly in the years to come, which will require frequent re-training of the involved personnel.

If participation of clinical staff in POC manufacture is at least considerable, then it is important to understand what the incentives for such participation will be. Hospital employees are frequently very busy with their routine medical and administrative tasks. Some workshop participants pointed out that in these conditions, additional training and manufacturing duties may end up being of little interest. They also argued that when the product at stake is not manufactured frequently, then there may be little incentive to acquire these new skills. This is particularly problematic for ATMPs, as some of them will target rare diseases with small, or very small, patient populations. Therefore, the issues of mobilization of staff, training, and workforce maintenance become pressing questions, as they can have decisive impacts on the final product's quality.

## Hospitals

For hospitals, it may be too challenging to follow all the guidelines involved in therapy production, especially the very strict GMP standards complied with by the industry. For some classes of products, including ATMPs, sophisticated processes will be necessary, such as the work with complex materials and the management of large supply chains which will frequently have an international scope. In clinical and academic settings, this expertise is often lacking [18,19], and it is not sure that it can be implemented without much initial effort.

As explained before, some ATMPS will target small patient populations. This may be a deterrent to some hospital administrators unable or unwilling to reserve resources, physical space, and staff for activities who do not seem beneficial to many patients. In some cases, then, it can be more useful to concentrate certain manufacturing activities in some strategically selected hospitals, which will produce for a relatively larger number of patients. This solution may also help solve the training difficulties described above.

The financial aspect of POC manufacture was also discussed by workshop participants. Hospitals may be asked to reimburse the sponsor company (or drug developer) for manufacturing activities happening on their premises. Alternatively, the hospital may receive part of the therapy's reimbursement, as is done in the MHRA's Specials scheme for unlicensed medicines. For companies, manufacturing schemes can prove less attractive if the hospital receives a significant portion of the reimbursement.

Finally, an issue that is likely to become crucial is distribution of liabilities between POC Manufacturing Sites and the Control Site. As noted above, there may be deviations from the original manufacturing protocol, bringing about additional and unforeseen risks. It will then be essential to receive specific guidance from the MHRA, so it is possible to know who is technically and legally responsible in manufacturing processes and systems that can reach high degrees of complexity.

Given that hospitals will play a crucial role as Manufacturing Sites, these issues need to be attended to. Otherwise, it is not guaranteed that the regulatory proposal will act as an enabler for POC manufacture as it is intended to do.


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## THE FUTURE OF POC MANUFACTURE IN THE UK

It has been claimed that current technological trends require "[…] smarter, more adaptive regulatory systems […] that are more proportionate to the levels of risk embedded in new technologies" [20]. Equally, it has been noted that emerging therapeutic approaches, such as cell and gene therapies, require "pioneering regulatory development" [21]. The MHRA's POC regulatory framework proposal seems to be in line with these considerations, as it brings about a regulatory approach aimed to be innovative, flexible, and proportionate. The flexibility of the proposed framework was indeed praised by our workshop attendees. Furthermore, the MHRA has been considerably successful in its dialogue with therapy manufacturers. This experience will likely be valuable in the years to come because, as noted before [22], ongoing dialogue between regulators and manufacturers is key when it comes to producing ATMPs at POC.

It is expected that the emerging regulatory framework will be approved by the end of 2021 or in the beginning of 2022, after completion of the public consultation and the Parliamentary process. When it becomes law, it can provide additional incentives for POC manufacture in the country, an MHRA's expectation. At the same time, the important challenges entailed by such expansion of POC manufacture, some of which have been highlighted in this Commentary, need to be considered. They manifest themselves at a very particular historical moment, after the legal transition represented by Brexit and the unprecedented medical demands generated by the Covid-19 pandemic. These circumstances make it particularly important to combine technical and medical requisites harmoniously or, differently said, to seek "[…] the right balance between following the hospital internal rules and directives and putting in place procedures in order to meet GMP and GCP compliance" [22].

On the one hand, it is important to go beyond the present situation, in which POC manufacture depends on solutions found or improvisations made in particular hospitals, with low levels of collaboration and technology diffusion [23]. On the other, it is also necessary to pay attention to the concentration of technical expertise or controlling force that may derive from highly standardized manufacturing systems.

Our workshop attendees believe that these challenges can be surmounted, and these potentialities fully explored, if the issues discussed above are properly attended to in an open, responsible, and continuous manner. To a large degree, this view is shared by the members of our FTH Manufacturing Hub who have conducted studies on several aspects of POC manufacture. With the continuation of such and similar discussions and research projects, the UK can become a pioneering country not only in terms of regulations for POC manufacture but also in terms of actual production of useful therapies in clinical settings.

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