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NUCLEIC ACID INSIGHTS

CONTENT PILLARS

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Engineering and manufacturing

NUCLEIC ACID INSIGHTS

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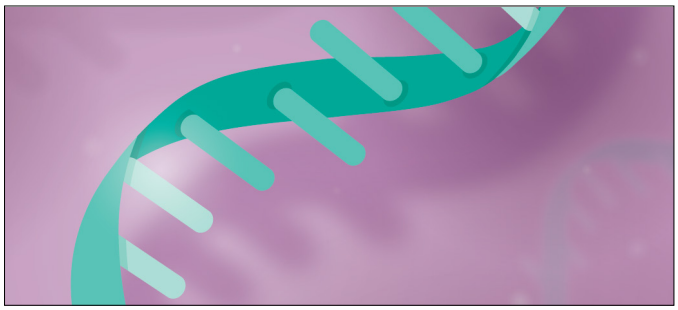

Producing low-immunogenicity, high-stability mRNA with dsRNA reduction to <0.005% (LLOQ)

Jen Dennin, Product Manager, CELLSCRIPT™

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Optimizing mRNA design for therapeutic applications is critical to ensure maximum protein expression, low impurities and a reduced immune response. This poster describes how to efficiently generate lower immunogenicity mRNA for downstream applications while also reducing dsRNA using the INCOGNITO™ mScript™ Complete mRNA Production Systems.

CONSIDERATIONS FOR OPTIMAL mRNA DESIGN

Optimizing mRNA design enables robust protein expression, minimizes immunogenicity, and provides mRNA stability and longevity in cells.

The most important design feature for therapeutic applications is nucleoside selection—particularly the incorporation of modified nucleosides, which can increase mRNA stability and reduce innate immunogenicity.

5′ capping provides stability and recognition by cellular translational machinery. Generally, either a Cap 0 or Cap 1 cap is used. The addition of Cap 1 marks the mRNA as 'self', thus reducing innate immune responses. Caps can be added either post-transcriptionally or co-transcriptionally. Post-transcriptional capping uses enzymes to cap *in vitro* transcribed (IVT) RNA by first adding Cap 0, which is then methylated to Cap 1. This process has a high capping efficiency of almost 100%. In contrast, co-transcriptional capping incorporates a cap analog during transcription, which results in a lower capping efficiency.

At the 3′ end, longer poly(A) tails protect from enzymatic degradation, thus improving mRNA stability. Exonuclease digestion in cells shortens the tail eventually, leading to instability of the 5′ cap and de-capping. Poly(A) tails can be added either post-transcriptionally, which can generate tails of over 300 bases, or co-transcriptionally, which limits tail lengths to approximately 60–120 bases.

INCOGNITO mScript Complete Kits provide all reagents to generate a functional post-transcriptionally capped and tailed mRNA with reduced immunogenicity due to incorporation of modified nucleosides and dsRNA removal.

dsRNA REMOVAL IS CRUCIAL FOR REDUCING IMMUNOGENICITY

dsRNA is an unwanted byproduct of IVT, and all IVT RNA and mRNA preps will contain some degree of dsRNA contamination. As cells have evolved pattern recognition sensors to detect dsRNA from viral infections, the presence of dsRNA will trigger an immune response. Removal of dsRNA is essential for reducing immunogenicity and has traditionally been performed using HPLC or chromatography columns.

A new highly efficient enzymatic and scalable method for dsRNA removal is provided by the Min-Immune™ Gold dsRNA Removal Kit, which reduces dsRNA to <0.005% LLOQ per sample **(Figure 1)**. This is achieved without any reduction in the ssRNA sample, unlike chromatographic methods. The INCOGNITO mScript Complete Kits includes a Min-Immune Gold module for dsRNA removal.

mRNA QUALITY ANALYSIS

To ensure a high quality of mRNA, 5′ capping efficiency and 3′ poly(A) tail lengths should be analyzed. The EZ-QC™ mRNA Assay Kits can be used to perform these analyses using polyacrylamide gel electrophoresis at a standard laboratory bench. The assays are cost effective and only require low input mRNA amounts at picomolar levels. **Figure 2** shows an example of the results from the EZ-QC mRNA Capping Efficiency Assay Kits.

SUMMARY

INCOGNITO mScript Complete Kits simplify the synthesis of ultra-low immunogenicity, high-stability mRNA. They provide high yield transcription as well as approximately 100% capping efficiency via post-transcriptional capping. Post-transcriptional tailing offers an expanded poly(A) tail length range with the standard protocol generating poly(A) tails of approximately 150–200 bases, and the modified protocol enables tails of over 300 bases. The Min-Immune Gold dsRNA Removal Module can remove dsRNA to less than 0.005% LLOQ per sample.

Figure 1. Comparison of Min-Immune Gold-treated 1.4 kb pseudouridine (Ψ)-containing RNA sample to an untreated sample (right) and dsRNA standards (left panel). Min-Immune Gold-treated dsRNA is reduced below the limit of quantitation (less than 0.005% LLOQ) after treatment.

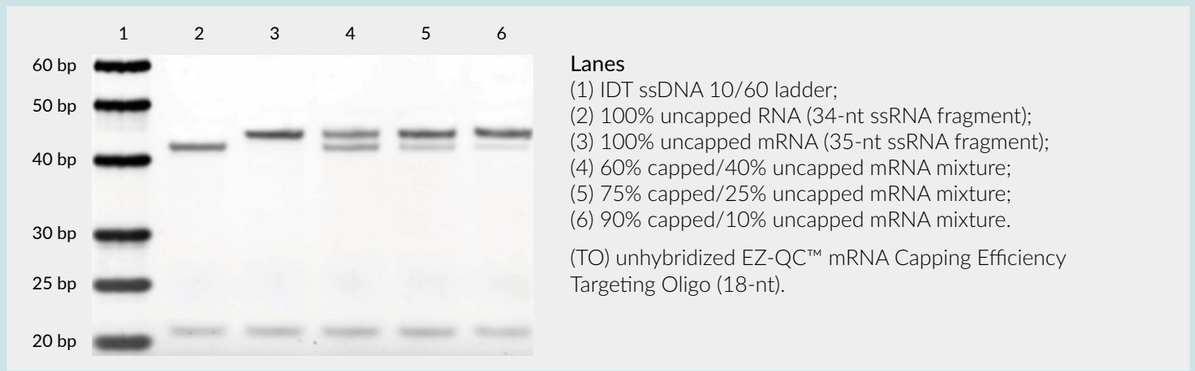

Figure 2. Mixtures of capped and uncapped mRNA were assayed using the EZ-QC mRNA Capping Efficiency Assay Kit and resolved on a polyacrylamide gel. The targeting oligonucleotide facilitates cutting the mRNA at a single, repeatable location allowing for greater confidence in determining the percentage of capped and uncapped content of the assayed samples.

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NUCLEIC ACID INSIGHTS

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PLATFORMING

SPOTLIGHT

INNOVATOR INSIGHT

Solving unique analytical challenges in mRNA-LNP bioprocessing with precise measurement methods

Nigel Herbert

UV-Vis spectroscopy is widely used for measuring sample concentrations in gene therapy development, although it poses challenges such as dilution errors and lengthy sample preparation. Variable pathlength technology (VPT), also known as slope spectroscopy, is a UV-Vis-based technique that can be utilized to overcome these hurdles by measuring absorbance changes over variable pathlengths, eliminating dilution and baseline correction requirements. In this article, three case studies are explored to confirm the specificity, accuracy, reproducibility, and linearity of SoloVPE® for encapsulated mRNA analysis.

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ADDRESSING CHALLENGES OF UV-VIS SPECTROSCOPY WITH VPT

UV-Vis spectroscopy is a widely used method for determining sample concentration, but it poses several challenges. Firstly, the pathlength is typically fixed at 1 cm, which requires dilution at certain concentrations, often leading to unreliable results due to errors, which typically range from 5% to 20%. Furthermore, baseline correction is required to remove background noise and interference. Lastly, sample preparation can be time-consuming, as samples are often sent from the manufacturing site to QC for analysis.

These challenges can be addressed by novel technologies, such as VPT-based spectroscopy, also known as slope spectroscopy. This method calculates the change in absorbance over the change in pathlength to produce a linear slope.

At-line instruments such as SoloVPE® and SoloVPE PLUS®, as well as in-line technologies such as FlowVPX® use slope spectroscopy to perform measurements of a wide range of concentrations without the need for dilution or baseline correction. The SoloVPE PLUS system has a micron-level pathlength resolution, meaning there is no need for dilutions because the light source is close to the detector. This enables the absorbance level to be kept below the

saturation level. Consequently, this allows for measuring a wide range of concentrations. Notably, the system has a two-step procedure, in contrast to traditional UV-Vis techniques with multiple steps.

The light travels from the Agilent Cary 60 UV-Vis spectrophotometer through the delivery fiber and into the single-use Fibrettes®, which are silica tubes inserted into the SoloVPE PLUS for each measurement. The Fibrette is lowered into one of the sample vessels, makes contact with the bottom, and then uses a search algorithm to collect three absorbance readings at different pathlengths to target a specific absorbance. The system then adjusts to the pathlength closest to the desired absorbance and takes readings, collecting a maximum of 10 data points or a minimum of 5 data points to generate a linear slope. Overall, this process provides results in <30 s, enabling a rapid analysis. Every measurement taken by the system includes an R^2 value, which indicates compliance with the Beer-Lambert Law. For a result to be considered reliable, the R^2 value must be greater than or equal to 0.999.

The system has a 2 µm step resolution and can measure from 2 µm to 15 mm, providing 7,500 pathlengths. FlowVPX, the in-line slope spectroscopy system, offers a 1 µm step resolution and can take measurements from 1 µm to 3–5 mm, depending on the chosen flow cell, providing 3,000 or 5,000 pathlengths to choose from. Both systems have a wide dynamic range and can measure very high concentrations. For instance, it is possible to measure highly concentrated samples, such as monoclonal antibodies that are >300 mg/ml, with high precision and accuracy.

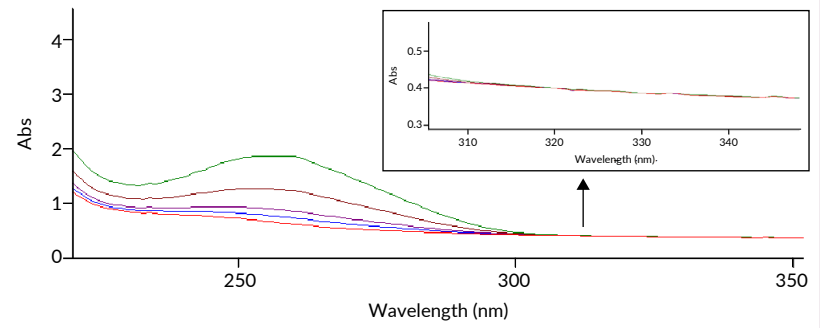
Additionally, ViPER, a Structured Query Language server database-driven software platform, can be used along with SoloVPE, SoloVPE PLUS, and the FlowVPX.

FIGURE 1

Spectral scans of (A) unencapsulated and (B) encapsulated mRNA using SoloVPE.

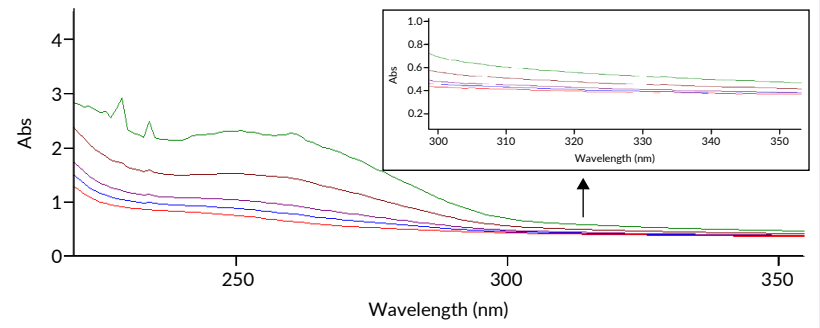
A Unencapsulated mRNA

- Clear lambda max at 260 nm
- No significant absorbance in the near UV range (300 nm to 350 nm)
- All pathlengths within the linear range
- Tail end of spectra pinches at ~300 nm



B Encapsulated mRNA

- Lambda max has increased absorbance using same pathlengths
- Increased absorbance in the near UV range due to LNP
- Top pathlengths start near the non-linear range



SLOPE SPECTROSCOPY APPLICATIONS IN GENE THERAPY DEVELOPMENT

Slope spectroscopy-based technologies can be used in various gene therapy manufacturing applications. For example, they can be used to monitor the growth of *Escherichia coli* cells. These technologies can also measure high concentrations and purity ratios of nucleic acids, including plasmid DNA, single-stranded DNA, RNA (unencapsulated or encapsulated), as well as oligonucleotides. For instance, FlowVPX can be used to measure approximately 80 mg/ml oligonucleotides, whilst SoloVPE PLUS can be used for concentrations such as 40 mg/ml. FlowVPX can also measure genome and viral vector capsid titer, as well as percentage of full AAV, allowing developers to make real-time decisions throughout the process. Overall, these systems can measure a wide range of concentrations with a high degree of accuracy and precision, ultimately saving time, increasing productivity, minimizing risk, and strengthening the control of the manufacturing process.

OVERCOMING HURDLES IN ENCAPSULATED mRNA ANALYSIS

Analysis of mRNA encapsulated in lipid nanoparticles (LNPs) can be challenging, particularly because the number of methods available for achieving accurate measurements are limited. One of the most common techniques is a fluorescent-based assay—RiboGreen™—which can be time-consuming, taking between 45 min to 1 hour. Additionally, LNPs significantly scatter UV light as they are typically smaller than the wavelength of light, meaning that an appropriate light scattering technique must be applied to determine an accurate concentration of the payload. Furthermore, most analytical methods have a high degree of variability since the RNA must be extracted. For example, with the RiboGreen® method, operator-to-operator variability can be as high as 10%. Lastly, fluorescence-based assays require LNP disruption. Typically, detergents such as Triton™ X-100 can reduce fluorescent signals by up to 8% since Triton itself also produces some fluorescence. Additionally, Triton-X is toxic and environmentally harmful, and has been banned in some regions such as the EU. The RiboGreen method also requires sample dilution to a level within the correct range for the assay, which can introduce errors. There may also be a discrepancy between the buffer of the standard and the sample.

In contrast, the SoloVPE PLUS operates a two-step procedure, where the sample is pipetted in, the method is set up using the software, and the collection is started. Therefore a process that typically takes up to an hour can be done in under a minute, without the need for sample dilution. In order to evaluate the efficiency of slope spectroscopy-based techniques to measure encapsulated mRNA concentration, three internal case studies were carried out.

CASE STUDY 1: SPECIFICITY

The aim of the first study was to assess the specificity to establish the best SoloVPE method for measuring the concentration of total mRNA encapsulated by an LNP.

Unencapsulated and encapsulated mRNA were both used at a concentration of 1 mg/ml. A spectral scan was taken on both samples to show the UV light scattering from the LNP, and the same pathlength was used in both scans. Notably, there was a significant change in the absorbance profile when comparing the two samples **(Figure 1)**.

During this test, it was confirmed that the dual scatter algorithm was the optimal function for correcting light scattering. This correction follows a similar profile to the LNP and is best suited for quantitation.

The absorbance profiles also helped determine the appropriate wavelengths to use. In this case, 300 nm and 350 nm were selected as scatter wavelengths because they correspond to the point where the absorbance profile pinches, and the tail end of the spectra stabilizes. Depending on the size and structure of the LNP, these wavelengths may also vary.

Overall, this case study demonstrates that SoloVPE can achieve specificity by distinguishing between encapsulated and unencapsulated mRNA through significant changes in absorbance profiles and optimal light scattering correction.

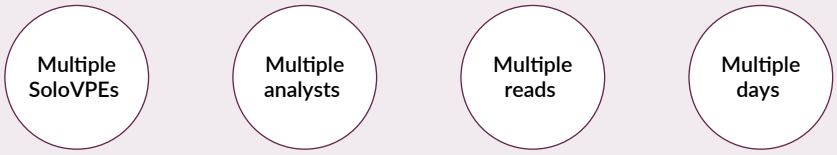
CASE STUDY 2: ACCURACY AND REPRODUCIBILITY

Another proprietary study was carried out to examine the accuracy and reproducibility of the SoloVPE method. The 1 mg/ml samples were measured in triplicate with two different analysts running different

FIGURE 2

(A) The analysis of SoloVPE precision across multiple days, analysts, and systems, and (B) the comparison of the SoloVPE System to RiboGreen in determining the concentration of mRNA encapsulated in LNP.

A



Day	Instrument	Analyst	Rep 1 (mg/mL)	Rep 2 (mg/mL)	Rep 3 (mg/mL)	Mean concentration (mg/mL)	%RSD
1	1	1	0.95245	0.94678	0.94627	0.9485	0.36%
		2	0.96969	0.96353	0.96518	0.96613	0.33%
	2	1	0.97705	0.96699	0.99298	0.97901	1.34%
		2	0.97658	0.95837	0.97384	0.9696	1.01%
Day 1: Mean and precision						**0.96581**	**1.41%**
1	1	1	1.03739	1.04023	1.05891	1.04551	1.12%
		2	1.05177	1.0501	1.04446	1.04877	0.37%
	2	1	1.00343	1.0143	1.01013	1.00929	0.54%
		2	1.01699	1.01295	1.01412	1.01469	0.21%
Day 2: Mean and precision						**1.029565**	**1.88%**

B

Method	Concentration (mg/mL)	Mean concentration (mg/mL)	%RSD
SoldVPE	1.01502	**1.01204**	0.58%
	1.00529		
	1.01579		
RiboGreen	0.99	**0.99**	0.71%
	0.98		
	0.99		

systems on two different days. Based on the results, the precision within the same system and analyst as well as across two different systems and two different analysts was <2% **(Figure 2A)**. These results confirm that the SoloVPE method can be easily transferred from site to site.

The experiments also showed that LNPs are very sensitive and easily agitated by pipette mixing or vortexing. Notably, the best results were achieved when the sample was inverted 10 times, pipetted into a vessel, and then left to sit for 2 min prior to measuring.

Accuracy was then confirmed by comparing the results to the orthogonal method, RiboGreen. The results were nearly identical, and the SoloVPE had excellent precision between replicates **(Figure 2B)**. Assuming RiboGreen was the expected value, the SoloVPE had a percentage error of approximately 2.2%, demonstrating that the method is reliable.

CASE STUDY 3: LINEARITY

In another internal study, linearity was tested to ensure that the SoloVPE method complies with the Beer-Lambert Law. A 1 mg/ml sample was taken and diluted into four different concentrations, giving five samples in total. The theoretical concentrations were 1 mg/ml, 0.8 mg/ml, 0.6 mg/ml, 0.4 mg/ml, and 0.2 mg/ml. Afterward, all samples were measured, and the theoretical concentration was plotted against the measured concentration, which generated an $R^2 > 0.999$ **(Figure 3)**. This not only confirms that the method is linear, but that it can be used across a wide range of concentrations.

INTEGRATION IN TANGENTIAL FLOW FILTRATION

FlowVPX can be integrated into both bench-scale and manufacturing-scale tangential flow filtration (TFF) systems, allowing developers to use concentration as a set-point control in their processes.

Instead of relying on mass or weight, concentration steps within the process can be controlled directly with FlowVPX. This method enables developers to measure the concentration of encapsulated mRNA and establish set points based on concentration.

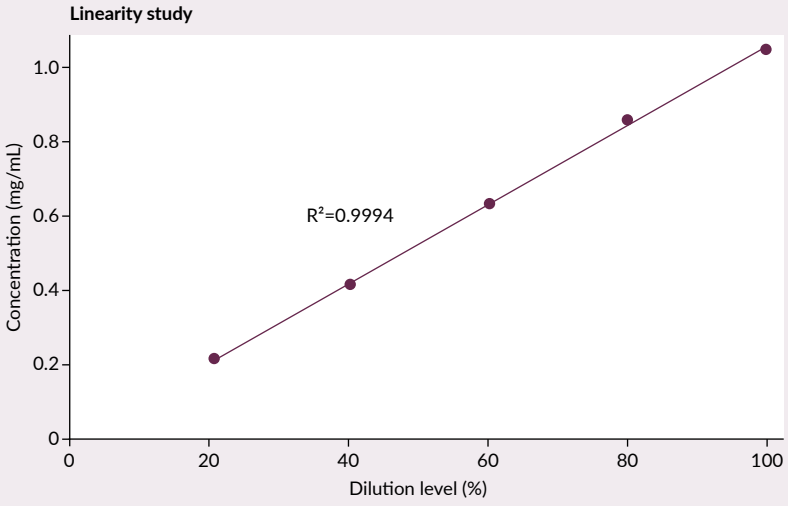
Furthermore, the technology is transferable from the at-line system to the in-line system, though it is recommended to first

FIGURE 3

Testing the linearity of the method used by the SoloVPE system.

Dilution level	Measured concentration (mg/ml)
100%	1.04227
80%	0.85308
60%	0.62828
40%	0.41159
20%	0.20607

- High linearity, R>0.999
- Accurate across wide concentration range
- Method complies with Beer's Law



define the method on the SoloVPE PLUS. Depending on the LNP formulation and particle size, adjustments may be necessary, particularly when selecting scatter wavelengths. Once method development is completed, it can then be transferred to FlowVPX.

SUMMARY

Slope spectroscopy enables precise concentration measurements without dilution or baseline correction. Case studies explored in this article demonstrate the specificity, accuracy, reproducibility, and linearity of this technology in gene therapy applications. These findings highlight slope spectroscopy's reliability for mRNA quantification, supporting real-time decision-making in bioprocessing. Overall, these technologies streamline workflows, reduce variability, and enhance precision, making them valuable for gene therapy and bioprocessing applications.

Q&A

Nigel Herbert

Q What is the highest concentration that has been measured using the SoloVPE PLUS for this particular modality?

NH The highest concentrations measured so far are approximately 2 mg/ml. However, significantly higher concentrations can be measured. The upper limit for this specific modality has not yet been tested, but the systems can certainly measure concentrations well beyond the shown data.

Q Is the SoloVPE system only applicable for mRNA, or can it also be applied to DNA?

NH This method is specifically designed for encapsulated RNA, but the SoloVPE system can measure any type of nucleic acid, including single-stranded DNA, double-stranded DNA, and both unencapsulated and encapsulated mRNA.

Q You mentioned that LNP formulation can affect the methods. Which specific LNP was used in this study?

NH For this particular study, we used a generic LNP formulation, which consisted of an ionizable lipid, a coagulated lipid cholesterol, and DOPE helper

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lipid. Depending on the specific LNP formulation, the method may need to be adjusted, particularly regarding the scatter wavelengths, which may need to be changed.

Q Does encapsulation efficiency impact the outcomes of the methods?

NH It potentially does—when we tested this method, the encapsulation efficiency was about 98%. This method assumes high encapsulation efficiency, as we are measuring total RNA and cannot differentiate between unencapsulated and encapsulated RNA. However, if your sample has poor encapsulation efficiency, there are likely more significant concerns than simply using SoloVPE for this method. To assess this, you would need to test different encapsulation efficiencies, compare the methods, and determine if there is a significant discrepancy between the results.

Q For mRNA-LNP samples with high light scattering, such as those with large particle sizes and high concentrations that result in high turbidity, can your correction algorithm still accurately measure the total mRNA?

NH For this particular sample, the particle size was about 67 nm, and the polydispersity index was 0.124. For this specific LNP, we were able to measure these concentrations without any issues. If the particle size were to change significantly, additional testing would be required to ensure the method works effectively.

BIOGRAPHY

Nigel Herbert has a BSc degree in Biomaterials Engineering and a minor in Chemistry from Alfred University, Alfred, NY, USA. He brings a wealth of skills to his role as a Senior Bioanalytics Applications Specialist at Repligen. Since joining our team in 2019, Nigel has been on the forefront of advancing our customers' analytical methods. He specializes in UV-Vis spectroscopy, analytical processes, and method validation, offering invaluable support to ensure the highest standards of precision and reliability. One of Nigel's remarkable achievements is his collaborative spirit, resulting in numerous published application notes. Through close partnerships with our customers, he has made significant strides in solving complex challenges and driving innovation in the biotechnology field. Nigel Herbert's unwavering commitment to enhancing your analytical capabilities exemplifies Repligen's dedication to your success.

Nigel Herbert, Senior Field Application Scientist, Process Analytics, Repligen, Bridgewater, NJ, USA

AUTHORSHIP & CONFLICT OF INTEREST

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COMMENTARY

Incorporating digitalization in the conceptual design, research and development of plasmid biomanufacturing

Duarte Miguel de França Teixeira dos Prazeres, Ana Margarida Azevedo, Sofia Oliveira Duarte, Ana Rita Silva-Santos, Krist V Gernaey, Carina L Gargalo, Rosa Hassfurther, Annina Kemmer, and M Nicolas Cruz Bournazou

The importance of large-scale production of plasmid DNA (pDNA) has increased steadily over the years due to the development of a growing number of direct and indirect applications. To meet the growing demand for pDNA, significant efforts must be made towards improving its manufacturing. In particular, the digitalization of pDNA manufacturing could enable faster process optimization, support data-driven decision-making, and contribute to waste reduction and more sustainable operations. In this commentary article, we further contend that the benefits of digitalization should be captured early on at the research and development stage of the manufacturing process. To support this vision, we present a conceptual framework for incorporating digitalization into pDNA process development, discuss technological enablers, explain how digital methods could overcome traditional limitations, and delve into implementation considerations.

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PLASMIDS AND THE DIGITALIZATION OF BIOMANUFACTURING

Plasmids are pervasive across the gene and cell therapy industry of today [1,2]. As biologicals, they are used to deliver genetic information to patient target cells or as vehicles to deliver the molecular components of gene editor systems. Moreover, plasmids serve as essential raw materials for the manufacturing of engineered cell products (e.g., CAR-T cells) or of other biologicals (e.g., viral vectors and mRNA). The ability to manufacture plasmids cost-effectively on a large scale is thus critical for many

biopharmaceutical companies and research institutions [3]. In other circumstances, for example, in the production of lentiviral vectors for cell therapies, the actual challenge may be to develop GMP-compliant scale-down models capable of producing pDNA in a cost-effective manner [4]. One approach to increase efficiency, throughput and scalability, conserve resources, and minimize environmental impact in pDNA manufacturing is to embrace digitalization [3].

A fundamental principle of digitalization is the mapping of the physical space in a digital object via a digital twin (DT) [5]. A DT is a continuously updated *in silico* representation of a real-world system or process that acts as an identical counterpart in the digital space. An essential feature of a fully functional DT is a two-way dataflow between the physical system and its digital counterpart [6]. Ultimately, the DT generates a dynamic or static profile of the process based on historical and near-real-time measurements across an array of dimensions [6]. DTs are valuable for system simulation, integration, testing, monitoring, maintenance and even training, and are an essential building block of model-based systems engineering. Furthermore, in conjunction with mathematical modeling, DTs are likely essential for the successful implementation of continuous biomanufacturing, as they enable real-time process control, predictive decision-making, and rapid optimization [7,8].

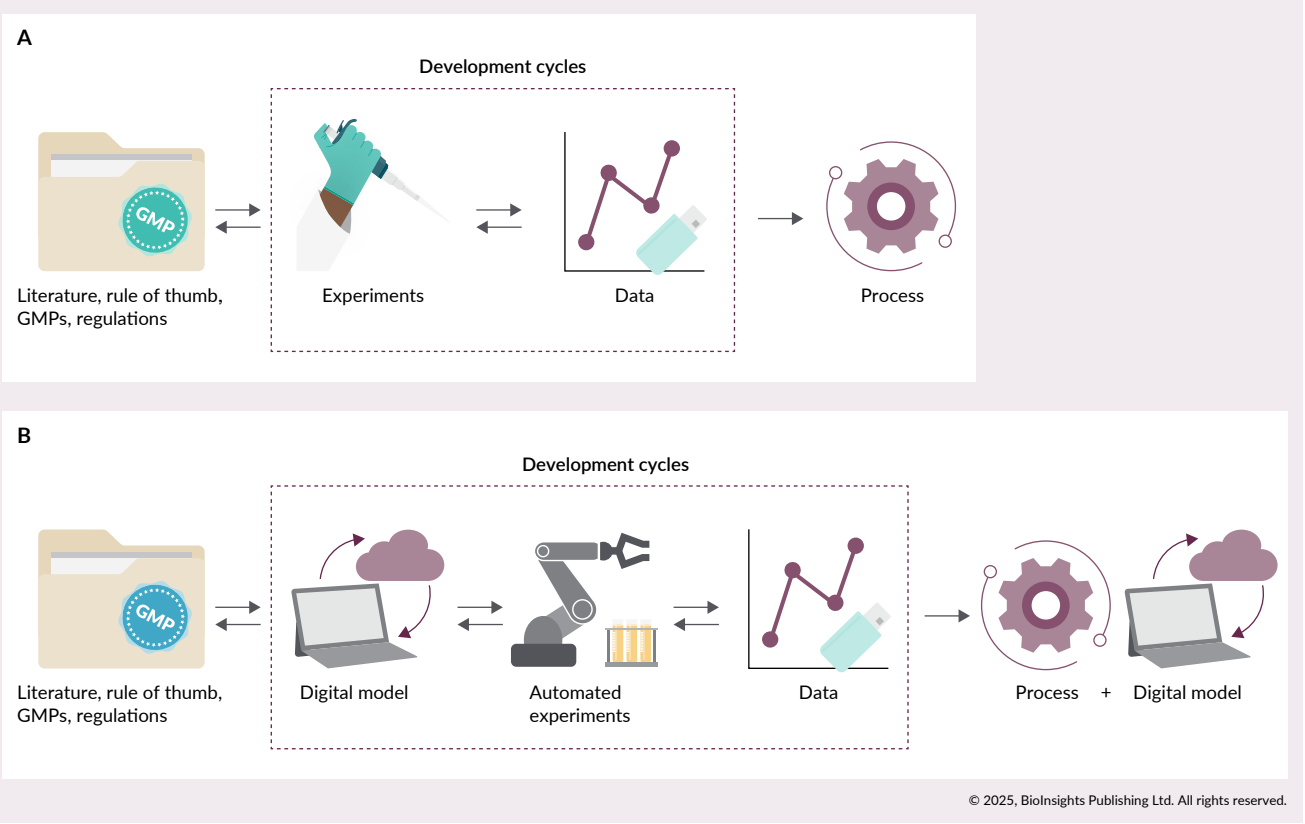
The creation of a DT of a biomanufacturing process has been advocated as one of the most compelling benefits of digitalization [6–9]. While as a first approach this will involve the digitalization of well-established manufacturing processes that are already in routine operation, several authors argue that the benefits of biomanufacturing should be captured early on at the research and development stage [10,11]. The development of a process compliant with Industry 4.0, which is characterized by the integration of digital technologies—such as the Internet of Things (IoT), artificial intelligence, data analytics, and automation—into manufacturing systems, inherently also demands the development of its DT [12]. The research question underlying this approach is therefore 'How to develop and incorporate a digitalization framework in the conceptual design, research and development of (pDNA) biomanufacturing processes?'. Such a framework is currently lacking, as most efforts are focused on digitizing established biomanufacturing processes.

THE LIMITATIONS OF TRADITIONAL PROCESS DEVELOPMENT

A real shift to a biomanufacturing scenario, where a physical process and its digital counterpart communicate, interact two ways, and operate in synchrony without interruption, requires digitalization to be embedded early in the biomanufacturing research and development stage [10]. This entails replacing the traditional process development pipeline, which follows a linear, step-by-step methodology known to be time consuming and laborious [10,11,13], with a digitally centered process development approach **(Figure 1)**. A process draft is usually designed based on the available literature, in-house experience, rules of thumb and GMPs. Key information to bear in mind pertains to final product specifications (e.g., pDNA topology, biological potency, impurity limits), some of which are established with guidance from regulations [14]. Examples of process-related impurities in pDNA manufacturing include host cell components (proteins, genomic DNA, RNA, endotoxins), residual reagents (solvents, salts, enzymes), and leachables from equipment, resins or filters. The final specifications will differ depending on the final application of the target pDNA [14]. For example, more stringent quality requirements regarding impurities will be in place if the pDNA is to be used in therapeutic applications, as opposed to cases

FIGURE 1

Replacing the (A) traditional approach to biomanufacturing process development by a (B) digitally centered process development.



where it serves as a raw material for the manufacture of a viral vector. Today, several pDNA manufacturing platforms have been developed, which can be readily adopted for the production of various pDNA molecules. Nevertheless, the introduction of new methodologies or process modifications targeted at generating more efficient processes will still require several process development cycles, relying heavily on human operators performing lab-based experimentation at small scale (typically 100 mL–10 L cell culture). The disadvantages of this approach are well recognized and include:

- Time and cost inefficiencies
- Limited process understanding and data utilization
- Poor scalability
- Human error and variability
- Regulatory compliance challenges

A digitally centered process development approach, paired with automated experimentation, could contribute to delivering new methodologies that generate more efficient processes, ultimately mitigating some of these limitations [10,11].

ENVISIONING A DIGITALLY CENTERED PDNA PROCESS DEVELOPMENT

A digitally centered approach to process development relies heavily on

incorporating digitalization concepts and computational tools at the early stages of process conceptualization, design, and development. The technological enablers of this approach include mathematical modelling (mechanistic, hybrid, and data-driven models), computational fluid dynamics, machine learning and AI, generative AI, automation and smart sensors, high throughput (HT) experimentation, workflow management systems, and edge and cloud computing **(Table 1)**. This approach offers several benefits, including accelerating development, reducing consumables by avoiding uninformative experiments, requiring fewer experiments, lowering error rates, and enhancing process understanding. Furthermore, by the end of the process development stage, digital models will be readily available to support technology transfer, process scale-up, and subsequently routine operation and control.

Here we present our view on how a digitally centered alternative can be utilized to

TABLE 1

Scientific and technological tools for digitally centered plasmid DNA process development.

Enabler	Description
Mathematical modeling	Mathematical process models of diverse nature (mechanistic, surrogate, data-driven, hybrid) are set up to provide information about key properties, variables and performance parameters/indicators (e.g., yields) of the different sub-processes (e.g., cell culture, unit operations), the interactions between process parameters, and product quality attributes (e.g., purity); these models are a key component of a DT, providing deep insights into the current state of the process through simulation
CFD	Software tools for performing CFD dynamics simulations can play a crucial role in bioprocess scale-up by enabling the simulation and analysis of fluid flows within bioreactors; this facilitates the optimization of mixing, mass transfer, and overall reactor design, which are essential for efficient scale-up
Machine learning and AI	AI and machine learning contribute to smart automation and analytics through the identification of optimal process parameters, automation of complex tasks, prediction of potential issues; enabling the shift to predictive rather than reactive process control
Generative AI (large-language models)	If trained on large and adequate datasets of bioprocess parameters, LLMs can suggest improvements to increase efficiency and product quality, or assist in designing more effective experiments, potentially reducing the number of iterations required in bioprocess development
Automation and smart sensors	Bioprocessing workflows can be optimized, monitored and controlled in real-time by integrating advanced technologies such as sensors and IoT devices for data acquisition, and AI and machine learning for predictive modeling and decision-making; automated systems can handle tasks such as sampling, analysis, and equipment maintenance and contribute to enhance process efficiency, improve product quality, and reduce variability by minimizing human intervention
HT experimentation	HT experimentation using robotic platforms enable rapid, parallel execution of numerous experiments, significantly accelerating process optimization and development; these systems can dispense reagents, mix solutions, and transfer samples, minimizing human error and increasing experimental throughput; it is thus possible to explore a broader range of parameters and conditions simultaneously, leading to faster identification of optimal production conditions; if integrated with advanced data analytics and computational modeling, HT experimentation can enhance decision-making capabilities and reduce development timelines
Workflow management systems (WMS)	By implementing WMS, processes can become fully documented, traceable and reproducible, allowing for reuse of the generated data; WMS enhance interoperability, thus enabling better collaboration between scientists; they allow for the seamless choreographing of tasks, ensuring that complex workflows are executed efficiently and in the correct sequence; additionally, WMS facilitate structured storage for data and metadata, preserving essential context for future analyses; built-in error detection mechanisms help identify issues early, triggering automated error handling procedures to maintain workflow reliability and data integrity
Edge and cloud computing	Edge computing enables real time data processing and control of the biomanufacturing facilities, empowering quick adjustments; cloud computing provides scalable storage, big data analytics, and collaborative platforms for long term data analysis, process optimization and predictive modelling

CFD: computational fluid dynamics. HT: high throughput. WMS: Workflow Management Systems.

aid, guide and accelerate the development and establishment of a pDNA manufacturing process. The overall goal is to develop an integrated model toolset that examines the entire biomanufacturing process, providing clarity on bottlenecks, highlighting optimization opportunities, and ultimately enhancing superior product quality and efficiency in laboratory operations. Specifically, we propose an approach that involves synergies between:

- Experimentation
- Digitalization
- HT model-assisted experimentation activities **(Figure 2)**

These intertwined collaborative research activities should cover the upstream and downstream processing stages of plasmid manufacturing.

Experimental setting up of a benchmark process

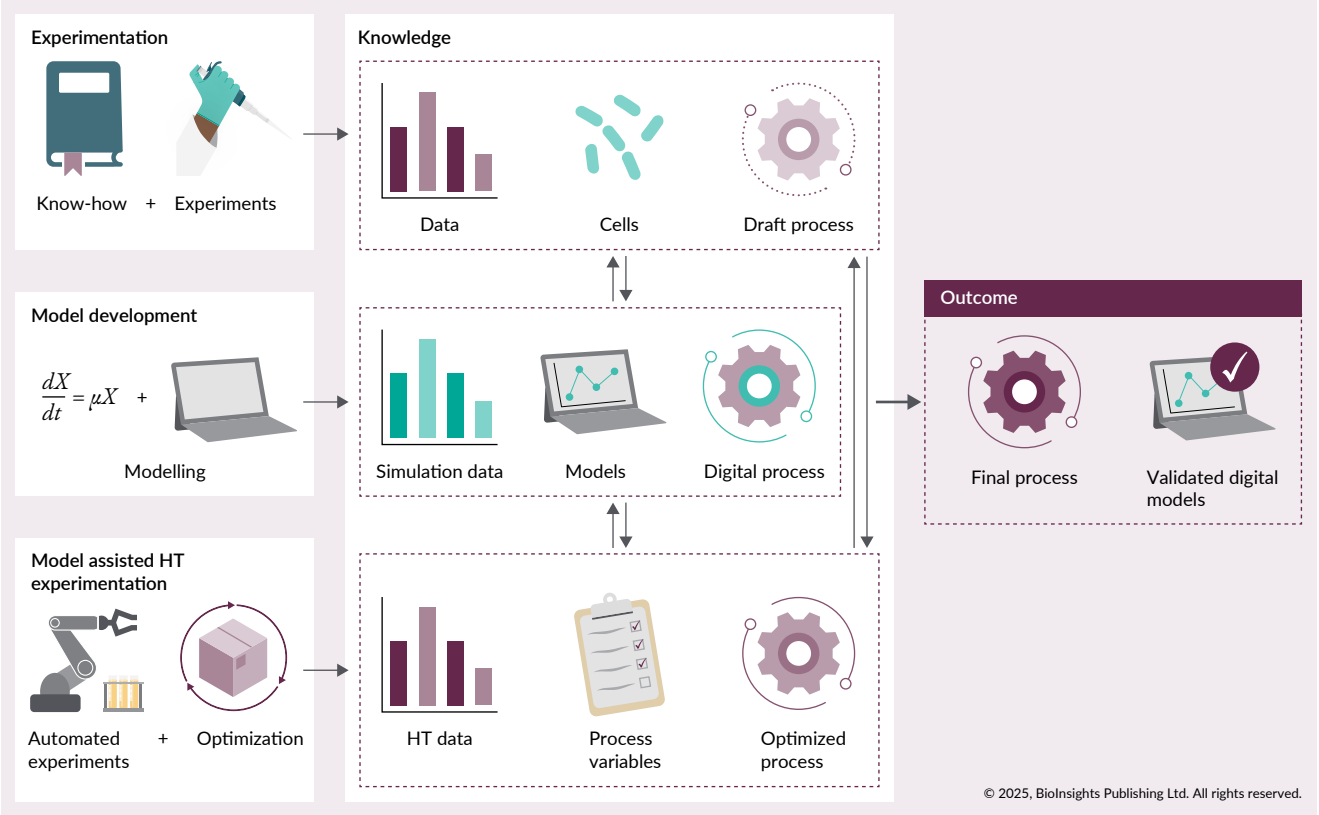
An experimental benchmark process is initially defined based on available knowledge and rules of thumb [15]. This heuristic approach involves the selection of a strain of the producer *Escherichia coli* with genotypes suitable for pDNA amplification, the preparation of banks of cells transformed with the target pDNA, and the set-up of key analytics (e.g., gel electrophoresis, HPLC, ELISA). Then, a working pDNA manufacturing process should be drafted and established at lab scale. This entails cultivating cells to amplify pDNA and then setting up a downstream processing train of operations to recover, isolate, and purify the pDNA. The goal is to quickly obtain initial datasets (e.g., time series data describing microbial cell culture and pDNA amplification, recovery yields of unit operations, etc.) that can be used to jump start and advance model development and guide HT experimentation for process optimization.

Modelling of upstream and downstream processing

Mathematical models are developed to represent, analyze, and predict the complex system surrounding pDNA manufacturing. This calls for selecting an appropriate model structure that aligns with the biological and physical nuances of the different operations in the manufacturing train. The models should be able to describe and predict the dynamics of cell growth and pDNA amplification. This requires the establishment of time course relations between variables such as the concentration of key nutrients (e.g., carbon source), biomass concentration, and pDNA titers [16,17]. Stoichiometric models of *E. coli* metabolism can also be useful in this context [18,19]. Models used to describe the isolation and purification of pDNA from the *E. coli* cells should predict the performance metrics of various operations (e.g., tangential flow filtration, precipitation, chromatography), especially in terms of yield and purification efficiency. Draft models for a particular operation are first tested using the corresponding initial data sets. Simulation results are then used to guide the design of additional experiments, such as model-based design of experiments [20–22]. The new sets of experimental data are further used to refine and validate the models. These experimental/modelling development cycles should be repeated until a satisfactory model is obtained. An illustration of this approach is provided by Muller *et al.* in the context of rAAV production [23]. Starting with shaker flask data, satisfactory process models were obtained after two to three iterative cycles combining high-throughput (HT) runs in a fully automated microbioreactor system with hybrid model refinement. Benchmarking this approach against a statistical Design

FIGURE 2

Synergies between (A) experimental setting up of a benchmark process, (B) digitalization, and (C) high-throughput model-assisted experimentation activities.



of Experiments method showed that the model-based experimental design consistently produced higher rAAV titers with fewer total experiments. Rigorous validation should be made by comparing model outputs against separate experimental datasets. The outcome is a set of robust models that reflect experimental observations, provide insights into the process and support subsequent process optimization.

High-throughput model-assisted experimentation activities

Optimization of pDNA manufacturing can then be performed by resorting to automated HT screening platforms [24–26], guided by the predictive models developed. Such platforms, which are being introduced into modern process development labs, can be used to screen process conditions and operating variables that maximize the performance of the unit operation being tested [27–29]. Experimental workflows can be integrated and automated in a laboratory environment by resorting to heterogeneous devices, including liquid handling stations, parallel cultivation systems, and mobile robots [30]. For example, advanced liquid handling stations with embedded parallel mini bioreactors can be used to run up to 48 parallel cell culture/pDNA amplification dynamic experiments in a process-wide design and optimization scheme [31,32]. The integration of a workflow management system [33] ensures the flexible yet reliable handling of complex HT experiments and FAIR data storage—findable, accessible,

interoperable, and reusable (FAIR) [34]. Here, model-based tools can enhance information gain and process robustness by enabling, for example, real-time process monitoring, the selection of the most relevant sampling times, and the optimization of process control. Many downstream processing unit operations used in pDNA manufacturing such as precipitation [35,36] and chromatography [37,38], can also be optimized using HT platforms [28,29]. One important aspect to mention is that HT experimentation critically depends on HT analytics to rapidly evaluate multiple conditions [39]. For example, an evaluation of 48 parallel cell culture/pDNA amplification experiments run on a mini bioreactor platform would undoubtedly involve assessing pDNA titers and topology. Since most scale-down reactor systems only incorporate DO and pH measurements, this would require collecting samples, performing miniprep isolation, and running agarose or capillary electrophoresis analysis in parallel, which is not trivial to implement [40]. Further challenges include the small volumes of scale-down reactors, which restrict the sampling frequency and volume, as well as the large number of samples generated. Reality shows that, unfortunately, analytical capacity often lags behind experimental throughput, creating a significant bottleneck [39]. This mismatch between experimental throughput and analytical capacity can slow down decision-making and delay process optimization, particularly in complex biological systems. Notwithstanding the analytical challenge, the large amounts of heterogeneous experimental data generated by HT platforms contain valuable information that can be explored using a wide variety of machine learning (ML) approaches [41–43]. Examples of data-driven methods that may be useful for optimization purposes include artificial neural networks [44], Bayesian optimization [45–47], deep reinforcement learning [48], and others [49].

Key benefits of HT experimentation include accelerated development, and the ability to perform a higher number of experiments while keeping the number of needed consumables low due to the smaller volumes. Applying model-based methods to design experiments with optimal information gain ensures that only the minimum number of experiments is performed [50,51]. The new data generated can be used to refine and validate the models that have been developed. The goal of these activities is to determine the optimal conditions for pDNA manufacturing and to develop a reliable digital model of the process. On the other hand, one should be aware that miniaturized systems may not replicate large-scale pDNA manufacturing (e.g., bioreactor dynamics and substrate heterogeneities/gradients [52]), and that analytical and data handling limitations can hinder the translation of results. Furthermore, the complexity of integrating automated platforms and the resources required to ensure regulatory compliance cannot be overstated.

Model integration and process validation

Ideally, models describing both upstream and downstream processing sections should be merged into a singular, unified model. This integration is still perceived as a bottleneck, often because upstream and downstream process models have focused on describing different sets of variables. Once integration is achieved, the consolidated model should be rigorously validated against lab-scale datasets (e.g., at the 1–2 L lab scale), ensuring it reflects real system dynamics, and that it is robust and reliable [53].

TRANSLATION INSIGHT

Embracing digitalization concepts and tools at the early stages of process conceptualization, design, and development

can accelerate development, reduce consumables and error rates, increase the number of informative experiments, and ultimately improve process understanding. In the context of plasmid manufacturing, this digitally centered approach to process development requires synergies and interconnection of:

- Experimentation
- Digitalization
- HT model-assisted experimentation activities

However, the field is in its infancy, with several areas requiring further study or pilot testing.

For once, many of the digitalization tools at our disposal **(Table 1)** are still underexplored in the context of process development. For example, there is clearly room for the development of LLMs tailored to the conceptual development of processes for the biomanufacturing of a particular class of bioproducts (e.g. nucleic acids, pDNA, mRNA), leveraging existing literature data and pre-existing knowledge (e.g., company data, expertise). Such dedicated LLMs could be invaluable, for example, in the initial drafting of a manufacturing process. The use of CFD in the context of process scale-up can also be considered sub-optimal due to its high computational cost, reliance on simplifications that may not fully capture complex interactions, and challenges in accurately predicting scale-dependent phenomena (e.g. turbulence, mixing, and heat transfer). Another important area that requires investment is the development of more advanced and refined mathematical models capable of accurately representing complex biological systems, for example, microbial cell culture and pDNA amplification. The importance of mathematical models in conjunction with the adoption of digitalization will be especially relevant in the context of continuous manufacturing, which is an industry trend likely to change the way plasmids are manufactured in the future [54–56].

Additionally, the full technical integration of the digitalization tools available **(Table 1)** in the context of process development is still a bottleneck. Clearly, we need to improve our ability to manage the loop of hypothesis formulation, model-based experimental design, high-throughput experimentation, data evaluation, model adaptation, conclusion, and new hypothesis generation, which still requires considerable human intervention. Although we are far from creating a 'Robot Process Development Scientist' designed to autonomously automate process development, akin to the Robot Scientist discussed by King *et al.* [57], the potential for digitalization to contribute to the generation of process knowledge is huge. The necessity to upgrade technological infrastructure for real-time data integration in process development laboratories is also imperative. Examples include the integration of HT experimentation and advanced analytics capabilities, the implementation of integrated Laboratory Information Management Systems (LIMS) or Electronic Lab Notebooks (ELN) [58], the replacement of legacy laboratory instruments with digitally enabled, IoT-compatible sensors and Process Analytical Technology tools (e.g., Raman, NIR, FTIR, and *in situ* microscopy) [59], and the installation of systems to ensure data integrity, traceability, and regulatory compliance in digital environments [60].

The implementation of digitalization in biomanufacturing—both in process development and operation—further requires a fundamental shift in how data are acquired and managed, aligning with the FAIR principles to ensure seamless integration, traceability, and utility across digital systems [61,62]. For example, this requires transforming heterogeneous data

formats (e.g., PDFs, Excel sheets) into structured, machine-readable formats (e.g., XML, JSON) to enable real-time synchronization between physical systems and their digital counterparts. Furthermore, the thorough tracking and recording of all tasks performed throughout experimentation at both experimental and computational levels is critical to ensure data reproducibility [33]. Another important aspect of digitalization is data safety, also known as cybersecurity, which involves managing data in a responsible manner to minimize the risk of a data breach. However, users are often not sufficiently aware of such safety aspects [63].

One significant challenge in embracing digitally centered process development is resistance to change among stakeholders. This can be addressed by demonstrating clear return on investment, ensuring data security, and fostering cross-disciplinary collaboration to build trust in digital innovations. This resistance may be exacerbated further by the lack of user knowledge—many potential users simply do not know how to use digital tools effectively or where to begin—as well as by the lack of tools specifically tailored for bioengineering. Clearly, a skilled workforce with competencies that differ from those of the past must be trained to understand the importance and value of digitalization tools, to utilize the new methodologies and associated devices in the laboratory, and to handle complex data outputs. This requires universities and research institutes to develop world-class educational programs in digital biomanufacturing, which are currently not widely available.

Although quantitative data on the digitalization of pDNA manufacturing is still scarce, it is reasonable to anticipate benefits comparable to those reported in other biomanufacturing domains where AI and advanced analytics have been integrated—such as improvements of throughput upstream (15–30%) and downstream (up to 60%) and significant improvements in resource efficiency and process robustness [64]. The digital shift in pDNA production is thus expected to enhance efficiency, sustainability, and decision-making in a similar manner.

Moving forward, academia can play a crucial role in exploring innovative digitalization approaches for early-stage biomanufacturing research, while industry should focus on pilot-testing digital tools in process development to assess their practical applications. Policymakers, on the other hand, must work to develop clear guidelines and regulatory frameworks that support the adoption of digitalization in biomanufacturing, ensuring both compliance and technological advancement. In conclusion, incorporating digitalization into manufacturing development is a strategic move towards efficiency and sustainability; however, its full potential depends on further research, industry validation, and supportive regulatory frameworks to ensure seamless integration and long-term impact.

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AUTHORSHIP & CONFLICT OF INTEREST

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NUCLEIC ACID INSIGHTS

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INTERVIEW

Nizar Y Saad, Research Assistant Professor, The Ohio State University College of Medicine, and Principal Investigator, Jerry R Mendell Center for Gene Therapy, Abigail Wexner Research Institute, Nationwide Children's Hospital

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Q Can you tell us a bit about your current role?

I am a Principal Investigator at the Jerry R Mendell Center for Gene Therapy at the Abigail Wexner Research Institute at Nationwide Children's Hospital and a Research Assistant Professor at the Ohio State University College of Medicine. My research focuses on translational science and the development of gene therapy approaches to treat genetic disorders, primarily neuromuscular diseases. I utilize both viral (AAV) and non-viral (extracellular vesicles) vectors to deliver our gene therapy products.

Q How did your academic background lead you to the nucleic acids space?

During my PhD, I focused on studying nucleic acids as regulatory elements in bacteria. This sparked my interest in pursuing a career centred around nucleic acids and using them to develop gene therapy approaches for rare genetic diseases.

Q What are your top predictions for the next five years in the nucleic acids field, and what developments do you most hope to see?

According to the RNA world hypothesis, nucleic acids were key elements that contributed to the origin of life on Earth. Therefore, it is not surprising to see nucleic acids play a tremendous role in shaping our current understanding of biology, from their function as genetic material to their applications in cutting-edge technologies such as CRISPR-Cas9, microRNAs, RNA vaccines, and therapeutic gene editing.

In therapeutics, nucleic acids are primarily used for information transfer, as seen in gene replacement strategies or as tools dependent on exogenously or endogenously provided enzymes (for example, guide RNAs in CRISPR-Cas9 technology or microRNAs in RNA interference). In the future, I hope to see nucleic acids used as independent effector molecules, particularly through the development of highly efficient ribozymes capable of autonomously regulating the expression of target genes. Additionally, I anticipate an expanded use of riboswitches to precisely control transgene expression in gene therapy applications.

Q What was your motivation for joining the board of *Nucleic Acid Insights*, and what do you most hope to see the journal achieve as we enter our second year?

Unlike many other journals, *Nucleic Acid Insights* provides a comprehensive perspective on the field of nucleic acids. It not only highlights new discoveries but also addresses the challenges the field faces, including those related to design, therapeutic efficacy, and manufacturing processes. By doing so, *Nucleic Acid Insights* brings together stakeholders from both academia and industry to foster collaboration, exchange ideas, and tackle critical challenges in nucleic acids research. I look forward to seeing *Nucleic Acid Insights* continue to strengthen the connection between academia and industry.

Q What was your song of 2024?

Les Cèdres by Ycare, Ibrahim Maalouf, and Hiba Tawaji is a song that reflects themes of longing, resilience, and a deep connection to heritage. The lyrics and music evoke a sense of emotional depth, with the iconic imagery of the cedars, often associated with Lebanon, symbolizing strength and endurance. The song blends elements from different musical genres, with Maalouf's trumpet and the vocals of Ycare and Tawaji adding a unique and poignant layer to the piece.

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