EDITION
2023

Analytical tools in bioprocessing

mabdesign
THE BIOTHERAPY EXPERT

ABOUT **MABDESIGN**

MabDesign, the French Association of the Biotherapy Industry

MabDesign, the French biotherapy industrial association, aims to support, federate and increase the visibility of the biopharmaceutical industry, foster exchanges, promote the development and competitiveness of companies, and stimulate innovation by encouraging the emergence of start-ups from academic research.

In order to carry out its development strategy and to adapt to changes in the industrial ecosystem, MabDesign's governance has evolved to meet the specific needs of the various companies working in the biotherapy industrial sector. Therefore, the Board of Directors of MabDesign already composed of DBV Technologies, Lyonbiopole, Pierre Fabre and Sanofi, has been strengthened with the arrival of ABL Europe, bioMérieux, Institut Pasteur, Thermo Fisher Scientific and TreeFrog Therapeutics as well as three Qualified Persons with Nicola Beltramineli (Innate Pharma), Hervé Broly (Merck), and Stéphane Legastelois (33 California). Their arrival to the Board of Directors reinforces MabDesign global vision of the current challenges and opportunities of the biopharmaceutical industry.

Moreover, to achieve its goals MabDesign sets up a coherent set of actions promoting exchanges, collaborations and skills development. In this dynamic MabDesign has developed a **national directory** that brings together industrial and academic players in biotherapy and allows to identify online the know-how available in France. MabDesign organizes high-level **international scientific events**, in collaboration with key ecosystem players, to highlight innovation and stimulate exchanges between companies in the sector. With the help of its Scientific Committee (**COSSF**), MabDesign writes summary reports (**ImmunoWatch and BioprocessWatch**) for the biotherapy industry. MabDesign offers specialized and **innovative continuous professional training** solutions to enable companies to adapt their skills to the market evolution and maintain their competitiveness. Finally, MabDesign offers its members a **wide range of services** to help companies of all sizes to optimize their positioning, protect and enhance their innovations, conquer new markets and raise public funds.

Operational since September 2015, MabDesign currently has over **280 member companies** and its diversity is its strength. MabDesign's dynamic network includes pharmaceutical and biotech companies, service providers (eg. CROs, CDMOs, etc), professional training actors, high-tech equipment suppliers and specialized consultants.

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INTRODUCTION

For several years now, MabDesign has been actively participating in national and regional programmes and organising scientific events and gatherings focusing on bioprocessing. In parallel, we have also been providing strategic consultancy services together with various training opportunities to key actors of this field, including academia, public bodies, SMEs and biotech and pharmaceutical companies, that are involved in the shaping of the bioprocessing industry in France through their R&D, innovation, technologies, services and products. In line with these past and current actions and to further our commitment and support to the French bioprocessing industry, MabDesign has launched in 2021 a second information-monitoring letter, the BioprocessWatch series.

Each edition of BioprocessWatch will focus on current challenges, a critical step or a recent innovation linked to the manufacturing of a specific biopharmaceutical product or affecting the whole field. BioprocessWatch will feature invited scientific contributions from academia and/or the industry, the most recent pipeline, economic and financial data (where applicable), insights into the intellectual property related to the theme and opinion articles and interviews from one or two experts working in the field.

Finally, we would like to acknowledge the continued support from the Centre-Val-de-Loire (CVL) region, through their Ambition Recherche et Developpement (ARD) CVL Biomédicaments programme, in making the launching of the BioprocessWatch series possible.

Editorial

Arnaud DELOBEL
Quality Assistance

Félix MONTERO JULIAN
bioMerieux

Alain BECK
Pierre Fabre

Navigating the Analytical Landscape in Modern Bioprocessing

The field of bioprocessing serves as a cornerstone in the biopharmaceutical industry, enabling the large-scale production of biological products for therapeutic applications. As products have evolved from simpler proteins to more complex entities like cellular therapies, monoclonal antibodies, viral vectors, and mRNA-based therapies, the analytical techniques used to characterize them have had to adapt accordingly. Accurate, reliable, and timely analytical data is imperative for making informed decisions at every stage of development—from discovery and process optimization to quality control and regulatory compliance. Particularly in early stages, analytical tools must be capable of generating valuable data without requiring large quantities of precious sample material.

Moreover, these analytical tools play a dual role. They are instrumental not only in characterizing the biological product itself, focusing on its purity, potency, and safety, but also in scrutinizing the process parameters. Such comprehensive analyses are pivotal for process optimization, scale-up, and ensuring the reproducibility and robustness of the manufacturing process. These was recently illustrated for next generation Antibody Drug Conjugates (Dumontent et al, 2023), generation of accelerated CMC data for antibodies and cocktails during Covid-19 pandemic (Broly H et al, 2023), use of Multiple-Attribute Method for Quality Control of mAbs (Butre C et al 2023), of state-of-the-art Mass Spectrometry methods for mAbs structural assessment and residual Host Cell Proteins identification and quantifications (Beaumal C et al, 2023), for biosimilar/ biobetter characterization (Douez E at al, 2023) as well as for many other Biologics discussed during the 10th Antibody Industrial Symposium (Antunes A et al, 2023).

Given the central role of analytical tools in this ever-evolving field, we are honored to serve as co-editors of this special issue, which is dedicated to highlighting some of the most recent advancements and applications of analytical technologies in bioprocessing and cell therapy

The issue opens with a paper from ABL Europe and BioTechne that discusses the Ella system's capabilities for quantifying Adeno-Associated Viruses (AAVs) and associated process impurities. Accurate measurements in this domain are crucial, especially as viral vectors become more central to emerging gene therapies.

ExcellGene contributes to this narrative by detailing the use of Biolayer Interferometry (BLI) in the analysis of monoclonal antibodies. The paper explores BLI's capabilities in measuring multiple critical attributes like protein concentration, ligand-based potency, and effector activities.

Quality Assistance adds another layer to the issue by focusing on the multi-attribute analysis of mRNA using Liquid Chromatography/Mass Spectrometry (LC/MS). The significance of reliable analytical methods for mRNA is heightened as it becomes increasingly vital in novel drug development.

Sartorius brings the spotlight onto Process Analytical Technology (PAT) through the lens of Raman spectroscopy. Their paper emphasizes how Raman spectroscopy methods can be standardized and

transferred across different applications, marking a step forward in the field of real-time process monitoring.

bioMérieus describes how the Analytical methods in Cell and Gene Therapy play a critical role for the control of the manufacturing process, raw materials, intermediates and the final product.

Finally, Merck's research delves into an often-overlooked but critically important aspect of bioprocessing—the role of excipients in reducing viscosity. Their paper offers new insights into formulation and the associated impact on drug delivery systems.

This special issue offers a comprehensive examination of the state-of-the-art analytical tools across various facets of bioprocessing. From viral vectors and monoclonal antibodies to mRNA, and from real-time process monitoring to drug formulation considerations, we hope this issue serves as a valuable resource for researchers and professionals alike.

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MAKING IT POSSIBLE

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Raw material

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Scale -up

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Low volume 1 – 10 L

High volume 10 – several hundred L

Clarification

DSP : Downstream Process

Concentration & Diafiltration

Viral clearance

Sterile filtration

Purification

Clarification

F&F : Fill & Finish

Sterilization

Filling

Distribution

Quality Control

scientific articles

Read the different inputs from the scientific community on various aspects of analytical tools

ANALYTICAL METHODS IN CELL THERAPY

Félix A. MONTERO JULIAN PhD
Global Scientific Director Pharma Quality Control Business Industry application Unit
BioMérieux

Introduction

Analytical methods in Cell and Gene Therapy play a critical role for the control of the manufacturing process, raw materials, intermediates and the final product. These methods are typically conducted in a quality control laboratory, following defined standard operating procedures (SOP's) and used to characterize cells, genes, and other biological components to ensure safety, efficacy, and consistency of these advanced therapeutic products. Analytical methods are also critically important in process development, where they are used to support manufacturing investigations, characterize processes and enable process changes. In both of these settings, a broad set of tools are needed to build the process understanding and product knowledge necessary to advance products through to the clinic.

The purpose of this article is to describe the critical role of analytical methods in the development and manufacturing of Cell and Gene Therapy products to ensure safety, efficacy and consistency of these advanced therapies for the wellbeing of patients.

Analytical methods must meet all regulatory requirements in terms of analytical procedures and analytical validation.

Here are some of the assays and quality attributes measured and their significance in the context of cell therapy products. Later, the key analytical methods/technologies used in Cell and Gene Therapy will be discussed.

Quality attributes

Here are some key analytical methods used in cell therapy.

Assays	Quality attributes	Definition
Cellular characterization	Identity/Phenotype	The identity of therapeutic cells relates to their specific phenotype or characteristics. This includes the surface markers, receptors, and other cell-specific features that define the type and function of the cells
	Viability	Cell viability is a lot release specification with a predefined acceptance criterion. Cell viability is controlled during the manufacturing and formulation process, and the percentage of total live cells.
	Potency	Beyond physical and genetic characteristics, cellular therapy identity may also encompass the functional properties of the cells. This is called potency and includes confirming that the cells can perform their intended biological functions, such as immune response (for immune cell therapies) or differentiation into specific cell types (for stem cell therapies).
	Purity	Purity refers to the proportion of the desired therapeutic cells in the final product. The identity of the cells is closely related to their purity. Contaminating cells or other cell types may compromise the identity and function of the therapeutic cells.

Assays	Quality attributes	Definition
Dose	Viable cell count/ anti-CD marker (e;g; CD3)/ CAR expression	In cellular therapy, the term "dose" typically refers to the quantity or number of therapeutic cells administered to a patient as part of the treatment. It specifies the amount of cellular material that is given to achieve a therapeutic effect. The concept of "dose" is essential in cellular therapy because the effectiveness and safety of the treatment often depends on delivering the right quantity of cells.
Safety assays	Sterility	Sterility testing in cellular therapy is a specialized quality control process used to assess the absence of viable microorganisms, including bacteria, fungi, and spores, in cellular therapy products. Sterility testing is a critical component of ensuring the safety and efficacy of these cellular products.
	Mycoplasma	The detection of mycoplasma contamination is a crucial quality control step in cellular therapy, as mycoplasmas are a type of bacteria that can infect cell cultures and compromise the safety and efficacy of cellular therapy products. Detecting mycoplasma contamination is essential to ensure that cellular therapy products are free from this type of microbial contamination.
	Endotoxins	Endotoxins are a type of bacterial toxin present in the cell walls of Gram-negative bacteria. They can pose a significant concern in the context of cell therapy, as they have the potential to trigger immune responses and adverse reactions in patients receiving cellular therapy products. Endotoxins are recognized as contaminants that must be controlled and monitored during the development and manufacturing of cellular therapy products.
Testing for RCR/ RCL	Replication-competent Lentivirus and Retrovirus	The potential pathogenicity of replication competent virus (RCR) or Lentivirus (RCL) requires vigilant testing to exclude the presence of RCR/RCL in retroviral vector-based human gene cell therapy products.
Vector copy number and genetic stability		Genetic identity is crucial in cellular therapy. It involves confirming that the cells used for therapy have the correct genetic makeup and that they have not undergone any unintended genetic changes during processing and expansion. Maintaining genetic identity is important to ensure the cells' intended function and to minimize the risk of adverse events, such as tumorigenicity. Vector copy number (VCN), a measurement of transgene copies within a CAR T cell products, is a product-specific characteristic that must be quantified prior to patient administration as high VCN increases the risk of insertional mutagenesis. Transgene integration in the drug product is an important safety parameter to measure for CAR T cell release. The VCN should be determined and reported on the Certificate of Analysis (COA) for each lot.
Impurities		Impurities can be classified into product-related (cellular impurities) and process-related impurities. Cellular impurities are those derived from leukapheresis material such as red blood cells, granulocytes, dead cells, and B cells/B-lineage lymphoblasts. Process-related impurities include ancillary materials and reagents that are not intended to be present in the final product.

Analytical technologies

Cell therapy products have specific characteristics, these products are manufactured at low volume, mostly in the range of 50 to 150mL. This has an impact on the QC technology choice. Thus it's important to use the minimum volume of sample, in order to make most of the product available for the patient. The cell therapy products are characterized to have short shelf life, meaning that the analytical method should provide results as fast as possible. Current cell therapy products are manufactured to treat very sick patients, thus every day saved on the QC testing matters. Adding rapid and easy-to-use analytical technologies brings overall efficiencies to the manufacturing process through both simplified workflows and accurate, actionable results to ensure patient safety.

On top of that, these products are manufactured with complex raw materials. Living cells are engineered and expanded and thus bring a lot of more complexity to the manufacturers. These products cannot be filtered or irradiated, making every single manufacturing step critical to avoid contamination and achieve more optimized and reproducible products. In a word the process must be "aseptic", and insure tracking, chain of custody and data integrity. Automation and integrated closed manufacturing solutions are leading the field towards lowering the cost of manufacturing processes.

Let's talk now about technologies.

Cellular Characterization: Flow cytometry is a key technology commonly used to analyze and characterize cell populations based on cell surface markers, allowing manufacturers to assess the purity, viability, and identity of the cells used in therapies.

As an analytical technique, flow cytometry can be used to characterize the physical and chemical attributes of a population of cells or particles qualitatively or quantitatively. The fundamental principles of the technique are well documented in the literature and have been summarized in pharmacopoeia texts (Ph. Eur. 2.7.24, USP <1027>). Efforts to establish best practices for performing flow cytometry in a regulated environment have been made. New compact and portable flow cytometers have now been proposed (Johnson, K., et al 2019).58

Flow cytometry is a widely used but technically complex tool; the practical application of which is recognized to be diverse and often challenging to standardize, so standardization is critical in supporting robust data generation, enabling data comparability between users and instruments. Also, when applied to development of cellular therapies it ensures reproducible product quality, safety, and efficacy throughout the entire product lifecycle.

For cellular counts and viability, specific cell counters using dyes and image-based analysis are commonly used, some technologies on the market also allow one to perform inline testing as well. Flow cytometry can provide viability and enumeration in appropriate settings, using counting beads.

Testing for RCR/RCL: The tools manufacturers currently use to detect RCVs, like the qPCR, pose several limitations due to its limited sensitivity that can be overcome by ddPCT technology. Cell-based assays can be used as well but it often requires considerable time to produce results. Though qPCR delivers results quickly, manufacturers cannot necessarily use it to guarantee batch safety (for example a CAR T cell). Commercially available p24 ELISAs are also used in some cases.

Genetic stability: Assessing genetic stability in cellular products is a crucial step in ensuring the safety and effectiveness of these therapies. The methods used to assess genetic stability can vary depending on the type of cellular product, its intended application, and regulatory requirements. Here are some common approaches and considerations for assessing genetic stability in cellular products:

1. **Karyotyping:** Karyotyping involves the examination of the number and structure of chromosomes within a cell. It is a classical cytogenetic technique used to identify numerical and structural chromosomal abnormalities. Karyotyping can be performed on a representative sample of cells from the product.

2. **Fluorescence In Situ Hybridization (FISH):** FISH is a molecular cytogenetic technique that uses fluorescent probes to detect specific DNA sequences or chromosomes within a cell. It is particularly useful for identifying numerical and structural chromosomal abnormalities.

3. **Comparative Genomic Hybridization (CGH):** CGH is a high-resolution molecular technique that can be used to compare the DNA content of a test sample to a reference sample. It is capable of detecting gains, losses, or imbalances in chromosomal material across the entire genome.

4. **Single Nucleotide Polymorphism (SNP) Analysis:** SNP analysis can identify genetic variations at the single nucleotide level. It is useful for detecting point mutations, copy number variations (CNVs), and other genomic changes.

5. **Next-Generation Sequencing (NGS):** NGS technologies, such as whole-genome sequencing (WGS) or targeted sequencing, can provide comprehensive genomic data. These techniques can detect a wide range of genetic alterations, including point mutations, insertions, deletions, and structural variants.

6. **Short Tandem Repeat (STR) Analysis:** STR analysis involves profiling specific repetitive DNA sequences in the cellular product's genome. It is often used for cell line authentication and to monitor genetic stability over time.

7. **Loss of Heterozygosity (LOH) Analysis:** LOH analysis is used to detect the loss of one allele at a specific genomic locus. It can be indicative of genetic instability, particularly in the context of tumor-derived cellular products.

8. **Functional Assays:** Assessing the functionality of the cellular product can indirectly provide insights into genetic stability. If genetic changes affect the product's functionality, it may indicate instability. Functional assays can include proliferation, differentiation, or specific cell function tests.

The choice of genetic stability assessment methods and criteria should be tailored to the specific cellular product and its intended therapeutic application. Developing a robust testing strategy in collaboration with experts in genetics, molecular biology, and regulatory affairs is essential to ensure the genetic stability and safety of cellular products.

Sterility: The sterility testing of cell therapy products is a critical quality control step to ensure patient safety and product efficacy. Because of the nature of the cell therapy products as indicated above, these products cannot be filtered or irradiated. The current sterility test requires a sample of a product to be placed into aerobic and anaerobic recovery media for a 14 day culture period during which no growth should be observed. This assay, while ideally suited for biopharmaceuticals, is not fit-for-purpose for cell and gene therapy products which are often formulated as drug products containing living cells with a

shelf life of several hours to a few days. In addition, the cellular products generate turbidity in the culture broth making it difficult or impossible to differentiate bacterial growth from the turbidity generated by the cellular product. In this case, cell-growth based methods are used and are considered as the gold standard for product release. The growth of microorganisms is monitored over time through the production of CO2 and the color changes of a probe located at the bottom of the growth vial. However, these methods still take 7 days, and there is a real need for a ultra rapid sterility testing to reduce this test to few hours. Technologies like solid phase cytometry combined with specific lytic reagents may improve the time to detection dramatically moving from 7 days to a few hours.

Mycoplasma Testing: To ensure product safety, cell and gene therapies are tested for endotoxin contamination and mycoplasma infection. Mycoplasma contamination is a significant concern in the development and production of cell therapy products. Mycoplasmas are a type of bacteria that lack a cell wall, making them resistant to many antibiotics and difficult to detect. When present in cell cultures used for cell therapy production, mycoplasma contamination can have detrimental effects on both the safety and efficacy of the final cell therapy product. Detection of mycoplasma contamination is crucial. There are various methods for detecting mycoplasma, including PCR-based assays, enzymatic tests, and staining techniques. Regular testing of cell cultures and raw materials for mycoplasma contamination is essential to ensure product safety. The most common analytical method is the PCR. New assays for mycoplasma detection called “Lab-in a pouch” provide results in one hour, with a minimum of hands-on time, and meeting the expected regulatory requirements in terms of Limit of detection, specificity (inclusivity, exclusivity), robustness and ruggedness, as required by the pharmacopoeias.

Endotoxin testing: Endotoxin detection is a critical aspect of ensuring the safety and quality of cellular therapy products. Endotoxins are a type of bacterial toxin found in the cell walls of Gram-negative bacteria, such as Escherichia coli (E. coli). When present in cellular therapy products, endotoxins can trigger strong immune responses and have detrimental effects on patient safety and product efficacy. The most common method for endotoxin detection is the Limulus Amebocyte Lysate (LAL) assay. New methods using the recombinant factor C, the protein responsible for the endotoxin detection starts has gained traction and provides equivalent results compared to LAL. Unlike LAL, this product is sustainable and is not derived from animals.

Assessment of Cell Functionality: Assessing the functionality of cells in cellular therapy is critical to ensuring the safety and efficacy of the treatment. The specific methods and assays used to assess cell functionality can vary depending on the type of cellular therapy, the intended therapeutic outcome, and the characteristics of the cells being used. Here are some general approaches and considerations for assessing cell functionality in cellular therapy.

1. **Functional Assays:** Functional assays are designed to evaluate the specific functions and properties of the therapeutic cells. These assays should be chosen based on the therapeutic mechanism of action and the intended clinical outcome. Some common functional assays include:

    • **Cytokine Release Assays:** These assess the ability of the cells to produce and release specific cytokines or growth factors. This is important for immune cell therapies, such as CAR-T cells.

    • **Cytotoxicity Assays:** For therapies involving cytotoxic cells, such as natural killer (NK) cells or T cells, cytotoxicity assays measure the ability of these cells to kill target cells or cancer cells.

- **Functional Activity Assays:** Assess the functional activity of cells in vitro, such as phagocytic activity for macrophages or the ability to form functional blood vessels for endothelial progenitor cells.

- **Cell Signaling Assays**: Evaluate signaling pathways within cells to ensure that the therapeutic cells are responding appropriately to their microenvironment.

2. **Surface Marker Expression:** Flow cytometry or immunohistochemistry can be used to assess the expression of specific cell surface markers, receptors, or antigens that are indicative of the desired cell phenotype and function.

3. **Proliferation and Expansion Assays:** Monitoring the ability of therapeutic cells to proliferate and expand in culture is essential for ensuring an adequate cell dose for treatment. This can be assessed through cell counting, cell cycle analysis, and measurement of population doublings.

4. **Immunological Assays:** In some cases, it may be important to evaluate the immunological properties of the cells, such as their ability to modulate immune responses, suppress inflammation, or promote tolerance.

5. **In Vivo Functionality:** For many cellular therapies, the ultimate test of functionality is their performance in vivo. Animal models or clinical trials are used to assess therapeutic efficacy, safety, and durability of response.

Conclusion

These analytical methods are continuously evolving and being refined to meet the unique challenges of cell and gene therapy development and commercialization, ensuring safe and effective treatments for patients with various medical conditions.

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ANALYTICS MADE SIMPLE: ADVANCING THE AAV MANUFACTURING PROCESS WITH PRECISE, EFFICIENT QUANTIFICATION AND CHARACTERIZATION USING ELLA™.

Mathilde ARNAULT and Violette VINCENT
Bio-Techne / ABL

Raising the bar in AAV characterization capabilities

Adeno-associated virus (AAV) has emerged as a leading vector for gene delivery for treating various diseases due to its safety profile and efficient transduction of various target tissues. AAV, like other viral vectors, are complex molecules leading to challenges in their characterization.

ABL's viral vector manufacturing site in Lyon, France is advancing its AAV process development and manufacturing platforms and is becoming an industry leader in AAV manufacturing and control on several serotypes (currently AAV2/5/6/8 and 9).

In recent months, ABL has been able to manufacture AAVs from the harvest stage (out of HEK293 cell line) through clarification, tangential flow filtration [TFF] (concentration diafiltration), capture and polishing chromatography. See Figure 1.

Those downstream process purification steps have allowed for a reduction of process impurities while enriching the ratio full/empty of AAV particles.

Capsid titer and process impurities are some of the main metrics in AAV manufacturing but can be challenging to evaluate. This article describes how ABL is using the Ella platform in its AAV manufacturing process and outlines some of its key benefits for AAV characterization and quantification.

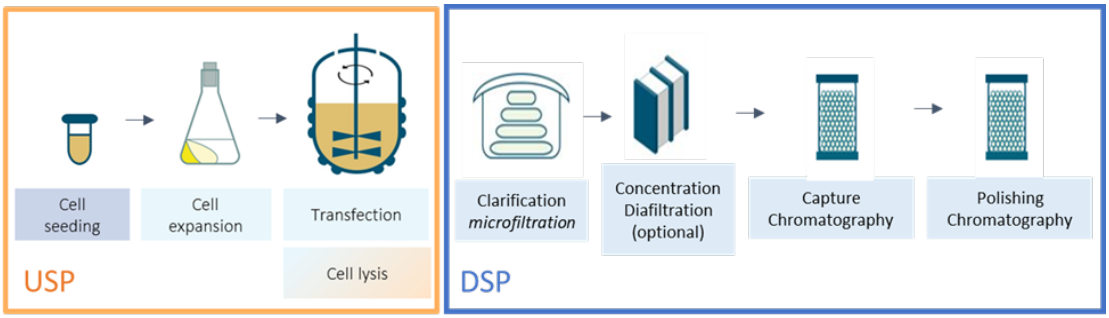
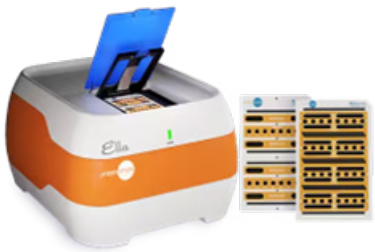


Figure 1: *AAV manufacturing – USP (Upstream) and DSP (Downstream) Steps*

Gaining a performance advantage



Ella, a Bio Techne instrument, is a fully automated ELISA platform that provides fast, accurate quantitation of many different targets.

Ella's cartridges contain fully validated Simple Plex™ assays powered by R&D Systems to deliver accurate, highly reproducible data in less than 90 minutes. Advanced microfluidic circuitry performs every step (reagent loading and wash) of the assay process, reducing manual steps and variability to help minimize user error. Ella assays generate triplicate data while requiring as little as 30µL of sample volume.

With Ella's Simple Plex cartridge-based assays, you get consistent, accurate results from lot-to-lot as well as a linear correlation to the available commercial kits, but on a much larger standard range (up to 4 logs of dynamic range).

ABL is using this advanced technology to assess its AAV capsid titers on several serotypes and to quantify process impurities such as endonuclease and host cell proteins. The automated Ella platform has allowed ABL to test a variety of AAV assays on its different manufacturing matrices and AAV products.

Measuring AAV capsid titers

Determination of AAV capsid titer is a key parameter to monitor the performance of the AAV-manufacturing platform and assess the full/empty capsids ratio over the process.

Initially, ABL used a standard ELISA Kit in its QC laboratory for capsid titer measurement. However, the limited dynamic range (less than 2 logs) and the higher variability of the assay due to the manual steps required to run at least three dilutions per sample resulted in suboptimal efficiency and reliability. With 13 samples per plate and a time to result of four hours per plate, the standard ELISA kits were unable to meet the speed and throughput demands ABL needed for its AAV analysis.

The Ella platform offers cartridges for serotype AAV2, AAV6 and AAV8 (Cat. Nos. SPCKB-OT-005905; SPCKB-OT-007971; SPCKB-OT-007970) and features up to 4 logs of dynamic range, with a lower limit of quantitation (LLOQ) from about 3E+06 vp/mL depending on the serotype. Another important advantage of the Ella platform is the ability to run up to 34 samples, with two dilutions, per cartridge.

Ella delivered consistent, accurate capsid titer measurements, confirming the results from the standard ELISA method while reducing variability, as shown below (see Table 1 and 2).

For the first AAV2 capsid titer test, results for the positive control (Vigene empty reference in AAV2: Reference RS-AAV2-ET by Charles River) showed a recovery of 95% versus the expected value (with CV between all 3 dilutions of 1,9%).

The ELISA Standard (Kit Control: KC) also showed a good recovery versus its expected titer (see Table 1 below).

Regarding samples from the various process steps, a recovery from 75% versus ELISA was obtained with Ella (see Table 2 below).

Expected titer (vp/mL)	Ella titer (vp/mL)	% CV (3 dilution)	Recovery versus Progen ELISA Xpress kit
2,30E+09	2,23E+09	3,8%	97%

Table 1: *Kit Control Empty capsid AAV2 Positive control*

Serotype	Sample Type	Titer (vp/mL)	% CV (3 dilution)	Recovery versus Progen ELISA Xpress kit
AAV2	Harvest	5,37E+11	1,2%	79%
	Harvest	1,88E+12	3,5%	81%
	Capture Chromatography : Elution	4,68E+12	5,6%	84%
	Capture Chromatography : Elution	1,99E+11	3,0%	80%
	Clarification	1,86E+12	1,9%	75%

Table 2: *AAV2 Capsid titer obtained with Ella and comparison with ELISA Progen kit*

The robustness of the Ella platform was confirmed on a large panel of samples from various serotypes (AAV2, AAV8, AAV6) and at different process steps, from unpurified to purified samples, without any issue with the microfluidics. Figure 2 represents the % CV between 2 dilutions per sample from crude harvest stage to post polishing chromatography. All the CVs were below 20% and mainly below 10%. With over 83 samples tested, the mean % CV between 2 dilutions obtained was 5%.

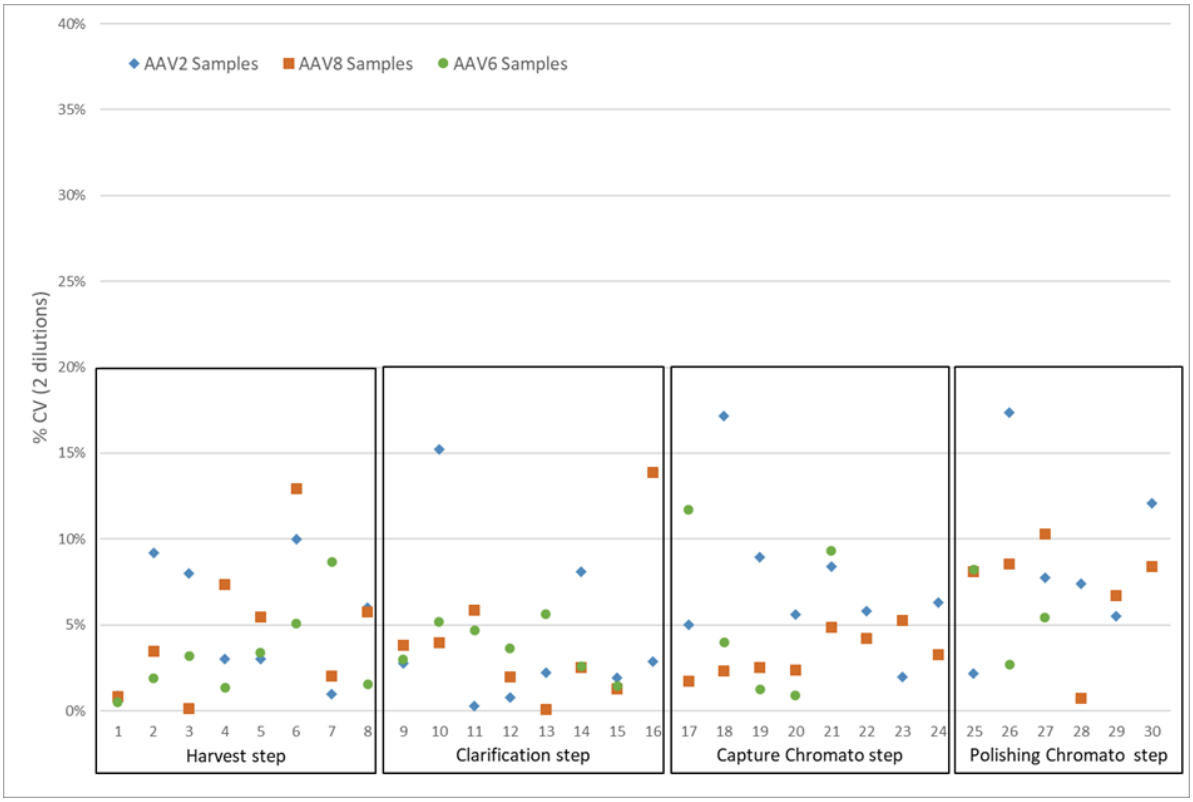


Figure 2: *CV inter dilution for AAV2, AAV8, AAV6 samples at various process stage.*

A Reference material composed of empty AAV from PROGEN was tested as positive control in each Ella run, with a recovery between 80% and 120% for all serotypes, as shown in Figure 3.

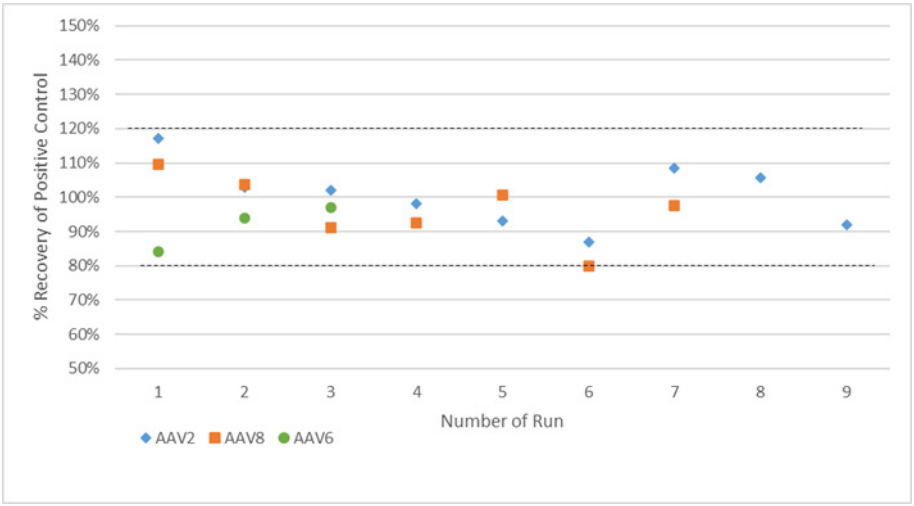


Figure 3: *Recovery of Positive control (empty AAV2, AAV8, AAV6) over the runs*

Ella offers a fast, efficient, and highly reliable screening tool for AAV process development. In Figure 4, a Design of Experiment (DoE) was performed to optimize the USP part of AAV6 manufacturing, by testing several concentrations of DNA (plasmids of interest); the ratio between the plasmid; and the ratio with the transfection reagent. Thanks to Ella, in complement to genome titer determination, several conditions were identified to improve the performance of the transfection process (See Figure 4 below).

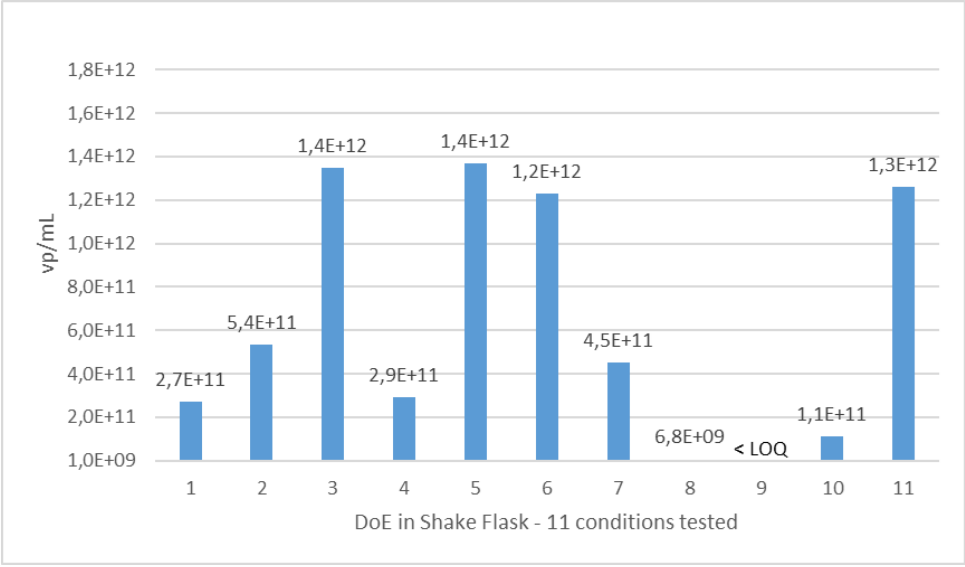


Figure 4: *AAV2 Capsid titer - DoE in Shakeflask*

Overall, for each of the three serotypes, transfer from ELISA to Ella routine testing has been performed in one run. The results obtained for Ella cartridges are comparable to results obtained with ELISA (positive controls and AAV samples) but the CVs are much lower (5% on average on Ella versus 15-20% for ELISA).

Residual endonuclease quantification

Endonuclease enzymes are widely used in AAV process development to degrade DNA coming from host cells or residual plasmids used to transfect the cells. This helps reduce the amount of residual DNA in the final product. Therefore, this is a regulatory requirement to verify that residual endonucleases have been removed though the purification process.

The Simple Plex Endonuclease assay on Ella provides a highly sensitive and specific solution for detecting residual impurities in bioprocess samples and detect both Benzonase® or Denarase® (Cat. No. SPCKC-OT-008612).

The assay offers a 4-log dynamic range with a lower limit of quantitation (LLOQ) at 1.97 pg. The quantification range was evaluated by testing serial dilutions of endonuclease positive control (Cat. No. 896993). Recovery between 86% and 106% were obtained for concentration between 7 to 11,250 pg/mL.

Various samples generated from the ABL AAV8 manufacturing platform were tested. Benzonase was added at the harvest stage to digest residual DNA and was quantified at several process steps: harvest, post clarification and post capture chromatography.

The results presented in Figure 5 indicate a reduction of residual Benzonase after a clarification step and a complete removal of the residues in the flow through of the capture chromatography: from a crude harvest containing 50,700 pg/mL of residual Benzonase, 11 pg/mL were quantified in the elution sample

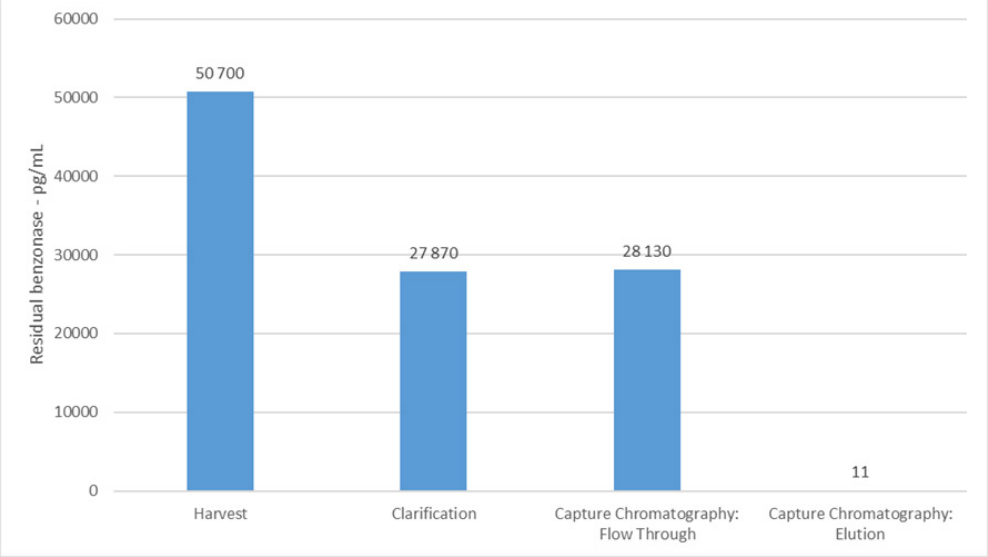


Figure 5: *Benzonase quantification in different bioprocess samples.*

All the quantifications were determined based on two dilutions (except for the elution samples that was tested pure) and a CV inter dilution. Less than 14% was obtained for all the samples tested in this study, showing the superior precision of the Simple Plex endonuclease assay.

No matrix interference was observed in various bioprocess samples: samples with a final dilution factor up to 1:20 in the cartridge were spiked with the positive endonuclease control. A recovery between 83% and 92% was obtained, as presented in the Table 3.

Serotype	Sample Type	Endonuclease spike recovery (%)
AAV8	Harvest	89 %
	Clarification	83 %
	Capture Chromatography : Elution	92 %

Table 3: *Endonuclease spike recovery in different process steps*

Residual Host Cell Protein quantification

With AAVs being manufactured into HEK293 cell line, Host Cell Protein impurities are created along the process. It is therefore necessary to quantify the remaining HCP impurities.
Regulatory guidelines require the verification of residual HCP removal though the purification process.

The Simple Plex HCP assay on Ella provides a highly sensitive and specific solution for detecting residual impurities and HEK293 Host Cell Protein in bioprocess samples. (Cat. No. SPCKB-OT-007066).

The assay offers a 3-log dynamic range with a lower limit of quantitation (LLOQ) at 1.64 ng/mL. The quantification range was evaluated by testing serial dilutions of HCP Cygnus positive control. Recovery between 88% and 122% were obtained for concentration between 10 to 200 ng/mL.

Positive controls from Bio-Techne (Cat. No. F6353-1-SPC) were also evaluated pure. Recovery between 96% and 108% were obtained for concentration between 5 to 255 ng/mL.

Various samples generated from the ABL AAV2, AAV6 and AAV8 manufacturing platform were tested. HCP impurities were quantified at several process steps: harvest, post clarification and post capture chromatography.

The results presented in Figure 6 indicate a reduction of residual HCP after a clarification step. Only results obtained for CVs < 20% are shown below. Higher CVs were obtained for post capture chromatography highlighting the high removal of those impurities after this stage and the matrix effect.

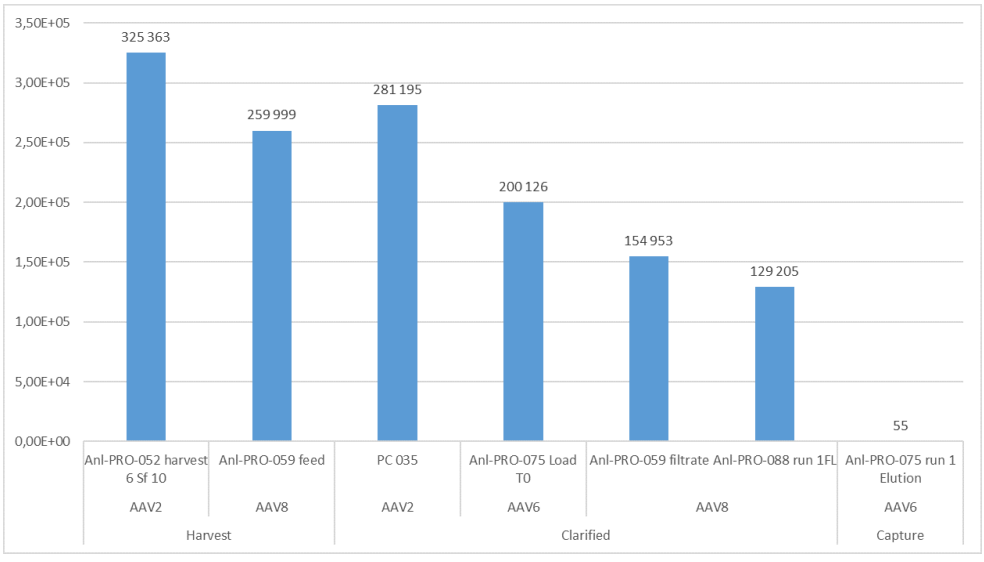


Figure 6: *HCP quantification in different AAV samples.*

All the quantifications were determined based on two-to-four dilutions. For dilutions above 5,000-fold, a CV inter dilution lower than 20% was obtained for all the samples tested in this study, showing the superior precision of the Simple Plex HCP assay.

However, for lower dilutions (up to 4-fold) CVs obtained were higher than 20%., Additional testing would be required to define the minimum dilution required to remove any potential matrix interference. See table 4 for detailed results.

Purification step	Serotype	Sample name	Dilution factor in cartridge	Sample [C] (ng/mL)	CV (%)	Validity
Clarified	AAV2	PC 035	5,000 to 40,000	2,81E+05	9%	valid
Clarified	AAV8	Anl-PRO-059 filtrate	5,000 to 40,000	1,55E+05	5%	valid
Harvest	AAV8	Anl-PRO-059 feed	5,000 to 40,000	2,60E+05	6%	valid
Harvest	AAV2	Anl-PRO-052 harvest 6 Sf 10	5,000 to 40,000	3,25E+05	11%	valid
Clarified	AAV6	Anl-PRO-075 Load T0	5,000 to 40,000	2,00E+05	10%	valid
Clarified	AAV8	Anl-PRO-088 run 1FL	5,000 to 40,000	1,29E+05	11%	valid
Capture	AAV8	Anl-PRO-088 Elution	2 to 4	7,66E+01	29%	not valid
Capture	AAV6	Anl-PRO-075 run 1 Elution	2 to 4	5,47E+01	16%	valid
Capture	AAV6	Anl-PRO-075 run 2 Elution	2 to 4	1,30E+01	66%	not valid
Polishing	AAV2	Anl-PRO-060 Load	2 to 4	NA	NA	NA
Polishing	AAV2	Anl-PRO-060 Mustang E2	2 to 4	NA	NA	NA

Table 4: *HCP quantifications and CV results for all samples*

No matrix interference was observed in various bioprocess samples diluted with a minimum factor of 5,000-fold: samples with a final dilution factor of 10,000 and 20,000 in the cartridge were spiked with the positive HCP control. A good recovery was obtained, as presented in Table 5.

Serotype	Sample Type	Dilution factor in cartridge	Spike recovery (%)
AAV8	Anl-PRO-059 feed (Harvest)	10,000	98%
		20,000	113%

Table 5: *HCP Spike recoveries*

Accelerating Processes through greater analytical speed and precision

Ella offers an efficient and highly reliable tool to quantify both capsid titer and process impurities, such as residual endonuclease and host cell protein along the AAV process.

Implementation of Ella for capsid determination has allowed ABL to increase the throughput in its laboratory, including testing 34 samples in less than two hours while improving the precision of the results, with a mean 5% CV between two dilutions, obtained over a large panel of AAV serotypes and samples (from harvest to post polishing chromatography). With a large dynamic range and no matrix impact observed on the microfluidics part, Ella offers a highly reliable and easy-to-implement analytical solution for samples coming from AAV manufacturing processes.

About ABL

ABL is a pure play CDMO specialized in the development and manufacturing of gene therapies, oncolytic viruses and vaccine candidates. Our mission is to provide GMP viral vectors from early-stage to market. Our services include manufacturing of bulk drug substance, fill-finish of drug products, process and assay development as well as regulatory support. ABL is a subsidiary of the Institut Mérieux and operates in Europe and the US.

We will be glad to give you more information, please contact us at contact@ableurope.com

SINGLE STEP QUANTITY AND QUALITY: ASSESSMENTS OF IMMUNE-EFFECTOR FUNCTIONS AND POTENCY OF ANTIBODIES BY BIOLAYER INTERFEROMETRY.

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Introduction

During the pre-clinical development of monoclonal antibodies (mAbs), biopharmaceutical companies need to be able to rapidly screen the quality and quantity of the antibodies they produce. This is especially important during early process development, when the leading clonally-derived cell populations (clones) need to be selected and when the upstream and downstream processes are being developed. The use of affinity chromatography (HPLC-Protein A) is the standard analytical approach for determination of mAb protein titers in Quality Control (QC) labs, and it allows for the screening of hundreds of clones within a day on a single HPLC instrument. However, the determination of other relevant Critical Quality Attributes (CQAs), such as the potency and effector function of the tested mAb, requires the use of more complex and time-consuming analytical techniques. Apart from the quantity (titer) of a mAb, the most important CQA is its biological activity. After binding to their targets, IgGs exert important downstream effector functions by inducing Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) and Complement-Dependent Cytotoxicity (CDC) through binding to multiple Fc-γ receptors (FcγRs) or Complement components, respectively.

EMEA and FDA require rigorous testing on Fc effector function for regulatory filings and potency lot release.

As a part of the testing, in vitro binding kinetics of therapeutic mAb candidates are routinely measured using functional cell-based assays, ELISA, Fluorescence Resonance Energy Transfer (FRET) and Surface Plasmon Resonance (SPR). Typically, these analyses take several hours or even days to complete and they require trained operators, specialized equipment and suitable reporter cell lines. Moreover, most of these require substantial quantities of highly purified protein, which are rarely available during the early stages of process development.

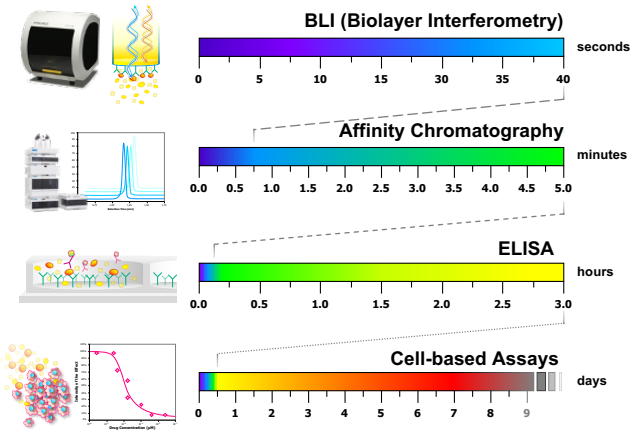


Figure 1: *Comparison of the runtimes of selected analytical techniques routinely used by QC laboratories for the assessment of protein titers in complex biological matrices and of the potency of the contained protein of interest.*

To tackle these challenges, ExcellGene has developed a novel, streamlined analytical approach that allows for the parallel determination of protein concentration, ligand-based potency, and effector activities of an IgG within two hours. This workflow utilizes Biolayer Interferometry (BLI), a technology that enables the determination of the specific binding of a ligand with a target molecule within minutes. BLI is not only faster than other techniques (Figure 1) but it also allows for parallel determination of the binding kinetics and quantification of the analyte. BLI's capacity to efficiently analyze small volumes of

both purified and non-purified samples, including culture supernatants (~ 40 - 200 µL), speeds up and simplifies early bioprocess development.

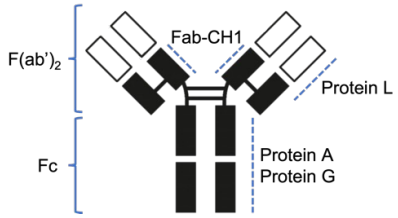
We present here a workflow for the determination of the concentration of a mAb in a sample, as well as its interaction kinetics with specific FcγRs and complement components responsible for activation of ADCC and CDC immune responses. In the next paragraphs, we summarize the test and present how this methodology can be used for early development of biosimilars.

Overview of the BLI method

Accurate antibody quantitation is critical for selecting top cell lines when developing and optimizing manufacturing. Traditional methods for measuring antibody concentration include HPLC, ELISA and densitometry, all of which are affected by long runtimes as well as lack of specificity and precision. BLI offers instead a versatile approach to measuring and characterizing molecular interactions and to quantify IgG with various biosensor chemistries, thus its use streamlines a variety of bioprocessing applications by providing precise results which require minimal sample handling and give rapid results.

A large portfolio of compatible biosensors (Figure 2) can be used to quantify mAbs in buffer solutions, in medium, or in any other complex matrices. The choice depends on the binding strategy, IgG type and the orientation of mAb capture on the biosensor's surface. All these options are available at ExcellGene to implement different optimized experimental designs.

(A)



(B)

Biosensor types	Binding area (chemistry)	Affinity			
		IgG1	IgG2	IgG3	IgG4
ProA	Fc (Protein A)	S	S	W	S
ProG	Fc (Protein G)	S	S	S	S
ProL	F(ab')2 (Protein L)	S	S	S	S
FAB2G	F(ab')2 (Fab-CH1)	S	S	S	S

S=strong W=weak

***Figure 2:* A.** *Simplified scheme showing the binding sites (dotted lines) of Protein A, Protein G, Protein L and anti-Fab-CH1 to the heavy and light chain regions of the antibody.* **B.** *Available biosensors for human mAb quantification and their chemistry and affinity for human IgG (S. Strong, W. Weak). Additional biosensors are available for different species and Ig isotypes.*

The analytical workflow is illustrated in Figure 3 (top). To determine mAb titers, the BLI system measures the rate of IgG binding to the surface of biosensors. Higher antibody concentrations result in faster binding rates. The analytical software calculates a concentration from each rate based on the values of a standard curve made starting from suitable reference material, either in terms of the nature of the IgG or the presence of known post-translational modifications (Figure 3). To reduce possible non-specific binding or matrix effects, it's advisable to have both the samples and the standard in the same matrix solution. All used biosensors can easily be regenerated using an acidic solution. These individual analytical steps integrate into an all-in-one global analysis approach, based on the regeneration of the same biosensors, to greatly reduce analysis time, costs and the number of required tests.

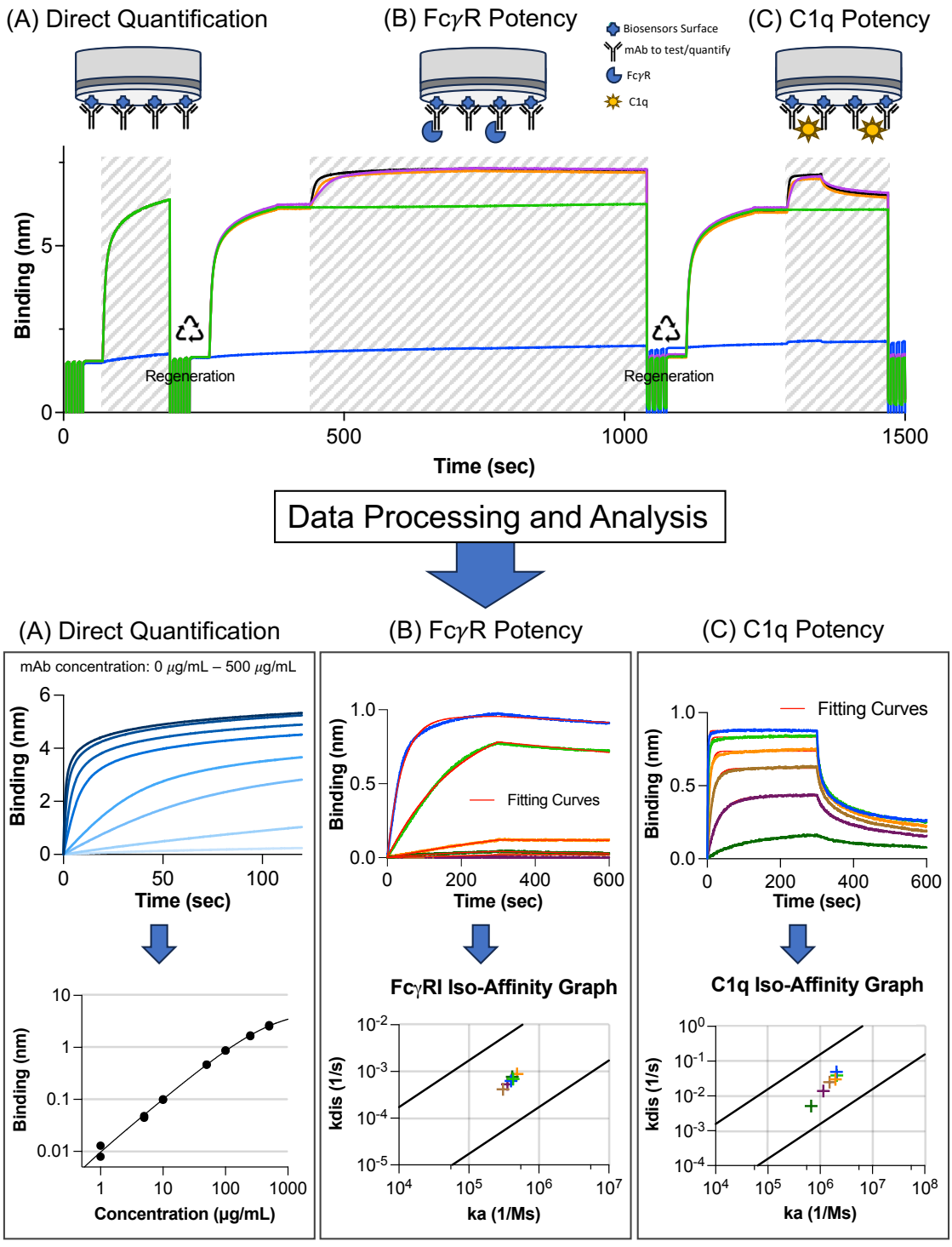


Figure 3: *An overview of the analytical BLI workflow for mAb characterization, while each step is detailed in the lower panels.* **A.** *Determination of the protein titers (FAB2G quantification of Ig1). Processed affinity curves (following baseline and biosensors reference subtraction) for known concentrations of a suitable reference material are used to determine a calibration curve and sample concentrations are interpolated from it.* **B.** *Determination of the ADCC effector activity. Processed affinity curves for different FcγRI (CD64) concentrations are used to determine the affinity constant (KD) of tested mAb (ka & kdis), represented as an Iso-Affinity graph (Black lines are the upper and lower limits for 10 log around the KD)* **C.** *Determination of the CDC effector activity. Similarly to ADCC, processed affinity curves are shown for different C1q concentrations and used to determine the affinity constant (kD).*

FcγR potency (ADCC effector activity) and C1q potency (CDC effector activity):

The ability of therapeutic mAbs to bind FcγRs can greatly impact their therapeutic efficacy, pharmacokinetics and safety. Binding affinities of FcγRs to mAb candidates can be determined with high sensitivity by high throughput analysis by BLI. The first step is the selection of the biosensor and thus the design of the interaction study. We chose to immobilize the mAb on the biosensor using the F(ab')2 region to expose the Fc fragment for interaction with various FcγRs to develop a fully-integrated label-free analytical approach.

The second step is the selection of the most suitable FcγR for routine mAb analyses. This choice depends on the nature of the tested IgG, as not all FcγRs have the same affinity for different IgGs (Table 1). The suitable concentration range also depends on the selected FcγR and its affinity for mAb. As with any kinetic assay, it is critical to perform proper assay development when analyzing FcγR binding interactions so that resulting affinity and kinetic constants are accurate, reliable and reproducible. The quality of the kinetic data depends on using optimal conditions for the biosensor format and the binding pair. Consideration must be given to assay components such as ligand loading density, analytes concentrations, buffer conditions, and assay step times. Here, all these parameters have been optimized to guarantee consistent analysis quality. For simple binding assays or qualitative analyses, measuring binding curves for a single analyte concentration can be sufficient. However, when accurate kinetic and affinity constants are required, a dilution series of the analyte at different concentrations must be measured in the association step (Figure 3). Running several concentrations also allows to highlight how well the fitted binding model applies over a concentration range around the KD. The optimal analyte concentration range also depends on the sensitivity of the assay and the affinity of the interaction.

		Affinity			
		IgG1	**IgG2**	**IgG3**	**IgG4**
FcγRI		Strong	NB	Strong	Strong
FcγRII	A	Medium	Weak	Weak	Weak
	B	Weak	Weak	Weak	Weak
FcγRIII	A	Med	Very Weak	Med	Weak
	B	Weak	NB	Weak	NB
FcγRn*		Medium	Medium	unknow	Med
C1q		Strong	Weak	Strong	NB

*At pH 6.0 only, no binding at pH 7.5 / NB= No Binding
Classification of affinity: Strong, KD in nM range; Medium, KD in □M range; Weak, KD in mM range

Table 1: *Affinity of human FcγRs for human IgG subclasses.*

IgGs also exert another important effector function by inducing the CDC immune response, the efficacy of which largely varies due to differential binding to C1q (Table 1). This is the initiation of the classical CDC pathway. Similarly to FcγRs potency testing, ExcellGene developed a test to evaluate and compare C1q potency with the same rigorous approach used for ADCC (Figure 3). The method was also able to discriminate between different IgG subclasses, as demonstrated by the expected absence of interaction between the IgG4 Pembrolizumab and C1q together with a slightly lower affinity for the FcγR CD64.

Quick facts in a bulleted form:

- Dynamic Range: 1-1000 µg/mL for most IgGs.
- Throughput: 8 samples in ~20 min (Quantification + FcγR and C1q potency) – each step can be run separately.
- Precision/accuracy: < 10%
- Limit of detection: IgG-dependent but typically 1-2 µg/mL
- Form crude extract to purified material – it allows to monitor each stage of process development.
- Possibility to test the effects of post-translational mAb modifications (Fc mutations, N-Glycosylation, etc.)

Application of the analytical workflow to biosimilars

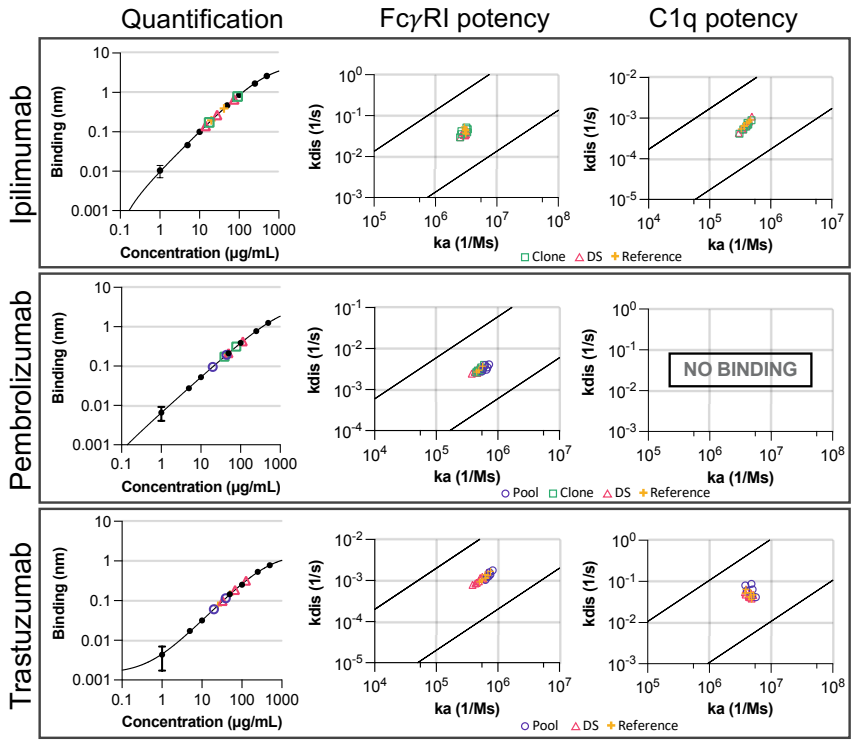
To explore the validity of the selected analytical approach, different lots of commercial therapeutic mAbs such as Herceptin® (Trastuzumab, IgG1), Yervoy® (Ipilimumab, IgG1) and Keytruda® (Pembrolizumab, IgG4) - were compared with biosimilars manufactured at ExcellGene and sampled at different steps of the bioprocess (Figure 4).

Briefly, the different biosimilars manufacturing intermediates at unknown concentrations were assayed using ProA/FAB2G biosensors (FAB2G presented in this article) to determine their protein titers later used as the loading quantity for the potency assays. For all antibodies, it is important to check that the selected concentrations are all within the application range of the test corresponding to the limits of the quantification range. The originators used as a reference material were also assayed to check that the quantification standard was correctly defined.

Each antibody was then tested against different concentrations of the FcγR CD64 and of C1q to assess the ADCC- and CDC-related potency of the Fc fragment, respectively. Identical curves were clearly observed for all the tested biosimilars at different stages of the bioprocess and were similar to the sensorgrams obtained for the corresponding commercial originator lots (Figure 4). The corresponding calculated affinity constants for the originators and biosimilars, as well as the biosimilars produced by pools and clones were equal within the standard deviation. This demonstrates the superior quality of biosimilar cell lines generated at ExcellGene using its proprietary CHOExpress® host cells.

Conclusions

The analytical workflow here presented proves to be faster than most of the standard orthogonal methods QC labs apply for the routine screening of clones in mAb pre-clinical development. It is robust and highly reproducible, doesn't require the prior purification of the protein of interest and it's a non-destructive assay which requires as little as 50 µL of diluted sample material. This already grants a striking advantage to ExcellGene's clients in facing analytical challenges and gaining the ability to take informed decisions very early in the bioprocess. Furthermore, the direct comparison of the measured potency and effector functions (ADCC, CDC) with the ones of historical commercial lots of the originator already present on the market provides reliable information on the bio-activity of the manufactured mAbs in support to the biosimilars development programs proposed to clients (Figure 4).



	FcγRI Potency K_D	C1q Potency K_D
Clone (ExcellGene product)	1.61 nM (±0.174 nM)	13.7 nM (±1.67 nM)
DS (ExcellGene Product)	1.73 nM (±0.221 nM)	12.7 nM (±1.41 nM)
Reference (Ipilimumab, Yervoy®)	1.80 nM (±0.097 nM)	14.3 nM (±2.20 nM)

	FcγRI Potency K_D	C1q Potency K_D
Pool (ExcellGene product)	5.40 nM (±0.572 nM)	NA
Clone (ExcellGene product)	6.08 nM (±0.353 nM)	NA
DS (ExcellGene Product)	6.24 nM (±0.509 nM)	NA
Reference (Pembrolizumab, Keytruda®)	6.19 nM (±0.312 nM)	NA

	FcγRI Potency K_D	C1q Potency K_D
Pool (ExcellGene product)	1.91 nM (±0.186 nM)	12.7 nM (±5.34 nM)
DS (ExcellGene Product)	2.04 nM (±0.149 nM)	10.5 nM (±1.98 nM)
Reference (Trastuzumab, Herceptin®)	2.02 nM (±0.167 nM)	10.2 nM (±2.12 nM)

Figure 4: *Determination and comparison of the binding kinetics of the indicated mAbs biosimilars developed at ExcellGene against the corresponding commercial originator lots with either the CD64, the Fc-γ receptor responsible for ADCC signal cascade initiation, or the complement component C1q responsible for the CDC effector response. Three different concentrations of FcγRI and C1q were tested for at least 2 dilutions of mAb at each step. The binding curves were analyzed as described above. The obtained association and dissociation constants were plotted for each biosimilar produced from clones or pools compared to the originators. The data acquired are summarized in the table where the average affinity constants are shown for each construct/step. The corresponding calculated affinity constants for the originators and the biosimilars produced at ExcellGene are highly congruous and within the experimental standard deviation.*

About ExcellGene

ExcellGene has been in cell line and process development for biologics for over 20 years (DNA to manufacturable process). The company successfully developed and optimized over 500 cell lines and processes, which have delivered high-quality materials for dozens of clinical trials. The in-house developed workflows and state-of-the-art CHO and HEK-293 expression platforms are able to produce cell lines within record time frames and reach for antibodies or antibody-like molecules titers of 5-10 grams per liter.

As shown here, our versatile platform accommodates a wide range of applications, including challenging to express therapeutics (fusion and multi-specific proteins, cytokines, etc.), antibodies and biosimilars, exosomes and viral vectors. If you are looking for assessment of manufacturability of multiple candidate proteins, our early-stage manufacturability assessment can provide you with all information you need in a matter of weeks in a very affordable way.

Furthermore, we provide protein and host cell engineering services, encompassing construct optimization, ADCC and CDC effector function enhancement, gene knock-in and knock-out, half-life extension and glycan profile modifications.

We will be glad to give you more information, please contact us at contact@excellgene.com

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MULTI-ATTRIBUTE MONITORING OF THERAPEUTIC MRNA BY LC-MS.

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Introduction

In recent years, therapeutic messenger RNA (mRNA) has emerged as a game-changer in medical science, [1] offering transformative approaches in the creation of prophylactic vaccines and gene therapies. Unlike traditional vaccines, which utilize attenuated viruses or antigenic proteins to elicit immune responses, mRNA-based vaccines deploy sequences encoding specific antigens to induce robust immunological defense against targeted pathogens. This innovative approach not only streamlines vaccine development[2] but also enables rapid adaptation to emerging epidemics and mutated virus variants. Beyond vaccines, the utility of therapeutic mRNA extends to gene therapy,[3] providing a strategy to correct or replace defective genes associated with genetic disorders, and in innovative approaches to treat certain cancers. Therapeutic mRNAs are commonly encapsulated in lipid nanoparticles to facilitate their delivery to cellular ribosomes, where they are translated into polypeptide sequences. Structurally, an mRNA molecule consists of a central open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs). Critical to mRNA functionality are two terminal features: the 5' cap and the 3' poly(A) tail.

The 5' cap serves multiple roles, including the protection of mRNA from exonuclease-mediated degradation and facilitation of ribosomal binding during translation initiation. This cap is primarily composed of 7-methylguanosine (m7G) connected via a 5' to 5' triphosphate linkage to the mRNA's first nucleotide. Depending on the methylation status of this first nucleotide, two distinct structures—Cap0 (unmethylated) and Cap1 (methylated)—may form. Cap1 mRNA, which is the expected structure, can be produced through either post-transcriptional enzymatic capping or co-transcriptional incorporation of a cap analog.[4]

The 3' poly(A) tail, a series of adenine nucleotides, protects mRNA from degradation and improves its translation efficacy by interacting with key proteins in the translation machinery [5]. This tail can be either directly encoded in the DNA template for in vitro transcription or enzymatically added post-transcription using poly(A) polymerase.[6]

Given their importance for obtaining a fully functional therapeutic mRNA, capping efficiency (i.e., the percentage of Cap1 structure obtained after in vitro transcription) and poly(A) tail length distribution are two critical quality attributes (CQAs) for mRNA.[7] While methods to analyze these CQAs exist, they often necessitate specific primers for hybridization and rely on RNAse H, [8] requiring adjustments for each cap and poly(A) tail to be analyzed and lacking a universal platform for assessment.

In this study, we introduce optimized methodologies for the comprehensive characterization of poly(A) tail length and capping structures. By refining sample preparation protocols, we have developed a single workflow that allows simultaneous assessment of both CQAs, thereby minimizing sample usage and reducing experimental time. Our approach leverages a combination of multiple RNases, a purification step, and liquid chromatography-mass spectrometry (LC/MS) for detailed analysis. Additionally, we have incorporated an optimized extraction step to facilitate the characterization of lipid formulations in drug products.

Results

Development of methods

We developed and refined analytical methods using a commercially available Cas9 mRNA as test subject. The manufacturer specifies that this mRNA is co-transcriptionally capped, resulting in the presence of Cap1 (m7GpppAm2) structures. However, no information was provided concerning the length of the poly(A) tail.

For capping analysis, Nuclease P1 was employed in the sample preparation stage, as it cleaves all phosphodiester bonds involving only one phosphate group, thus leaving the triphosphate of the cap intact. To achieve effective separation of various cap species from nucleotide monophosphates (NMPs)—the most abundant molecules produced post-digestion—we utilized a di-butylaminoacetic acid (DBuAA) gradient. As depicted in Figure 1A and 1B, this gradient successfully segregated all potential cap species from NMPs. Using cap standard curves and UNIFI's automated process, we were able to compute mRNA sample cap abundancies and quantify capping percentages. The analysis revealed that 90.2% of the Cas9 mRNA was capped with Cap1 (Figure 1C), a result consistent with expectations based on the co-transcriptional capping method employed by the manufacturer.

To assess the distribution of poly(A) tail lengths, we utilized RNAse T1, which cleaves at the 3' end of guanine (G) nucleotides. This enzymatic reaction generated short oligonucleotides and poly(A) tail, which were then subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. Due to limited chromatographic resolution for long oligonucleotides under these conditions, all poly(A) tails were visualized under a single chromatographic peak. This simplified the mass spectrometry data processing, as it necessitated the analysis of only a single mass spectrum (Figure 2A). Deconvolution of the obtained mass spectrum (Figure 2B) allowed us to determine the molecular weights of the various poly(A) tails present (Figure 2C). We utilized an automated Python script for peak identification and poly(A) tail length calculations. Our analysis revealed that the most abundant poly(A) tail was A123, which accounted for 13.1% of the total population, as shown in Figure 2D.

Analysis of a drug substance

Recognizing that sample availability can often be a constraint, especially during early developmental stages, and given the high demand on mass spectrometers and technical personnel, we sought to improve the efficiency of sample preparation and reduce the time commitment required for experiments. Our optimized methodology minimizes both the sample volume and the overall time needed for the comprehensive analysis of the two critical quality attributes (CQAs) of interest.

Our enhanced strategy employs oligo(dT) beads to capture mRNA through the hybridization of adenosine residues in the mRNA tail with thymidine residues on the beads (Figure 3A). The supernatant, which may contain residual nucleotides co-purified with the mRNA, can be examined using a technique similar to cap LC-MS analysis. During this stage, mRNA is anchored to the beads, allowing for digestion by RNase T1 into smaller fragments, including one carrying the cap, that are then released into the supernatant. Importantly, the poly(A) tails remain undigested and attached to the beads.

The uncaptured fraction was analyzed to identify any residual nucleotides that were not incorporated into the mRNA during synthesis or removed during purification. For the Cas9 mRNA, we detected high levels of residual ATP (18% of the main cap intensity) and 3.7% of residual GTP (Figure 3B). The capping analysis validated our initial findings, showing that 91.5% of the mRNA was capped with Cap1, a result closely aligned with the 90.2% observed in our preliminary experiments (Figure 3C). However, we noted

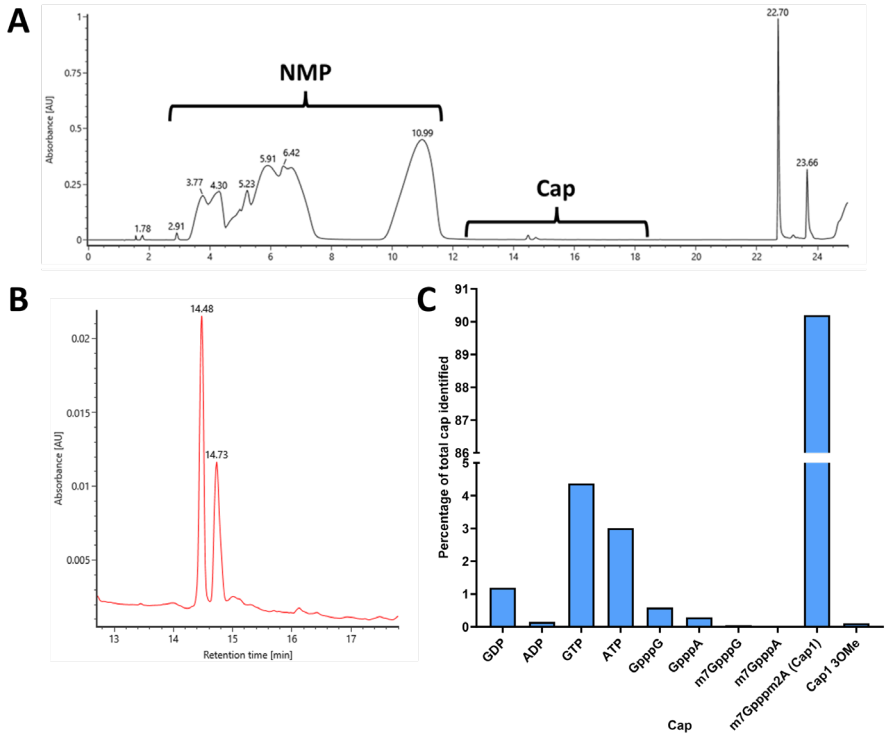

Figure 1: *Capping identification and relative quantification for Cas9 mRNA.* **A)** *UV chromatogram of Nuclease P1 digest.* **B)** *Zoom on Cap elution region.* **C)** *Relative quantification of cap.*

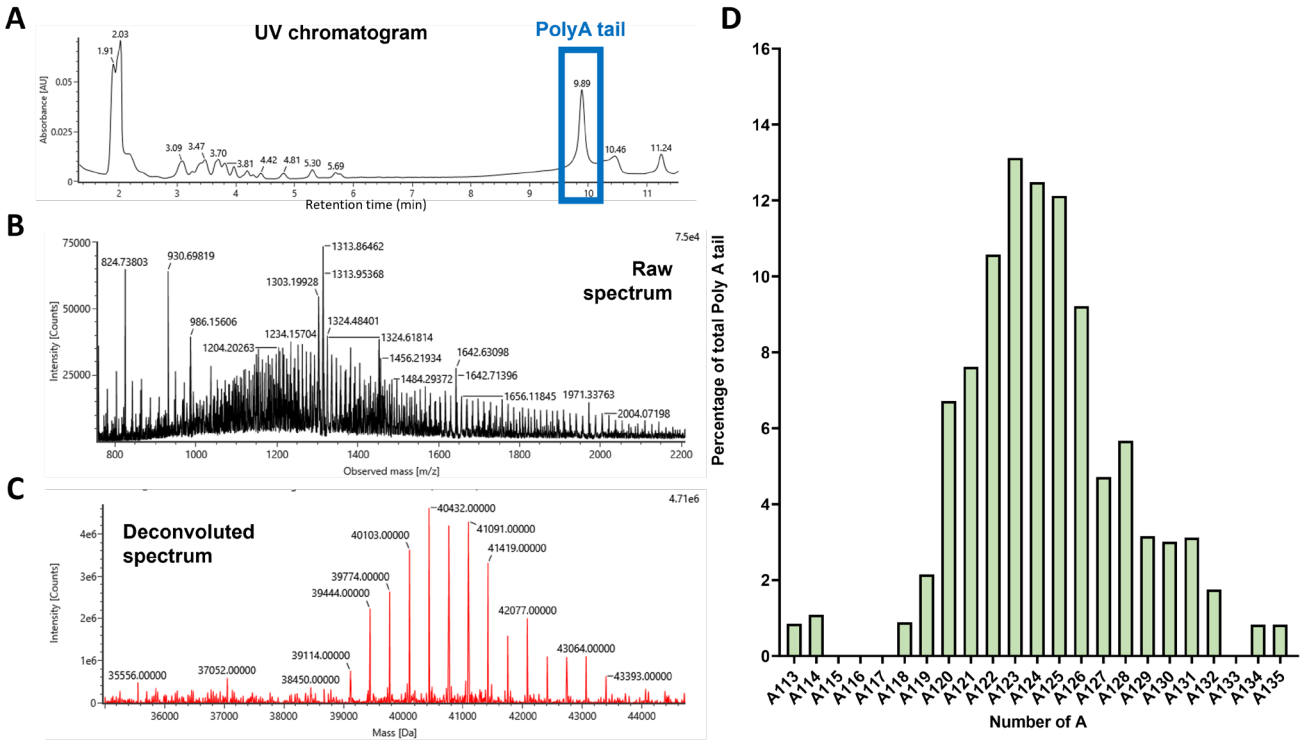

Figure 2: *PolyA tailing distribution for Cas9 mRNA.* **A)** *UV chromatogram of RNAse T1 digest.* **B)** *Raw mass spectrum of polyA tail peak.* **C)** *Deconvoluted mass spectrum of polyA tail peak.* **D)** *Size and abundancy distribution of polyA tails.*

an increase in ADP abundance (from 0.2% to 2.6%), which can only be explained by a low abundance resulting in a wrong quantification, and a decrease in GTP (from 4.4% to 2.3%), which can be explained by the potential residuals that have been removed thanks to the mRNA immobilization on beads. The poly(A) tail analysis mirrored the initial results, identifying the most abundant poly(A) tail as A123, accounting for 13.5% of the population compared to 13.1% in the initial tests (Figure 3D).

This refined approach significantly streamlines the characterization of mRNA CQAs by reducing sample size requirements and optimizing the use of mass spectrometers and personnel. Moreover, the time spent on mass spectrometry analysis can be further optimized since the mobile phases are compatible. However, this does necessitate the use of a quaternary pump coupled with a column manager and column selector to facilitate the simultaneous overnight analysis of both types of experiments.

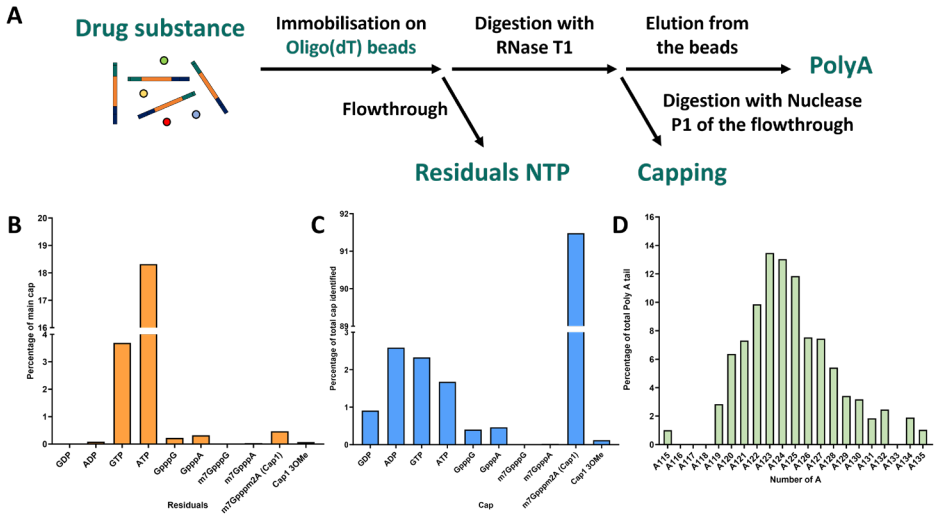

Figure 3: *Residuals and capping identification and polyA tail distribution for Cas9 mRNA after purification.* **A)** *Workflow of the multi-attribute monitoring sample preparation and analysis of mRNA drug substance.* **B)** *Relative quantification of residuals.* **C)** *Relative quantification of cap.* **D)** *Size and abundancy distribution of polyA tails.*

Application of the method to a lipid-nanoparticle encapsulated mRNA

A distinguishing feature of mRNA-based drug products is their encapsulation within lipid nanoparticles. Due to this unique formulation, direct capture of mRNA on beads is infeasible, necessitating an additional extraction/precipitation step. Following extensive testing with various organic solvents, we chose isopropanol precipitation as our method of choice. This two-step procedure is both rapid and convenient, and avoids the use of hazardous solvents. After precipitation and solvent removal, the mRNA is reconstituted in water and immobilized on poly(dT) beads, following the same workflow used in drug substance analysis (Figure 4). We then applied this optimized protocol to a lipid nanoparticle-encapsulated mRNA, thereby validating our methodology on an actual drug product. It is worth noting that the required sample volume is larger than what would typically be used for drug substance analysis. This can be explained by an incomplete precipitation process, resulting in a reduced mass spectrometry signal. However, any additional prepared sample can be repurposed for other types of assays, such as purity analysis via capillary gel electrophoresis (cGE) or anion-exchange chromatography with UV detection (AEX-UV).

Our sample preparation workflow yielded four distinct fractions: lipidic, residual, cap, and poly(A), each of which was analyzed using its respective method. The lipidic fraction revealed the presence of the four anticipated lipid types constituting the liposomal formulation: an ionizable lipid, cholesterol, a

PEGylated lipid, and a helper lipid (Figure 5A). High concentrations of this lipidic fraction can also be injected to scrutinize potential impurities and degradation products.

In our residual fraction analysis, we detected low-abundance species, suggesting that the mRNA was encapsulated using a highly purified drug substance (Figure 5B). Capping analysis identified ARCA (Anti-Reverse Cap Analog) as the dominant cap, accounting for 74.4% of the total population (Figure 5C). Interestingly, elevated levels of GTP (13.8%) were also detected. This is likely attributable to the post-transcriptional capping process utilized, which involves multiple enzymes and reaction intermediates, suggesting the reaction may be incomplete.

Finally, spectral data processing for poly(A) tail analysis revealed a predominant species containing 99 adenosine residues along with two additional residual nucleotides: N(1)-methylpseudouridine and cytosine. However, their sequential arrangement could not be determined (Figure 5D).

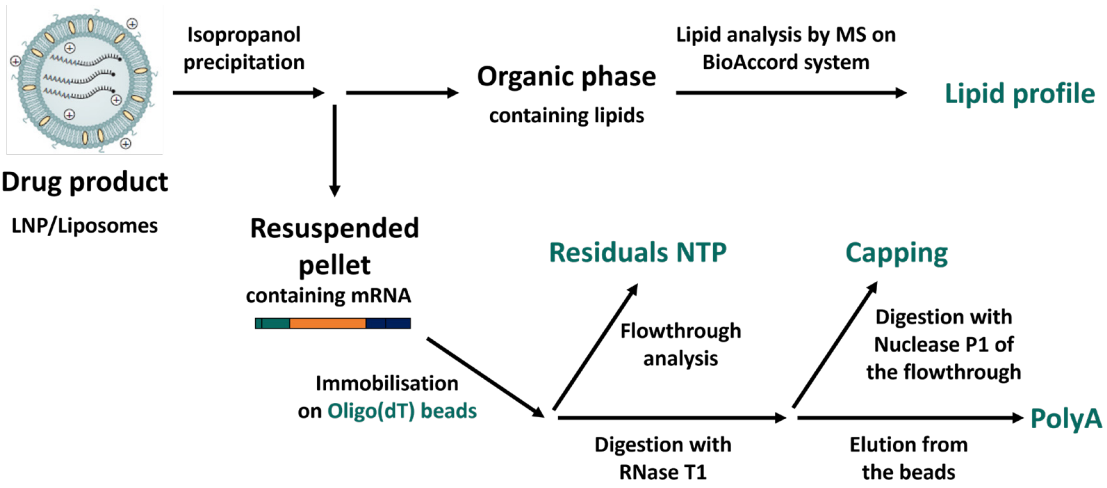

Figure 4: *Workflow of the multi-attribute monitoring sample preparation and analysis of mRNA drug product*

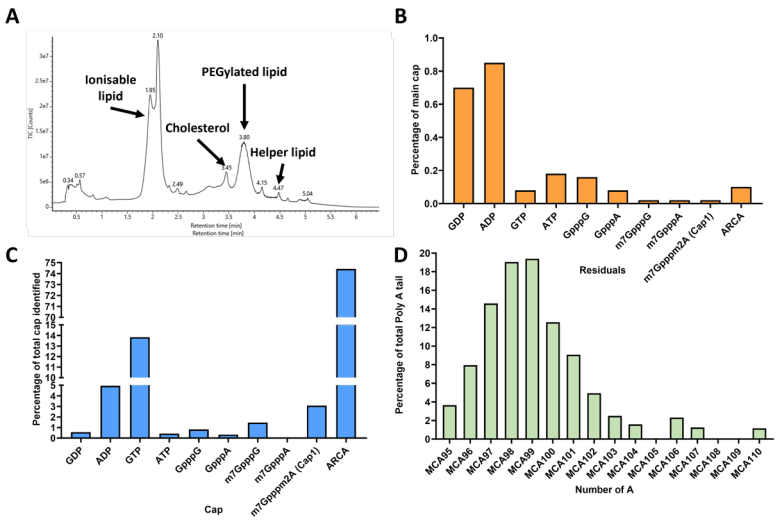

Figure 5: *Analysis of a lipid-nanoparticle encapsulated mRNA.* **A)** *Identification of lipids composing the lipid nanoparticle.* **B)** *Relative quantification of residuals.* **C)** *Relative quantification of cap.* **D)** *Size and abundancy distribution of polyA tails*

Conclusions

In summary, the methodologies we have developed offer comprehensive characterization of two essential quality attributes of mRNA, demonstrating versatility as they can be applied to a wide range of mRNA types without any a priori conditions. Our optimized workflow seamlessly integrates the analysis of both drug substances and drug products while minimizing sample requirements, sample preparation effort, and analysis time. This streamlining is of particular importance given the often-limited availability of sample quantities and the high demand for mass spectrometry resources.

Furthermore, our approach can be flexibly extended to integrate additional sample preparation steps or alternative analytical techniques. For instance, the proportion of polyadenylated mRNA could be accurately gauged by comparing bead-purified fractions against the flow through, thereby enabling the assessment of an even broader range of critical quality attributes for mRNA-based therapeutics. Therefore, the methods outlined in this study not only offer robust solutions for current challenges but also provide a foundation for future advancements in the analytical characterization of complex mRNA-based therapeutic systems.

Materials and methods

Extraction of mRNA from drug product

mRNA is extracted using isopropanol precipitation. mRNA sample is diluted in isopropanol (IPA), vortexed thoroughly and centrifuged. Supernatant is collected for lipid analysis. Pellet is mixed with IPA, vortexed and centrifuged. Pellet is resuspended in RNAse-free water for further preparation steps.

Immobilization of mRNA on poly-d(T) beads

Extracted mRNA drug product (DP) or mRNA drug substance (DS) is hybridized with poly-d(T) immobilized on magnetic beads (Invitrogen) at room temperature. The flow through of this step is used for the analysis of the residual triphosphate nucleotides in the samples from the mRNA synthesis (using the LC-MS analysis method of 5'-cap).

On-bead mRNA digestion

First digestion is performed using RNAse T1 (Thermo Scientific) during 2h at 37°C under agitation. Flow through is collected and further digested using Nuclease P1 (New England Biolabs) during 30 min at 37°C. This digest is analyzed using LC-MS analysis of 5'-cap poly(A) tails are eluted by heating the beads and the flowthrough is analyzed.

LC-MS analysis of 5'-cap

Samples are loaded on a Waters Premier BEH Oligonucleotides C18 (100 x 2.1 mm, 1.7 µm particles) and eluted using a 20 min gradient (Solvent A: 5 mM dibutylammonium acetate – Solvent B: acetonitrile) on a Waters Acquity H-Class bio. Waters Xevo G2-XS QTof is coupled on line with the UPLC system and is operated in MRM mode.

LC-MS analysis of 3'-poly(A) tail

Samples are loaded on an Agilent PLRP-S 1000Å (50 x 2.1 mm, 5 µm particles) and eluted using a 10 min gradient (Solvent A: 15 mM dibutylamine, 25 mM hexafluoroisopropanol in water – Solvent B: 15 mM dibutylamine, 25 mM hexafluoroisopropanol in methanol) on a Waters Acquity H-Class bio. Waters Xevo G2-XS QTof is coupled on line with the UPLC system and is operated in MSE mode.

LC-MS analysis of lipids

Samples are loaded on a Waters Premier CSH Phenyl-Hexyl (50 x 2.1 mm, 1.7 µm particles) and eluted using a 6 min gradient (Solvent A: 0.1% formic acid in water – Solvent B: 0.1% formic acid in ACN) on a Waters BioAccord system (refer to Waters Application Note 720007844).

Data processing

Data processing is performed using UNIFI integrated identification and quantification modules. Poly(A) tailing deconvoluted spectra are further processed using homemade python script.

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TRANSFERABILITY OF RAMAN CALIBRATION MODELS WITH PROCESS CHANGES IN CHO CULTIVATIONS

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Abstract

Over the past two decades, the biopharmaceutical industry has diligently pursued the implementation of process analytical technology (PAT) as a means to achieve quality by design (QbD) in biological drug manufacturing. Raman spectroscopy has been demonstrated to offer non-invasive, real-time monitoring of critical process parameters (CPPs) and critical quality attributes (CQAs), revolutionizing bioprocess monitoring. Accurate calibration models are essential for successful Raman-based monitoring and control. However, developing these models can be time-consuming and costly, due to the need of generating a number of calibration data from multiple cell cultivations to adequately cover process variability. A generic modeling approach, that allows the incorporation of additional data, specific to new applications, and facilitates calibration model transferability, has been proposed to address this issue. Here, the transferability of Raman calibration models was studied by changing process conditions during CHO cell cultivations. Results show successful transfer of glucose calibration models without significant accuracy losses, even without a generic modeling approach. Titer calibration models, in some cases however, required a generic modeling approach to achieve effective transferability. The generic modeling approach emerges as a practical and efficient method to ensure calibration model transferability, reducing time and cost associated with creating new models for varying process conditions. This study contributes to the broader understanding of Raman-based PAT applications, emphasizing the importance of reliable calibration models for efficient and adaptable bioprocess monitoring and control.

1. Introduction

Ever since the FDA's initiative in 2004,[1] the biopharmaceutical industry has been continuously striving for the implementation of process analytical technology (PAT) as a means to achieve quality by design (QbD) in biological drug manufacturing. This endeavor requires technologies that can be implemented in an in-line or on-line fashion to allow for real-time monitoring and control of the process.

Raman spectroscopy has been presented as a promising solution, as it enables non-invasive, real-time monitoring of critical process parameters (CPPs),[2] critical quality attributes (CQAs),[3] and other key performance indicators in the bioprocess, like for example titer.[4, 5] Traditionally, these are obtained by off-line analysis of samples taken from the process, resulting in a limited ability to influence an active process. Raman-based approaches offer increased process insights due to greater data availability and faster reaction times. This is particularly interesting and beneficial for monitoring process performance indicators like titer and quality-related attributes, such as mAb glycation and glycosylation, whose off-line analysis results are typically not available in real-time and often only after the process has already ended.

Going beyond monitoring, Raman spectroscopy has been successfully used to control the feeding of cell cultures with feedback control loops.[6, 7] In this way, constant glucose concentrations were achieved throughout the process, resulting in increased mAb product quality.[8] Specifically, increased glucose

concentrations have been associated with increased production of toxic metabolites like lactate,[9] higher protein glycation,[8] and elevated oxidative stress through the generation of reactive oxygen species,[10] underlining the importance of strict (and sufficient) glucose level control.

Accurate calibration models are essential for Raman-based monitoring and control in bioprocess applications. They are needed to translate spectral data to specific analyte concentrations, through the use of advanced statistical methods, like orthogonal projection to latent structures (OPLS) regression.[11] However, calibration model development is costly and time-consuming due to the need of running multiple cell cultivations under varying conditions to cover process variability adequately. Further, calibration models usually demonstrate lower accuracies when used in environments not covered by the calibration (training phase), for example when critical process conditions have been altered. This often requires application of advanced model transfer approaches and, in case these fail, the development of completely new calibration models.

An efficient method to mitigate model transfer limitations is the generic modeling approach. This approach incorporates additional data into the calibration model that is specific to the new application (e.g., cultivations from another cell line). For example, it has been shown that Raman models built on CHO, HeLa and Sf9 cultivations are transferable to HEK-293 cultures by including a single batch of HEK-293 data into the initial model (representing just 2.75% of the total sample size).[12] This demonstrates that a marginal addition of experimental data is needed to achieve model transferability, underlining the great efficiency and practicality of the approach.

To study the transferability of Raman calibration models upon process changes in CHO cultivations, twelve fed-batch cultivations of a mAb producing cell line were performed within a design of experiments (DoE) framework. Specifically, varying production media systems, feeding strategies, and inoculation densities (two levels each) were tested. Following the automated data generation in an Ambr® 250 HT system, local Raman calibration models were developed for glucose and titer. Their prediction accuracies were investigated when deployed against cultivations of different process conditions, meaning the respective other DoE levels.

2. Experimental Section

To study the transferability of Raman calibration models between different process conditions, a mAb producing CHO DG44 cell line (Sartorius) was cultured using a Sartorius Ambr® 250 HT system. Conditions were established according to a DoE framework, as shown in Table 1, where three factors with two levels each were covered: production medium system (1 and 2), feeding strategy (1 and 2) and inoculation density (1: 0.3 · 106 cells/mL and 2: 0.6 · 106 cells/mL), with four duplicates resulting in twelve fed-batch cultivations in total. The cultivations were performed using the Ambr® 250 HT system equipped with the Ambr® Analysis Module and the BioPAT® Spectro (all Sartorius). Raman spectra were recorded automatically twice per day using the Raman Rxn2 system (Endress+Hauser AG). Respective samples were analyzed with the BioProfile® FLEX2 system (Nova Biomedical Corporation) for various cell culture parameters along with the Cedex® Bio HT analyzer (Roche CustomBiotech) for titer measurements (starting on cultivation day 6).

For data analysis and model building, SIMCA17 software (Sartorius) was used. The Raman spectra were pre-processed using Asymmetric Least Squares (AsLS) baseline correction[13] and water-band normalization. Following that, a principal component analysis (PCA) was conducted on the spectral data to study variability and remove outliers for subsequent regression modeling. An orthogonal projection to latent structures (OPLS) regression for single-Y calibration modeling was employed, utilizing the pre-processed Raman spectra (450-1800 cm-1, centered) and the measured reference values (scaled to unit variance).

To study the model transferability, local models were built, focusing on glucose and titer, for each level of a DoE factor (e.g., production medium system 1) and evaluated for their prediction accuracy against the respective other level (e.g., production medium system 2). To quantify the accuracy before and after the model transfer, the internal cross-validated Root Mean Square Error of Cross Validation (RMSECV%) and the external Root Mean Square Error of Prediction (RMSEP%) were used, calculated by dividing the RMSECV and RMSEP by the average measured reference value (and multiplying by 100). This increased the comparability between internal and external prediction errors, as it accounts for varying mean concentrations in training (internal) and test (external) datasets.

To minimize the transfer limitations of titer models between different production media systems, generic OPLS models were developed by systematically adding a single cultivation of production medium system 1 (cultivations C1-C6) to the local model of the production medium system 2 (cultivations C7-C12). This was done for all cultivations of production medium system 1, resulting in six generic titer models. The respective remaining cultivations of production medium system 1 served as external prediction sets. To compare the performance upon transfer between local and generic titer models, the external prediction errors (RMSEP%) were computed for the generic models obtained from predicting on cultivations that were not used for calibration.

Cultivation	Inoculation Density	Production Medium System	Feeding Strategy	Duplicates
C1	2	1	2	C3
C2	1	1	2	
C3	2	1	2	C1
C4	1	1	1	C6
C5	2	1	1	
C6	1	1	1	C4
C7	1	2	2	C9
C8	2	2	2	
C9	1	2	2	C7
C10	2	2	1	C12
C11	1	2	1	
C12	2	2	1	C10

Table 1: *Overview of the DoE set-up covering three factors: production medium system, feeding strategy and inoculation density, with two levels (1 and 2) each. The 'Duplicates' column links the cultivations performed under equal DoE conditions.*

3. Results and Discussion

3.1 PCA on Raman data

The PCA model developed to investigate the variability within the Raman data captured 67.5% and 18.3% of the spectral variance within the first and second principal components, respectively. The scores plot of the first two components, as depicted in Figure 1, showed a strong overlap between the spectra of inoculation densities 1 and 2. Slightly different trajectories were found for the production media

systems, with production medium system 1 demonstrating a larger spread in the second component. Further, cultivations performed with feeding strategy 2 showed larger score values in the second component compared to feeding strategy 1, resulting in clustering along the second component. The results indicate that model transfer between different inoculation densities could work seamlessly, while there might be limitations for transfer between different production media systems and feeding strategies.

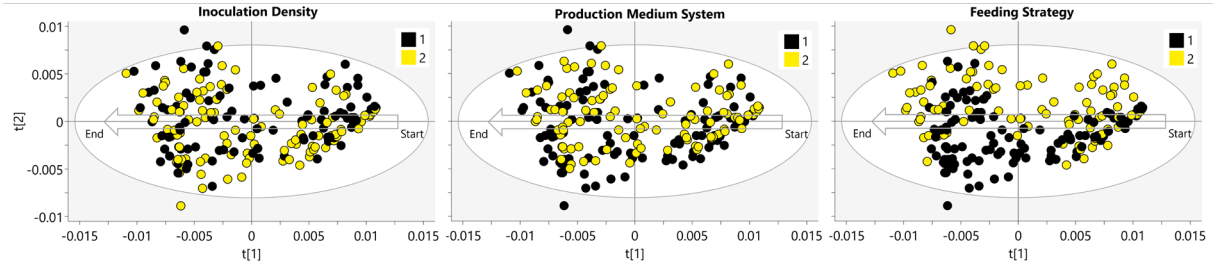

Figure 1: *Scores plot showing the first and second principal components of the PCA model developed on the Raman spectra and colored according to inoculation densities, production media systems, and feeding strategies (N = 205) with the arrows indicating the batch maturity.*

3.2 Transferability of Local Glucose Models

The local glucose models showed excellent model fit regardless of the specific DoE level that was used for calibration, as displayed in Table 2. The internal prediction errors were comparable for all models, ranging from 7.0% to 9.2%. Further, the models showed high linearity of predictions (R2CV ≥ 0.96).

DoE Factor	Level	Cal. Range [g/L]	LV	R^2CV	RMSECV% [%]	R^2P	RMSEP% [%]
Production Medium System	1	1.3 - 12.7	1+3	0.96	8.3	0.97	8.6
	2	1.0 – 12.5	1+4	0.97	8.3	0.97	8.2
Inoculation Density	1	1.0 – 12.7	1+4	0.96	9.2	0.97	7.4
	2	1.3 – 11.7	1+3	0.97	7.6	0.96	9.0
Feeding Strategy	1	1.3 – 12.5	1+4	0.96	8.7	0.97	8.4
	2	1.0 – 12.7	1+4	0.97	7.0	0.96	9.6

Table 2: *Summary of key parameters and performance of local glucose models. Cal. Range: Calibration range; LV: Number of latent variables used in OPLS model; R^2CV: Linearity of cross-validated predictions; RMSECV%: Internal cross-validated prediction error; R^2P: Linearity of external predictions; RMSEP%: External prediction error.*

The local glucose models were transferable to cultivations with different production media systems, feeding strategies, and inoculation densities without any significant prediction accuracy losses (Figure 2). This was demonstrated by the low external prediction errors ranging from 7.4% to 9.6%, which were very comparable to the respective internal prediction errors. The difference between external and internal prediction errors never exceeded +2.6%, demonstrating that the local models retained high prediction accuracy even after changing the cultivation conditions. The specific direction of transfer (e.g., production medium system 1 → 2 or 2→ 1) did not have a notable impact on the prediction accuracy. It is noteworthy that the transfer between different feeding strategies worked seamlessly, despite its strong impact on the process trajectory as seen on the PCA scores plot of the spectral data and discussed before.

The results demonstrate that Raman glucose models are transferable between different process conditions without any considerable prediction accuracy losses. This knowledge is crucial as it shows that developing a new glucose model upon changing process conditions might not be necessary at all, eliminating the need for additional experiments.

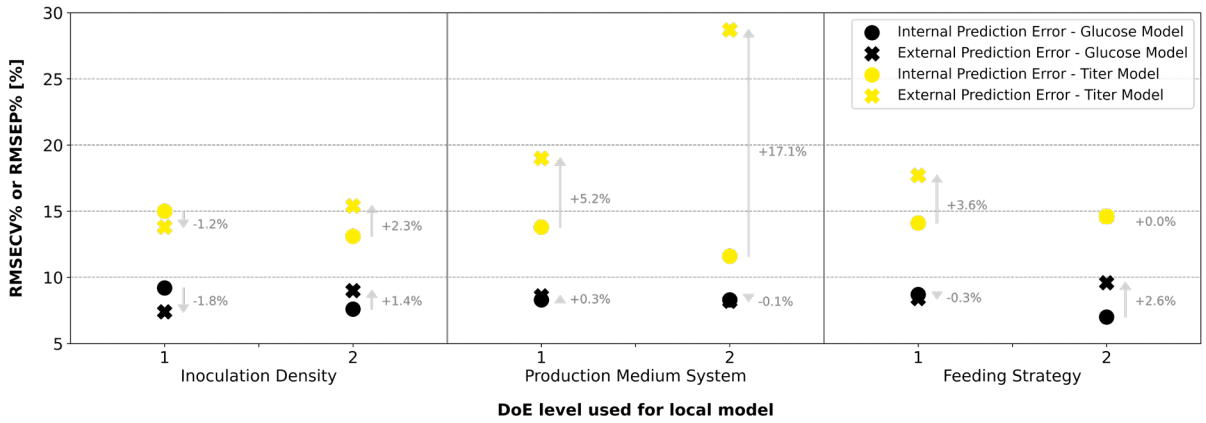

Figure 2: *External prediction errors (RMSEP%), internal cross-validated prediction errors (RMSECV%), and their differences for local glucose and titer models. The external prediction errors were obtained by predicting from Raman data from the respective DoE level not used for model calibration. Exact values are given in Table 2 and Table 3 for glucose and titer, respectively.*

3.3 Transferability of Local Titer Models

The local titer models demonstrated good model fit regardless of the specific DoE level that was used for calibration, as shown in Table 3. The internal prediction errors were comparable between the different local models, ranging from 11.6% to 15.0%. All models showed good linearity of predictions with $R^2CV \geq 0.83$, except for the model based on feeding strategy 1 (0.76). As compared to the local glucose models, local titer models exhibited slightly increased internal prediction errors, which can be explained by less specific Raman bands of the mAb molecule as compared to the ones observed for glucose.

DoE Factor	Level	Cal. Range [g/L]	LV	R^2CV	RMSECV% [%]	R^2P	RMSEP% [%]
Production Medium System	1	1.3 – 4.2	1+3	0.85	13.8	0.78	19.0
	2	1.2 – 4.2	1+3	0.91	11.6	0.83	28.7
Inoculation Density	1	1.2 – 4.2	1+4	0.84	15.0	0.87	13.8
	2	1.3 – 3.8	1+3	0.86	13.1	0.84	15.4
Feeding Strategy	1	1.2 – 3.0	1+3	0.76	14.1	0.82	17.7
	2	1.4 – 4.2	1+3	0.83	14.6	0.75	14.6

Table 3: *Summary of key parameters and performance of local titer models. Cal. Range: Calibration range; LV: Number of latent variables used in OPLS model; R^2CV: Linearity of cross-validated predictions; RMSECV%: Internal cross-validated prediction error; R^2P: Linearity of external predictions; RMSEP%: External prediction error.*

The local titer models demonstrated external prediction errors ranging from 13.8% to 28.7% when transferred to the DoE level not used for calibration. Comparing these to the internal prediction errors,

differences of up to 3.6% were found for the local models based on feeding strategies and inoculation densities. The slight increases in external prediction errors might be associated with model extrapolation due to different titer ranges in calibration and prediction sets, particularly seen for local models for different feeding strategies. Nonetheless, the obtained external prediction errors were still relatively low and comparable to the internal prediction errors, demonstrating the feasibility of seamless model transfer between different feeding strategies and inoculation densities.

The local models from different production media systems, however, exhibited significantly increased external prediction errors as compared to the internal prediction errors. Depending on the direction of transfer, differences of 5.2% and 17.1% were found, respectively (Figure 2). This indicates that the local titer model based on production medium system 2 was not transferable to cultivations of production medium system 1 without a considerable prediction accuracy loss. This cannot merely be explained by extrapolation as the cultivations of both production media systems covered almost the same titer range. Further analysis revealed that the predictive loadings of the local models (describing the correlation between Raman and reference data) exhibit considerable differences depending on the production medium system. This indicates that the models lack specificity for the target mAb molecule, which could be due to overlapping Raman bands of mAb molecules and media compontents.

3.4 Transferability of Generic Titer Models between Production Media Systems

To overcome the transfer limitations found for the local titer model based on production medium system 2, six generic models were developed as described in the methods section. Independent on the specific single cultivation of production medium system 1 that was added to the local medium 2 model, for all generic models significant improvements in the external prediction accuracies were seen. This could be seen by consistently reduced external prediction errors upon model transfer, as compared to the local model based on production medium system 2 (19.3% ≥ RMSEP% ≥ 15.0%) vs 28.7%, Figure 3). The results demonstrate the great advantage of the generic modeling approach: improvement in prediction accuracy upon model transfer was achieved with small additional experimental effort. It is expected that adding more experiments would further improve the performance of the final model, but also increase costs of model generation significantly. Hence, it needs to be investigated how the trade-off between model performance and costs could be reached.

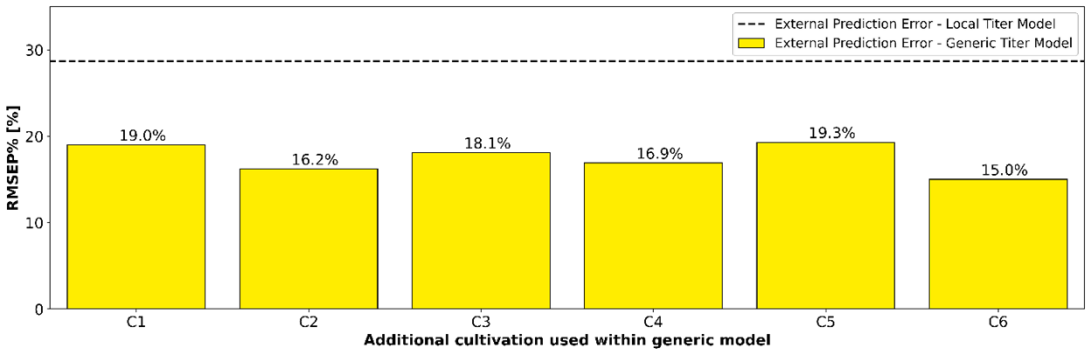

Figure 3: *External prediction error (RMSEP%) of the local titer model based on production medium system 2 (dashed line) and of the generic titer models (yellow bars). Six generic titer models were obtained by systematically adding a single cultivation of production medium system 1 (C1-C6) to the local model based on production medium system 2. The respective remaining five cultivations served as external prediction sets.*

4. Conclusions

This work presents an assessment on the transferability of Raman calibration models upon changes in crucial process conditions of CHO cultivations, like different production media systems, feeding

strategies, and inoculation densities with a focus on modeling glucose and titer.

The results show that glucose calibration models were transferable upon changed conditions without any significant accuracy losses. For titer models, this only held true for the transfer between different feeding strategies and inoculation densities, while the production medium system had high impact on titer model transferability. This was expected, as different production media systems have different chemical compositions, directly impacting the Raman spectra. However, the same transferability limitations for production media systems were not found for the glucose models. This is best explained by more specific glucose Raman bands with less overlap with media components as compared to the Raman spectra of mAb molecules.

The generic modeling approach was used as a practical and efficient method to ensure model transferability, leading to significantly reduced prediction errors of titer models upon changes in the production medium system. Already with the addition of a single cultivation to the model, significant improvements have been achieved. Further improvements are expected after inclusion of more experimental data, but a trade-off between experimental effort and model performance is necessary in order to keep the costs of model generation within reasonable limits.

About Sartorius

The Sartorius Group is a leading international partner of life sciences research and the biopharmaceutical industry. With innovative laboratory instruments and consumables, the Group's Lab Products & Services division focuses on laboratories performing research and quality control at pharmaceutical and biopharmaceutical companies as well as academic research institutes. The Bioprocess Solutions division, with its broad product portfolio focusing on single-use solutions, helps customers manufacture biotech medications and vaccines safely, rapidly, and economically. The company, based in Göttingen, Germany, has a strong global reach with around 60 production and sales sites worldwide. Sartorius delivers significant organic growth and regularly expands its portfolio through the acquisition of complementary technologies. In fiscal 2022, the company generated sales revenue of around 4.2 billion euros. At the end of 2022, around 16,000 employees were working for customers around the globe.

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THE VISCOSITY REDUCTION PLATFORM: VISCOSITY-REDUCING EXCIPIENTS FOR PROTEIN FORMULATION

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Abstract

This article evaluates viscosity-reducing capacities of excipient combinations. It shows the over-additive effect of using two excipients together, highlighting the platform's ability to balance viscosity reduction with protein stability. Additionally, the case study presented evaluates the effect of the excipients in the Viscosity Reduction Platform on ultrafiltration processes used to produce a highly concentrated formulation of a monoclonal antibody (mAb).

Introduction

Protein viscosity is one of the major obstacles in preparing highly concentrated protein formulations suitable for subcutaneous (subQ) injection. SubQ application of highly concentrated protein formulations enables a higher patient convenience and might thus lead to reduction in health costs. This can be achieved by reducing the time requirement of the patient to administer therapy, e.g. by enabling self-injection at home and reducing time required to do so. However, highly viscous protein solutions would require a significant force to be applied to the syringe for injection. As a result, the patient could experience a considerable amount of pain. In many cases, injectability would not be possible.[1,2]

When characterizing protein viscosity behavior, one can differentiate two different concentration regimes as shown in Figure 1.

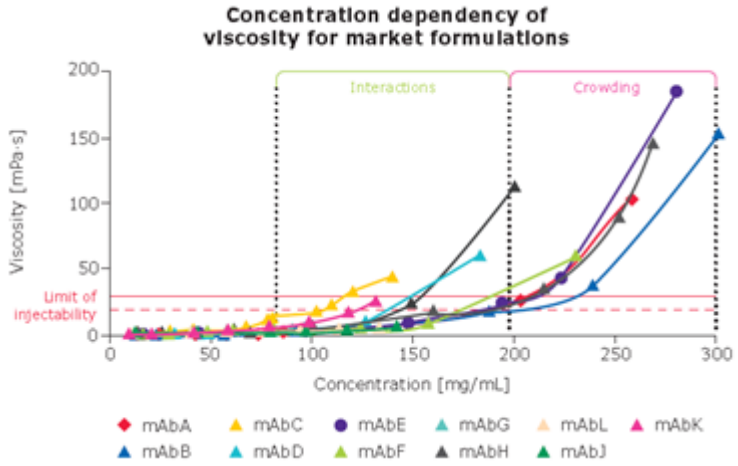

Figure 1: *Dependency of viscosity on antibody concentration and likely underlying causes. Red lines show injectability limit.*

At concentrations below 75 mg/mL, proteins are rarely viscous. When increasing the concentration to between 100 and 200 mg/mL, some proteins exhibit elevated viscosity exceeding the limit of injectability, which is typically between 20 and 25 mPa·s. At this concentration regime, several proteins exhibit an affinity for self-interaction, i.e. forming transient clusters that give rise to elevated viscosity. At concentrations above 200 mg/mL, the nearest neighbor distance between the protein molecules shrink so that without a specific affinity for self-interactions said protein-protein interaction take place. While viscosity-reducing excipients can alter either of these interaction patterns, they are likely to be more efficient at protein concentration regimes below 200 mg/mL.[3,4]

These intermolecular interactions between proteins have the same molecular origin as the intramolecular interactions that structurally stabilize the proteins. Thus, viscosity-reducing excipients that affect protein-protein interactions can potentially also destabilize proteins. As such, it is essential to balance an excipient's viscosity-reducing ability against its potential to destabilize a protein. For some excipients, a concentration-dependent effect on protein stability is well-documented. At lower concentrations, the excipients act as stabilizers, but this behavior changes to a rather adverse effect with increasing concentration.[5] Excipient concentration is thus a critical factor in managing protein stability.

These two aspects can be better balanced by using an excipient combination of an amino acid and an anionic excipient. When used in combination, excipients are more efficient in reducing viscosity and may even do so in an over-additive manner. Consequently, lower concentrations of the individual excipients can be used, which is more favorable for protein stability.

Excipient	Abbrev.
L-Arginine EMPROVE® EXPERT Ph Eur,ChP,JP,USP	Arg
L-Ornithine monohydrochloride EMPROVE® EXPERT DAB	Orn
L-Phenylalanine EMPROVE® EXPERT Ph Eur,USP	Phe
Thiamine phosphoric acid ester chloride dihydrate EMPROVE® API	TMP
Benzenesulfonic acid EMPROVE® EXPERT	BSAcid
Pyridoxine hydrochloride EMPROVE® EXPERT Ph Eur,BP,JP,USP,FCC	Pyr

Table 1: *Excipients and abbreviations*

Buffer	Buffer Components
Phosphate buffer	Sodium dihydrogen phosphate monohydrate EMPROVE® EXPERT BP,USP
	di-Sodium hydrogen phosphate heptahydrate EMPROVE® EXPERT DAC,USP
	Optional addition: Sodium chloride EMPROVE® EXPERT Ph Eur,BP,ChP,JP,USP

Table 2: *Materials used for base buffer preparation*

Results & Discussion

Table 1 summarizes the excipients that are part of the Viscosity Reduction Platform. For clarity reasons, abbreviations mentioned in Table 1 are used in the following. L-Arginine (Arg) is the industry standard for viscosity reduction using a single excipient and consequently serves as the benchmark excipient. Figure 2 shows the viscosity of 120 mg/mL infliximAb formulated at pH 7.2 (phosphate buffer). The materials used to prepare the base buffers are listed in Table 2. Amicon® Ultra-4 Centrifugal Filter Unit, Ultracel®-30 were used for the buffer exchange and concentration processes via diafiltration.

Using excipient combinations to reduce protein viscosity

As individual excipients may not be effective enough to reduce the viscosity of a highly concentrated protein formulation on their own, the Viscosity Reduction Platform is based on the use of excipient

the benchmark excipient, which by itself is only able to slightly reduce the viscosity. In several cases, combining Orn, Arg or Phe with an anionic excipient leads to a more substantial reduction in viscosity – including below the injectability limit, most importantly. With each amino acid, there are multiple combinations that would allow for injectability of infliximab:

- Orn is particularly effective with Pyr
- Arg can be combined with TMP
- Phe is best combined with BSAcid or TMP

In summary, different excipient combinations are efficient for infliximab. However, not all excipients may be suitable for every route of administration due to potential tissue-specific reactions, which is why using an excipient portfolio is beneficial. There are conditions where arginine is not desirable because of the route of administration or a local reaction to the excipient. The Viscosity Reduction Platform presented here provides a range of alternatives. In summary, the data with infliximAb as a model antibody shows that using excipient combinations can reduce viscosity more effectively than the leading industry standard. Combined excipients are also more efficient than highly concentrated single excipients and can even perform synergistically. Moreover, an excipient portfolio gives formulation scientists greater flexibility. Depending on the nature of the antibody, the desired pH, or the route of administration, having a variety of options at hand can be beneficial when developing the final formulation. The most suitable choice of excipients will depend on the type of protein and the formulation conditions.

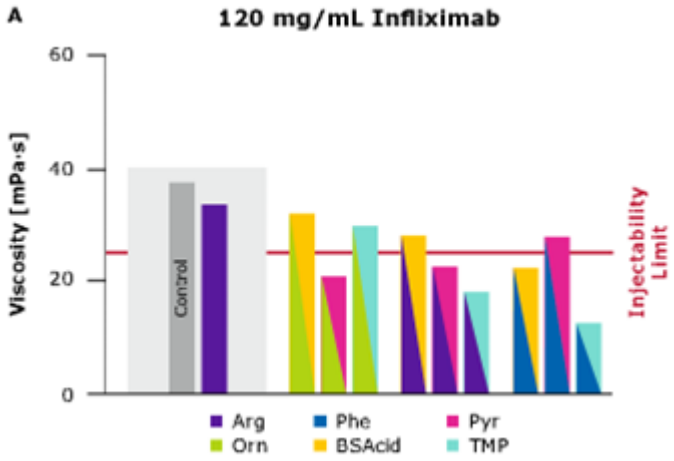

Figure 2: *Combinations of Viscosity Reduction Platform excipients compared to experiments without a viscosity-reducing excipient (grey bar), with sodium chloride as control (green bar) and the industry standard L-arginine (Arg) as benchmark (purple bar). The color codes of the split bars indicate the excipient combinations used. Model antibody infliximab.*

Addressing protein stability with the Viscosity Reduction Platform

As previously discussed, the balance between protein viscosity and protein stability is rather delicate.

Focusing on protein stability, a forced degradation study was performed using combinations of excipients with varying concentrations of the individual components. Excipient combinations can overcome the adverse effect of using an anionic excipient alone. The formulations used were not optimized further after addition of the viscosity- reducing excipients.5 Instead, the stability of infliximAb was investigated over a longer period at 2–8 °C and 25 °C/60% relative humidity.

Figure 3 shows for all selected excipient combinations that infliximab was able to retain a high monomer

content after 24 weeks at 2–8 °C. This high stability was achieved without further optimization of the formulation and could thus be potentially improved even more if the antibody were to undergo thorough formulation development. It is particularly noteworthy that the combination of Phe and TMP is able to maintain a high monomer content at 2–8 °C compared to 25 °C. At 25 °C, formulations containing TMP showed a strong destabilizing effect up to a total loss of monomer. This further supports the hypothesis that the decrease in protein stability is due to the decomposition of the excipient molecule. When stored under accelerated conditions, i.e. 25 °C/60% rH, a high monomer content (even above 95% in some cases) was observed for selected excipient combinations. Overall, it was shown that using viscosity-reducing excipients in combination with each other can maintain formulation stability under relevant storage conditions.

Detailed study data on the impact of protein stability of single excipients, the effect of protein formulation pH on excipient performance, impact of reduced protein viscosity on syringeability and the use of the Viscosity Reduction Platform on plasma-derived protein therapeutics shows that this strong viscosity reduction tool is not only for utilization in highly concentrated mAb products but also for other biologics formulations.[5]

The Viscosity Reduction Platform allows for a better balance of protein stability versus protein viscosity. This platform therefore enables subcutaneous delivery while preserving long-term stability. It also makes the application through a device both more patient friendly and more economical. The Viscosity Reduction Platform provides formulation scientists with a variety of options for formulation development that take the route of administration and the requirements of the protein into account.

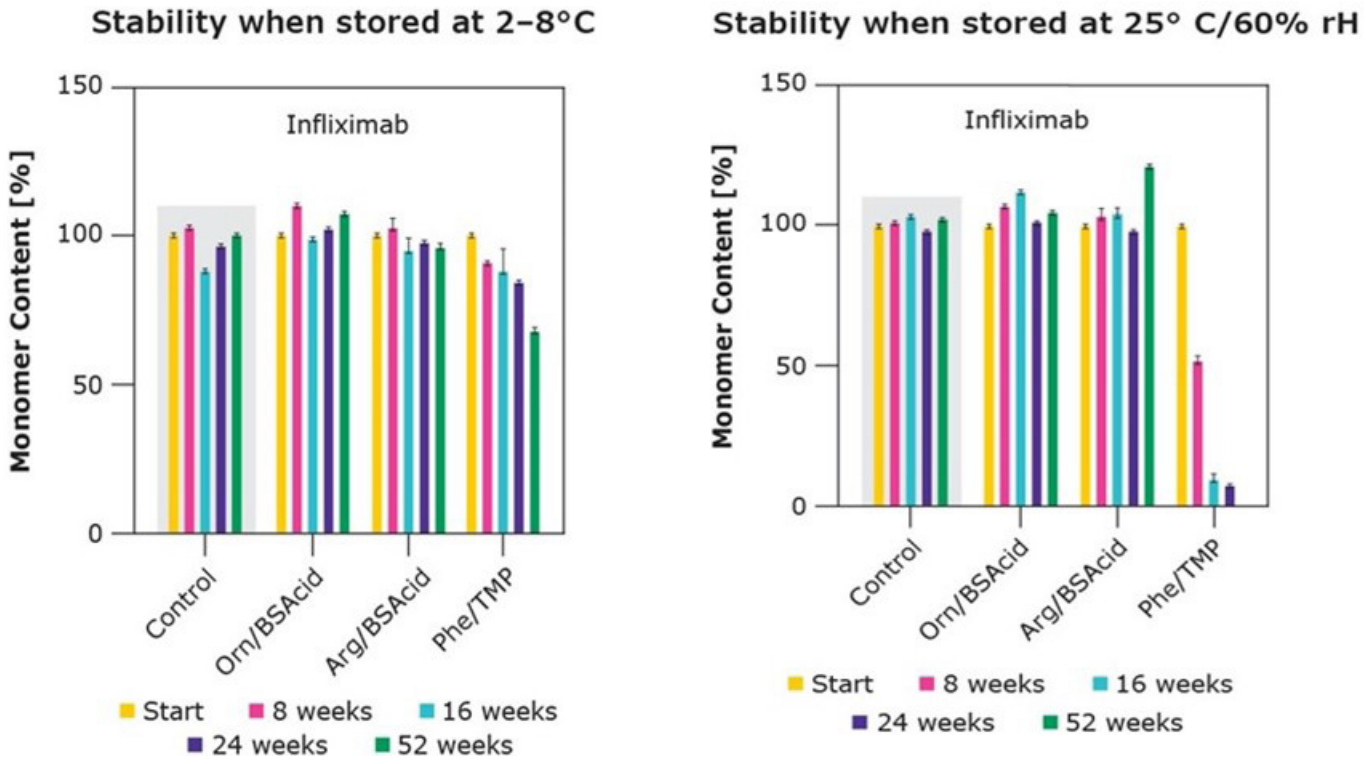

Figure 3: *Long-term stability of selected formulations with excipient combinations that successfully reduced viscosity. A) Stability at 2–8 °C and B) stability at 25 °C/60% rH.*

Viscosity-reducing excipients for improvement of filtration processes

The challenge arising from highly viscous proteins becomes more difficult particularly when advanced downstream processing (DSP) methods are used. As described in literature, an increasing protein

concentration can promote attractive interactions between proteins that can ultimately lead to high viscosity.[6,7] The resulting decreased flux rates due to the higher flow resistance may lead to protein gelation and potentially membrane fouling.[8]

Reducing processing time, footprint and aggregate formation are critical challenges in the production of highly concentrated mAbs.[9] Typically, tangential flow filtration (TFF) is used for the final formulation step in order to exchange the process buffer for the formulation buffer (diafiltration), clear small molecular impurities and concentrate the protein to the desired final concentration. The sizing of the unit operation and ultimately the process economics rely on the mass transfer/permeate flux during the concentration and diafiltration steps.

In this chapter, the effect of the excipients in the Viscosity Reduction Platform on ultrafiltration processes used to produce a highly concentrated formulation of a mAb are evaluated. The benefits of the Viscosity Reduction Platform in DSP are discussed with particular emphasis on TFF.

For the TFF process a Pellicon® XL Cassette with Biomax® Membrane, 0.005 m², NMWCO 30 kDa was used. A 0.1 M sodium hydroxide solution was used to clean the cassettes after each run. A 5 mM phosphate buffer at pH 7.2 containing 200 mM sucrose was used as the basis buffer. The excipients were used at a concentration of 75 mM in each case. The pH was adjusted using hydrochloric acid 1 mol/L EMPROVE® EXPERT hydrochloric acid and sodium hydroxide solution 32%, EMPROVE® EXPERT. A solution containing 10 mg/mL Infliximab in basis buffer was used as starting feed.

Critical process parameters can be improved by adding excipients

The filtration process as presented herein can be divided into two different sub-steps – the diafiltration, wherein the buffer is exchanged, and the concentration phase, where the target concentration is adjusted. The diafiltration phase in particular is relevant for the overall process time due to the high volumes used in this process step. This phase can make up 80 to 85% of the total process time and is thus critical when optimizing process economics. Figure 4 compares the different process durations for infliximab in the presence and absence of the respective excipients at 8 °C and at ambient temperature. As expected, the diafiltration time is responsible for a major part of the process time. The addition of viscosity-reducing excipients can reduce this time by up to 31%. In spite of the lower volume resulting in a higher concentration factor, the time needed can also be reduced. At ambient temperatures, a similar trend can be observed. However, as pointed out earlier, the overall process duration is reduced at higher temperatures due to the temperature dependence of the filtration process itself.

To identify the underlying cause of this reduction in process time, the permeability of the membrane was investigated in the presence and absence of excipients at both temperatures. It was observed that adding excipients causes the permeability of the Biomax® membrane to remain higher and also more constant throughout the diafiltration. If a more viscous solution is filtered, which is the case when no excipients are added, the membrane permeability is reduced over time, indicating a progressive fouling of the membrane itself.[8,10,11] The use of viscosity-reducing excipients prevented this membrane blocking and thus allowed for consistently high permeability. This ultimately led to the improved performance during diafiltration that was observed and discussed previously.

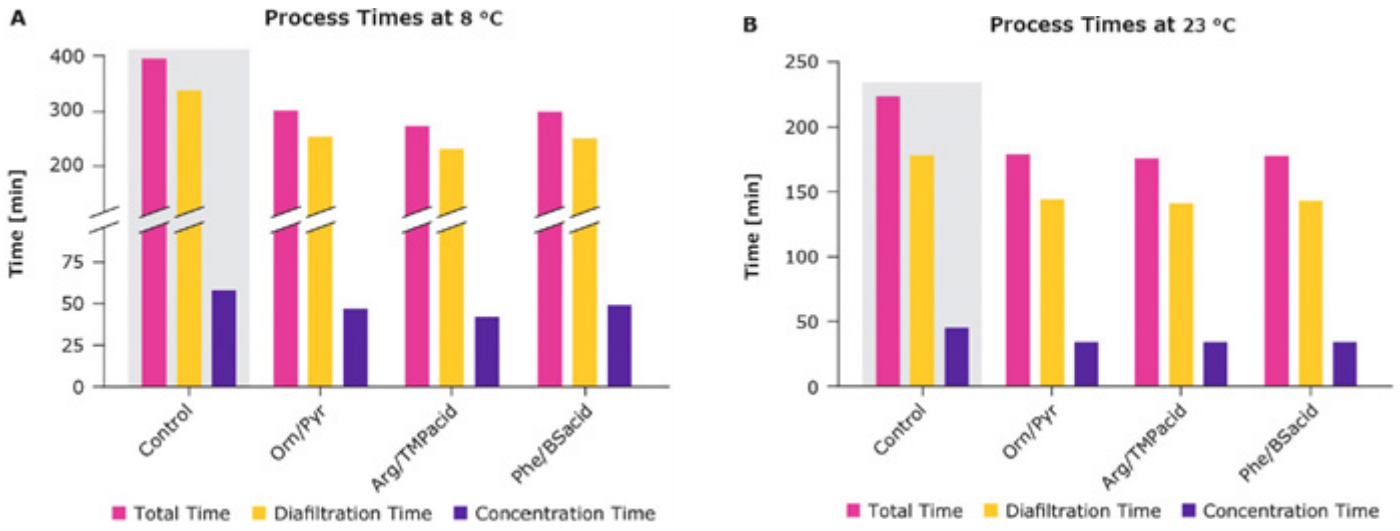

Figure 4: *Effect of excipients on process time of diafiltration and concentration step using a 10 mg/mL infliximab solution and a Pellicon® XL (Biomax®, 30 kDa) cassette. Concentration was increased to a maximum inlet pressure of 43 psi. A) Process times at 8 °C. B) Process times at 23 °C.*

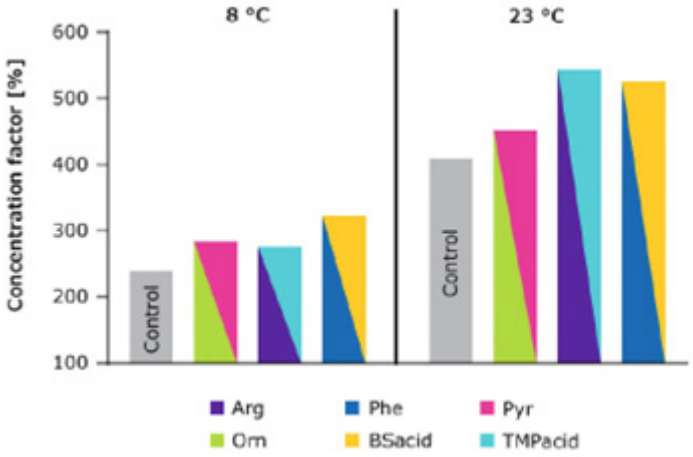

Figure 5: *Concentration factor of a 10 mg/mL infliximab formulation (100 g) after diafiltration process with or without excipients when reaching an inlet pressure of 40 psi (back pressure valve fully open).*

Looking at the system when an inlet pressure of 40 psi was reached (close to the process termination point of 43 psi), this benefit translates to a higher concentration factor (Figure 5). At 8 °C, a factor of 320% was reached, in contrast to the basis buffer with 240%. Likewise, 540% compared to 410% were found at ambient temperature. Since higher concentrations can be reached at lower pressure, polarization effects can be reduced.[12,13]

Similarly to the diafiltration step, the concentration process was also improved. Throughout this step, permeability was higher when excipients were used. It would thus be possible to perform the diafiltration step at higher protein concentrations while maintaining the same permeability as in the control. As such, starting volume and thus the total diafiltration volume required for 5 diavolumes can be reduced. In the case of 100 g starting solution used here, the diafiltration buffer volume could have been reduced by 33–53%.

By reducing protein viscosity while maintaining protein stability and membrane permeability[13], the

process economics of manufacturing mAbs can be rendered more attractive. While optimization of large-scale processes is still required, the combination of a very efficient TFF cassette such as the Pellicon® D Screen with the addition of excipients of the Viscosity Reduction Platform can be a promising approach. Pellicon® D Screen and the Viscosity Reduction Platform are thus suitable tools for creating highly concentrated protein formulations in an economically attractive manner.[10]

As discussed previously, the Viscosity Reduction Platform enables the user to better balance protein viscosity with protein stability by using excipient combinations.[5] The Viscosity Reduction Platform can therefore not only enable the formulation of conveniently administrable drug products but can also improve the process economics in filtration steps.

Finding the right combination for a protein of interest:

The six excipients from the Viscosity Reduction Platform can be combined in up to nine functional combinations. As every protein is different, it is important to experimentally screen for the most promising combination for a protein of interest.

This evaluation can be outsourced to Merck via the Feasibility Testing Service, or customers can perform their own screening, which can be facilitated with the VRP Excipient Finder.

The VRP Excipient Finder is a machine-learning based digital application, which minimizes the experiments needed during the evaluation, thus reducing time and material consumption.

For more information regarding the Viscosity Reduction Platform and the above mentioned service offering, please visit: sigmaaldrich.com/viscosity-reduction

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