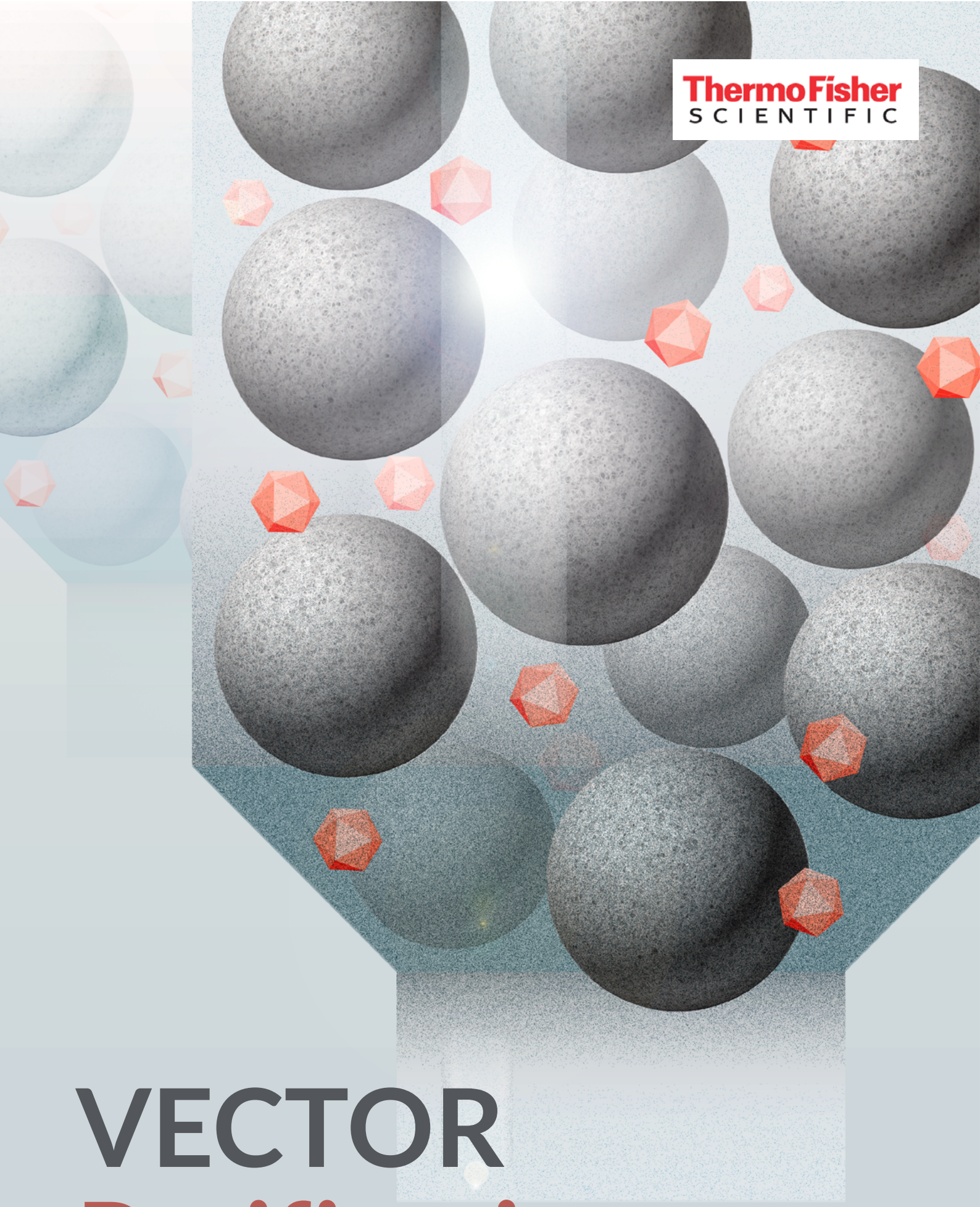
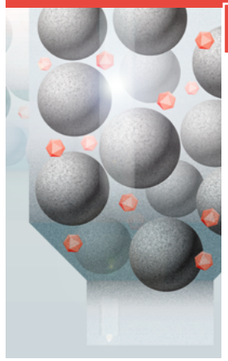


VECTOR Purification



CELL & GENE THERAPY INSIGHTS

VECTOR Purification

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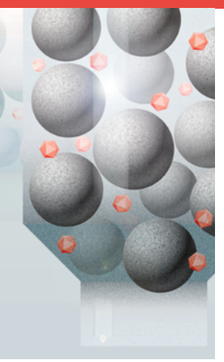


Evolution of vector purification

Charlotte Barker, Editor, BioInsights

As the cell and gene therapy industry expands, efficient vector manufacture is of more importance than ever.

FOREWORD

HOW CAN WE ACHIEVE EFFICIENT, REPRODUCIBLE, SCALABLE, AND COST-EFFECTIVE VIRAL VECTOR MANUFACTURE?

As the cell and gene therapy industry expands, efficient vector manufacture is of more importance than ever. In this eBook, *Cell & Gene Therapy Insights* and Thermo Fisher Scientific explore recent advances and ongoing purification challenges for adenovirus, retrovirus, lentivirus, and adeno-associated virus (AAV) vectors.

AAV is among the most commonly used vectors in cell and gene therapy, and we open the eBook with a top-level view on the evolution of AAV process development. Three leading industry experts join us for a wide-ranging roundtable discussion, covering everything from regulatory uncertainty to pandemic pressures (Chapter 1). Panelist Michael Mercaldi draws attention to the migration of technologies developed for the established recombinant protein sector into the growing cell and gene therapy field; notably, affinity resins.

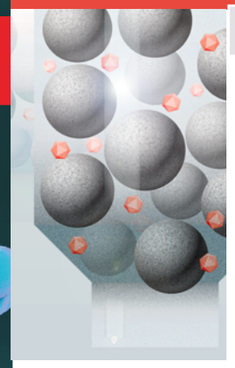
In Chapter 2, Mikhail Goldfarb and Orjana Terova describe a series of viral clearance studies conducted by clinical-stage biotech REGENXBIO, showing that an affinity resin (Thermo Fisher Scientific POROS™ CaptureSelect™ AAVX) provides robust performance, even under "worse case" processing parameters.

CDMOs have unique needs revolving around flexibility, robustness, and productivity, making universal tools essential. Vincent Ravault (Yposkesi) and Nicolas Laroudie (Thermo Fisher Scientific) describe the use of POROS CaptureSelect AAVX resin as a pan-affinity tool for the universal capture of AAV vectors in Chapter 3.

Plasmid DNA (pDNA) is a critical component of commonly used viral vectors and in the production of mRNA therapeutics, but purification can be challenging. Scientists are working to develop more efficient processes and in Chapter 4 Alejandro Becerra (Thermo Fisher Scientific) and Johannes F Buyel (Fraunhofer IME) describe a design of experiments study to optimize process conditions, maximize purity and recovery, and confirm optimal operating parameters when using POROS™ AEX affinity exchange resins for pDNA purification.

Following the immense success of mRNA vaccines in fighting the COVID-19 pandemic, there has been a rapid rise in the number of mRNA-based therapeutics in development. Kelly Flook explores how affinity resins could help speed up large-scale purification of mRNA-based therapies in Chapter 6.

We hope readers will find this eBook helpful in exploring the key factors for a successful viral manufacturing pipeline and the role of new and established purification technology.



CHAPTER 1

Evolution in AAV process development: 2022 and beyond

Elisa Manzotti, Founder, *Cell and Gene Therapy Insights,* speaks to **Alejandro Becerra, Matthais Hebben** and **Michael Mercaldi**

ALEJANDRO BECERRA
Principal Applications Scientist and Global Purification Technical Lead, Thermo Fisher Scientific

Dr Alejandro Becerra is a Principal Applications Scientist and Global Purification Technical Lead. Alejandro has over 14 years of experience in downstream processing and customer support having worked as Purification Team Manager and other bioprocess engineering roles prior to joining Thermo Fisher Scientific in 2018. Dr Becerra is a subject matter expert in preparative chromatography with expertise in the development, optimization and scale-up of antibody, recombinant protein and viral vector purification processes. Alejandro holds a PhD in Chemical Engineering from Cornell University.

MATTHIAS HEBBEN
Vice President of Technology Development, LogicBio Therapeutics

Matthias Hebben has been serving as vice president of technology development at LogicBio Therapeutics since February 2019. In his role, he is leading the CMC efforts, including vector core, capsid optimization, process development, analytical development and clinical product manufacturing. Before that, he served as director of technology development and head of bioprocess development at Genethon for 6 years. Before that, he occupied several positions at Vivalis (Valneva), Intervet Shering Plough and Virbac. Matthias has a PhD in molecular biology and a MSc in bioprocess engineering

MICHAEL MERCALDI
Senior Director of Downstream Process Development, Homology Medicines

Michael is the Senior Director of Downstream Process Development at Homology Medicines. He is responsible for leading the development of Homology's purification and drug product manufacturing processes for their gene therapy and gene editing programs. He has held positions in process development throughout his career at MedImmune/AstraZeneca, Merrimack Pharmaceuticals and Codiak Biosciences before joining Homology. He holds a BS in Chemical Engineering from the Rensselaer Polytechnic Institute and a PhD in Biochemical Engineering from Tufts University.

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DOI: 10.18609/cgti.2021.157

In 2019, BioInsights hosted a popular roundtable video and article with leading experts in AAV vector process development. Two eventful years later, we're bringing our panel back together to discuss how viral vector manufacture is evolving and what we can expect in the future.

Q Let's begin with regulation: what are the key areas of uncertainty for the gene therapy industry at present, and how have these changed since our last discussion?

MM: Overall, we have been very pleased with the regulatory agencies and how they are approaching gene therapy. It's a very rapidly evolving space, and they're trying to learn and work with manufacturers to make these therapies work better.

We would like to see a little bit more guidance on impurity levels – what are the impurities we should be looking for, and what are the acceptable levels that we need to achieve? For example, what is a safe level of empty capsids? That's an area where companies like us can proactively work with the agency to help figure what is a safe level of these impurities.

We would also like a bit more structure and guidance on CMC activities during development. Gene therapy moves very fast, and if you're treating a pediatric condition or ultra-rare disease, you may only try to register at Phase 1 data. The agencies are very proactive and want to work with you on getting that done, which is great, but we'd like to see more definition on what CMC activities need to be pulled in earlier. That will help us as a CMC organization to plan better, meet those demands more easily, and ultimately get these therapies out faster.

MH: There is more and more guidance for gene therapy products, which is always very useful. For me, the big changes that occurred for the last two years were the severe adverse events that have happened in several clinical trials. This is certainly going to change the scope of what is acceptable in terms of purity and product quality, as Michael mentioned.

It is going to be very important to understand what is happening in clinical trials and what could be causing toxicity – whether it be from capsids, impurities, or other sources. It's an open question today.

AB: As a vendor, we don't get deeply involved in regulation, but we need to be aware of it. For example, if guidelines change around empty/full capsid analysis, we need to understand if the current tools we offer are sufficient.

Q Potency is one issue that has grown dramatically in significance since we last spoke. What for you are the key learnings for the field from the various tribulations suffered by industry players, and what would be your advice regarding the timing of potency assay development in particular?

MH: The big challenge is that the infectivity of AAV is totally different between *in vitro* and *in vivo* and even between different animal species. This makes a potency assay a challenge – it's very complex to be able to identify or characterize the mechanism of action when you have to use massive amounts of vectors to be able to transduce a cell *in vitro*. That raises questions about the sensitivity of a potency assay. Again, because you have to use such a huge amount of vector *in vitro*, it is not necessarily representative of what is going to happen in a human body. It's very difficult to know whether a small change in the vector efficacy from batch to batch can be detected in your potency assay.

Over the last two years, we have seen several examples of companies that have faced some setbacks with regulatory agencies because of some issues with potency. From what I understand, most of these issues were associated with changes in the manufacturing process. That means there are still unidentified critical parameters in manufacturing processes. We need to perform a lot of investigations to be able to better characterize these processes.

It seems obvious that developing a potency assay should be done very early in development. However, it's difficult to be comfortable developing such an assay before you know if your intended mechanism of action will work in patients. It's difficult to know what parameters you will need to follow *in vitro* in your assay. Developing an assay before you know what is going to be the effective dose in patients can be also very tricky.

To sum up, while it is important to start developing the potency assay as soon as possible before the clinical trial, people need to understand that the assay will be a living protocol and will need to be adjusted over the course of clinical development.

> "...we have platform methods that we can quickly develop infectivity and gene expression assays for any kind of construct."
>
> - Michael Mercaldi

MM: It's becoming clear that agencies, especially the FDA, are now asking for multiple potency assays – infectivity, gene expression, and a cell-based potency assay. They feel that the three assays tell different parts of the story. There is also an increasing expectation that these assays will be available earlier in the lifecycle.

We're prepared for that at Homology – we have platform methods that we can quickly develop infectivity and gene expression assays for any kind of construct. Cell-based potency does require construct-specific work and, as Matthias was saying, that needs to happen very early in development. Once we identify a construct, we start developing the assay, so we have it in hand for our critical IND-enabling lots. That means we have all the data that the agencies are expecting and gives us a lot of confidence that we're making a quality product.

Q Turning to challenges for process development and product characterization stemming from reduced development timeframes – for instance, in the expedited regulatory pathway scenario: what are the implications for process and assay development and what steps may be taken to help avoid issues at the BLA stage?

MM: If your organization is trying to pursue an accelerated pathway, there are going to be expectations from any regulatory agency that you need to pull in some later-stage activities earlier in the development than you normally would. Organizations need to be ready for this – if you think you're going to be trying to register on Phase 1 data, you need to prepare to do a lot of these BLA-enabling activities at your IND stage. This puts a lot of pressure on a CMC organization because you need to do all this work plus balance timelines. How we've addressed this at Homology is by investing heavily in our platform and building an excellent analytics team with a large suite of analytical assays and deep knowledge of our constructs.

We are then able to leverage this platform and analytical knowledge, to pull in a lot of these activities very quickly or leverage previous construct knowledge that can give the agencies information that they need.

MH: I think this expedited regulatory pathway is a breakthrough in the field of regulation. It's a fantastic opportunity, most importantly for the patients, but also the industry.

But as Michael said, it means that you must set up your company as a commercial-phase company from the start. When you're a very small company trying to develop new technology, you don't know if you're going to be successful in the clinical trial. It requires money and time to be able to have everything ready before you start your clinical trials.

AB: From our side, we're seeing the same trend toward greater investment in process development. Organizations are spending more time upfront, building stronger analytics and process characterization.

Q What have been the key advances in AAV manufacturing technology over the past two years, and how might they continue to reshape vector bioprocess moving forward?

AB: I think in terms of technologies, progress has been a little slower, but we are seeing the utilization of different approaches with the existing tools. For example, potentially using shorter residence times and shorter bed heights for capture. And similarly, utilizing different approaches to make full capsid enrichment separation easier than with traditional gradients.

MM: I wouldn't say progress has been slow but I agree with Alejandro that the focus has been on taking existing tools developed for the recombinant protein or other therapeutic space and making it work for AAV. For example, Thermo Fisher Scientific developed the excellent affinity resins that are now used quite widely.

Other advances in the past two years include a more aligned process across the industry, consisting of a harvest step, an affinity step, an anion exchange step, and final formulation. When more companies use that same basic process, I think we're going to get a lot more learning and understanding about what those products are and how they work in that process. Plus, they will be more scalable.

Another thing that I've noticed a lot of companies do, including Homology, is to transition from ultra-centrifugation to anion exchange chromatography to remove empty capsids. For me, this is a key step to bringing these therapies to a broader patient population. Because now we have a scalable manufacturing process that can be executed well with our existing toolset. It means we can support larger bioreactor sizes and make more vector for broader patient indications. At Homology, we can now run a 2,000-liter bioreactor and have a very scalable high-throughput process using hematographic techniques.

Where I think the field needs to focus next is trying to develop new technologies for virus and nanoparticle separations. Vendors are already working on this, which is great. They're being very collaborative, and we work with a lot of great vendors on trying to develop these technologies.

MH: There have been some advances in transient transfection of HEK293 cells, which remains the most popular and reliable process to manufacture AAVs. Notably, new transfection reagents have improved yields significantly.

As mentioned by the other panelists, affinity chromatography is a fantastic tool to achieve high recovery and high purity of AAV capsids.

Enrichment of full capsid is still a challenge – some serotypes are very easy to purify but others are much more challenging. It's something that everyone is focusing on today, so certainly the next big step will be in that area I believe.

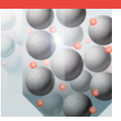
Q Toxicity issues have been much discussed in the AAV field of late – what do you see as the key pathways forward on the bioprocess side if the field is to address these concerns?

MH: As I mentioned earlier, there have been several recent toxicity events that have led to serious adverse events or even deaths. What shocked me most is the very early toxicity described by Jim Wilson's team recently, which seems to be correlated with complement activation, meaning that the capsids appear to immediately induce a toxic event. For me, I think that suggests the industry should consider decreasing the total amount of capsids in gene therapy products. That means, on one hand, removing empty capsids but also working with more effective serotypes or engineered AAV capsids that can be used at lower doses and are more tissue-specific.

At LogicBio we are putting a lot of effort into developing different methods to enrich for full capsids, and we have implemented a capsid engineering platform called sAAVy, which allows us to decrease the effective dose of our vectors.

MM: I think Matthias gave a great overview of what the field is experiencing right now. For us, we have one product in the clinic, HMI-102 for phenylketonuria, and so far, we have found that to be very well tolerated.

> "...we are putting a lot of effort into developing different methods to enrich for full capsids, and we have implemented a capsid engineering platform called sAAVy, which allows us to decrease the effective dose of our vectors."
>
> - Matthias Hebben



> "Plasmids … have their own challenges in terms of supply. From our side as a supplier of these tools, we want to ensure we can provide the materials necessary at the right time."
>
> - **Alejandro Becerra**

Beyond our observed clinical data, we leverage a lot of understanding of toxicology and immunogenicity from our colleagues across the organization, outside of CMC. For example, we've used mouse models to study the impact of empty capsids on potency, and through *in vivo* testing, we are starting to understand the impact of our capsids on immunogenicity. That allows us to tailor our processes to ensure a safe and effective product for our patients.

AB: Matthias mentioned full capsid enrichment as an important issue to address, and that's something we continue to work on internally and in collaboration with our partners, whether it's with existing tools or trying to develop new ones.

Q Can you comment on how and why the outsourced versus in-house vector manufacturing picture has developed over the past two years, and what future trends do you foresee in this regard?

MM: At Homology, we've taken an in-house manufacturing path, and we believe this has allowed us to quickly build up our pipeline.

Besides just manufacturing, internal process development and analytics also play a huge role, in that we're able to spend a lot of time, effort, and energy on developing and understanding our platform and products. This has allowed us to make significant improvements in both overall yield and purity. With internal capability, you have better control over your development pipeline and can dedicate more resources towards the internal manufacturing and development model.

AB: During the last roundtable, one of the challenges that came up was the restriction in the amount of AAV that can be produced globally. Two years on, we are seeing more organizations taking a similar approach to what Michael described. It's certainly a trend to see more and more in-house manufacturing, not only for the AAV but also plasmids. Plasmids are certainly one of the critical raw materials and have their own challenges in terms of supply. From our side as a supplier of these tools, we want to ensure we can provide the materials necessary at the right time.

MH: I have very mixed feelings about this question. It really depends on the indications that the company has in its portfolio. We are seeing more and more improvement in the manufacturing process. If you target a very small patient population for ultra-rare disorders, maybe you can supply the patient population with a very limited number of batches per year and it may not be the highest priority to have a manufacturing facility that is not going to be busy all year long.

But if you want to address a broader population and you need to manufacture one batch per week, clearly that's a different story. I'm not overlooking the fact that today the CDMOs are very busy, and the queues are very long. But I think there are more and more players in that field.

Q The COVID pandemic means the world is a very different place today compared to last time we spoke – what for you are the key ongoing issues for the gene therapy field that relate (directly or indirectly) to the pandemic, and what best practices have you sought to introduce at your respective organizations to counter them?

AB: It's definitely a different environment than two years ago. The pandemic put a significant strain on the suppliers, as some of the tools that are important for the cell and gene industry are being used for vaccines and therapies against COVID. It has prompted lots of suppliers to invest in expanding those manufacturing capacities, but since it wasn't planned it has taken some time, and it varies for specific products.

For the products I work with, namely chromatography resins, we were fortunate that we haven't really seen any impact on affinity resins, but other types of chromatography have been affected.

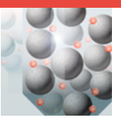
Fortunately, we had already been investing in an expansion of our existing facility and the build-up of a new one. We have accelerated those activities but it's still going to be sometime early next year before we can get back to normal delivery times. For now, we are working closely with our customers to improve delivery times where we can.

MH: It has been very complex for everyone, with shortages of everything from pipette tips to filters. In R&D that is manageable because you can always change your material and switch from one supplier to another. In GMP, it's a different story. You cannot do change control for every single raw material you would have to modify at the last minute so it can be a critical issue.

We have been very lucky at LogicBio that we have not been impacted at all in terms of GMP manufacturing. I know others that have been in a very bad situation as a result of shortages. I think this pandemic has shown how vulnerable the field is in terms of the supply chain. I hope there will be a solution to the shortages soon, but also that there will be a lot of work to anticipate the next big events and prevent shortages in future.

This pandemic has not just had huge impacts in the lab and the manufacturing space. For clinical trials it has been a real challenge – due to the risk of hospital systems becoming overwhelmed, clinical trials have been put on hold. At LogicBio we have been very lucky because we have been able to maintain very close contact with the clinical specialists, and able to continue to identify patients and have smooth enrollment of participants as soon as hospitals were able to be open. Thanks to that, we were able to start our first clinical trial, for pediatric patients with methylmalonic acidemia, this summer.

MM: I think we have a little bit of a different perspective because we have so much internal capability. We definitely



experienced some supply chain issues, but we have a great supply chain team that anticipated this to some degree, and they were able to work with the vendors proactively to make sure we were able to have the supplies we need to continue manufacturing. Consequently, we had no interruption to manufacturing, which was great.

In addition, our process development teams built a lot of redundancy into our process, to allow us to use alternative chromatography resins filters and so on. That means if we do have any supply chain issues, we're not dependent solely on one vendor.

I think the pandemic has highlighted weak points and forced companies to plan more for worst-case scenarios. Organizations will now aim to anticipate these shortages and make sure they have enough material in stock to ensure that manufacturing is uninterrupted, so clinical trials or commercial supply are not impacted. I think our organization has been able to do that pretty successfully.

MH: I agree that companies need to anticipate more in advance in this new world. But the issue remains – how do you manage the expiry dates of some ingredients when you have to stockpile things? Unfortunately, anticipation cannot solve all these problems.

MM: Absolutely. We do development stability for two years – in fact, we have enough material to go out to 36 months or longer. It comes back to the internal manufacturing, where we can plan out what batches we want to do and when, to align with clinical trials and expiry.

Thank you – it's interesting to get two contrasting perspectives from companies who have adopted different manufacturing models. It's clear from our conversation today that AAV process development has evolved significantly since we last met in 2019, and this has been a valuable update on the emerging advances and challenges.

AUTHORSHIP & CONFLICT OF INTEREST

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

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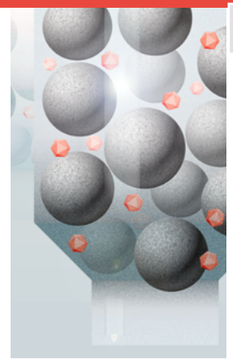

CELL & GENE THERAPY INSIGHTS

VECTOR Purification

CHAPTER 2

Downstream purification of adeno-associated virus for large-scale manufacturing of gene therapies

Mikhail Goldfarb & Orjana Terova

As the growth of the gene therapy field continues apace, it is crucial that the production of high yield, high quality viral vectors is achievable at a larger scale to meet the industry's growing needs. Validated viral clearance approaches are a key step in the manufacture of safe gene therapy products, and Thermo Fisher Scientific's combined CaptureSelect™ and POROS™ technologies offer an affinity chromatography platform that can simplify the vector purification process while also maintaining yield. A series of viral clearance studies conducted by REGENXBIO using the POROS™ CaptureSelect™ AAVX affinity resin demonstrated that the resin provided robust viral clearance, even under 'worst case' processing parameters outside of typical manufacturing conditions, as demonstrated using typical model viruses expanded to include human viruses. Additionally, minimal to no non-specific interactions were observed between the viruses and the AAV capsid, as well as between the viruses and POROS™ beads or V_HH ligand.

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INTRODUCTION

Significant advancements have been made in the gene therapy field in recent years, as evidenced by the increasing number of clinical trials and significant investments being made in the sector. With two therapies having already received FDA approval – Luxturna® (Novartis), in 2017 and Zolgensma® (AveXis) in 2019 – momentum in the field is building, further underpinned by the FDA

announcement last year that it expects that by 2025, approvals for cell and gene therapies will rise to 10 to 20 per year [1].

Vectors such as the non-enveloped adeno-associated virus (AAV) have played a key role in this clinical success and are being engineered and used to deliver DNA to target patient cells for the long-term treatment of rare diseases. Recombinant AAV subclasses have become the vector of choice for such therapies, with excellent clinical outcomes achieved at least in part due to their particularly strong safety profile and low immunogenicity.

However, there are still challenges for the field to address. The utilization of viral vectors in the clinic risks being derailed by manufacturing issues. While the field is able to produce high quality and high yield viral vector to meet current clinical needs, as more gene therapies become commercially available and their indications expand, there is an inevitable requirement to increase the scale and productivity of viral vector manufacturing. As such, there are many efforts underway to increase titer productivity in the manufacture of AAV, including attempts to optimize cell lines and achieve cost-effective scale-up by working with higher volumes.

As these improvements occur upstream, it is also of key importance that downstream purification solutions offer the high capacity, specificity and throughput that is needed to obtain AAV products with high yield and purity. Viral clearance is a crucial consideration within downstream purification – as clearly mandated by the FDA, and ICH Q5A [2,3], "all biotechnology products derived from cell lines have to demonstrate that the products are safe, by implementing appropriate testing, and demonstrating that the manufacturing process is capable of clearing any endogenous or adventitious viruses". Validated viral clearance approaches are therefore an essential step in ensuring the safety of gene therapy products. The importance of viral clearance studies is further emphasized by the need to understand the degree to which each individual step contributes to total viral clearance. Affinity chromatography, an established platform in both mAb and AAV capture spaces, is one such step that can contribute to viral clearance.

ENABLING A PARADIGM SHIFT IN VECTOR PURIFICATION

Affinity chromatography utilizes specific ligands which are more or less redundant coupled to a solid chromatographic support, allowing products to be captured from crude material. This approach offers several benefits including highly specific separation and provides the ability to perform one-step purification from crude material to a product with high yield and purity.

To bring the benefits of affinity chromatography to the gene therapy field, Thermo Fisher Scientific combined CaptureSelect™ and POROS™ technologies (see **Box 1**) to provide a process for the purification of viral vectors. CaptureSelect™ technology, which has already transformed the purification of antibody derivatives, and recombinant proteins, is now being applied to viral vectors; it can provide a simplified approach with lower cost and complexity while delivering the high purity and high yield products that the cell and gene therapy industry needs.

Resins currently available are the POROS™ CaptureSelect™ AAV8 and AAV9, which have been specifically designed and commercialized for AAV8 and AAV9 purification, and the AAVX affinity resin, which has been demonstrated to have specificity and affinity for a wide range of AAV serotypes. POROS™ CaptureSelect™ AAV affinity resins offer superior performance in terms of specificity and binding capacity, as shown through data obtained with various collaborators in both industry and academia [4].

For example, one end user reported that their standard purification process using three anion exchange chromatographic steps resulted in low yield and long process development times. When utilizing the POROS™ CaptureSelect™ AAV affinity resin, their product yield increased from 20% to 60%, and reduced costs by a factor of six.

BOX 1

CaptureSelect™ Affinity technology

The proven CaptureSelect™ affinity technology enables the purification of antibodies, antibody fragments, recombinant and plasma proteins, and viral vectors. These products enable increased purity and yield in a single purification step and are designed to simplify workflows and reduce time and cost in biopharmaceutical drug development. CaptureSelect™ affinity resins are manufactured in an animal origin-free production process making these resins suitable for process-scale bioseparations for a wide variety of biotherapeutic compounds.
The technology is based on a variable domain of Camelid heavy-chain only antibodies, or V_HH **(Figure 1)**. V_HHs are the smallest antigen binding fragments, of only around 15 kilodaltons, which allows binding to epitopes that may be difficult to reach, leading to unique affinity for the target molecule. These ligands are very robust, and can withstand the various conditions used during chromatography. Several V_HH ligands have been identified and developed against a broad range of AAV subtypes, as well as chimeras, allowing for effcient AAV purification.

The POROS™ backbone

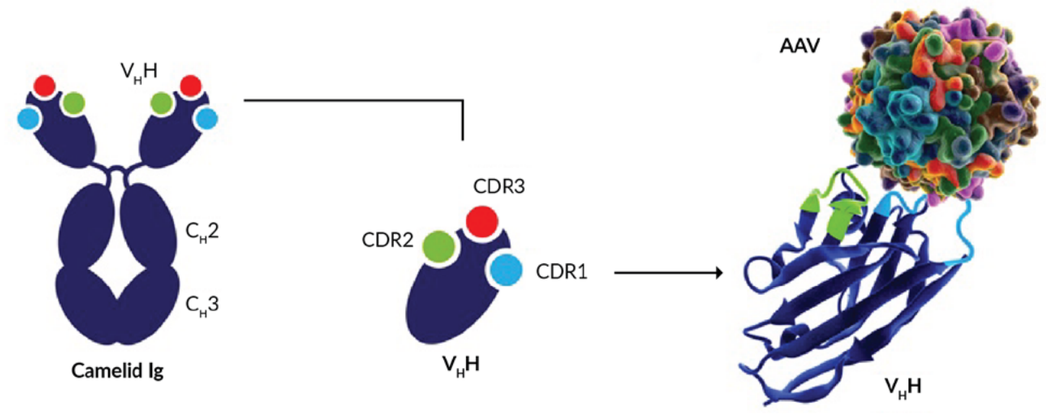
In order to make this purification process suitable for the capture of large biomolecules, such as AAV vectors, a large through-pore matrix support is used. The POROS™ technology offers three key advantages:

- **Poly(styrene-divinylbenzene) backbone:** resulting in linear and scalable performance. The beads are highly robust and chemically stable allowing for stringent cleaning when needed.
- **Large throughpores:** resulting in a reduced mass transfer resistance compared to other available resins. Capacity and resolution are maintained over a wide range of linear velocities, making the purification process more efficient.
- **50-micron bead size:** resulting in superior resolution. The smaller particle size (50 micron) results in tighter peaks and smaller elution volumes, helping to overcome tank size limitations at larger scale.

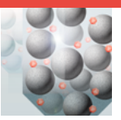
Therefore improving the ability to separate proteins and obtain effective impurity removal. For additional literature see [6].

FIGURE 1

CaptureSelect™ technology is based on Camelid-derived single domain [V_HH] antibody fragments, the smallest antigen binding molecule.



The small size of V_HH fragments (~15kD) allows binding at difficult to reach epitopes. Overall, V_HH fragments offer high specificity, affinity and stability.



ENSURING VIRAL SAFETY

A viral safety program focuses on three core areas: prevention, detection and removal. Prevention concentrates on avoiding adventitious viruses from entering products by selection of virus-free cell lines and raw materials, and by using closed process steps and single-use systems where possible. However, it is impossible to guarantee that no adventitious viruses have entered a process: closed processes are never completely closed, and you cannot fully control raw materials in your supply chain.

Therefore, an important component of a viral safety strategy is to implement a testing program to detect viruses at critical points in the production processes, such as in cell substrates, harvest, and bulk drug substance. Testing for specific viruses that have a high likelihood of being present in the product, such as any endogenous viruses which are known to be in the cell lines, or testing for general viruses such as retroviruses, are all required. However, there is an issue of limit of detection for each assay, and viruses may fall beneath that limit and thus enter the final product.

The third focus of the viral safety strategy is aimed at demonstrating that the purification process can clear any residual viruses possibly ending up in the product despite implementing prevention and detection strategies. In this step, the capacity to remove viruses is demonstrated by executing viral clearance studies which evaluate the removal of spiked viruses via downstream steps such as chromatography, filtration, or other steps that have the capacity to inactivate or remove viruses.

The importance of viral clearance: a case study from REGENXBIO

REGENXBIO is a gene therapy company that has developed a proprietary NAV® technology platform utilizing AAV vectors for delivery of various gene targets. Additionally, REGENXBIO has multiple in-house therapeutic programs predominantly utilizing AAV8 and AAV9 serotypes for gene delivery. As such, continuous development efforts are ongoing to optimize platform processes for AAV8 and -9 production, targeting high yield and high product purity. The processes utilize HEK293 producer cells adapted for suspension culture, modified to generate AAV with a gene of interest in a triple transfection process.

Rationale for choosing an affinity resin

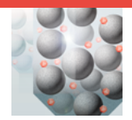
Effective downstream purification to generate a clinical product of high titer, potency and purity is essential while maintaining high AAV capsid recovery. AAV capsids comprise less than 0.1% of the proteins and nucleic acids that are generated by HEK293 cells, with most non-product related impurities being host cell proteins, host cell nucleic acids, plasmid DNA, media and feed components [5]. Therefore, in order to achieve such significant impurity clearance an effective affinity resin is required.

As discussed above, demonstrating effective viral clearance during the purification process is an integral part of an overall viral safety program. In order to better assess the effectiveness of the POROS™ CaptureSelect™ AAVX affinity resin, a series of viral clearance studies were conducted.

Study design

Viral validation for chromatography procedures is routinely performed by spiking a model virus into the load material and performing the chromatography procedures under scaled-down conditions that are considered representative of manufacturing scale. Both the load and the step products are evaluated for virus titers and the viral titer reduction across the purification step is measured in terms of the log reduction value (LRV), which can be expressed using the following equation:

$$LRV=\left[\left[Log\right]\right]\_10 \frac{\text{(Total virus in Load)}}{\text{(Total Virus in Product)}}$$



Due to assay variability, an LRV of less than one is considered negligible and cannot be counted towards the overall viral clearance. Clearance levels of 1 to 3 LRVs are thought of as contributing, and anything larger than 4 represents a robust and effective viral clearance step.

As no known viruses are present in the cell line and raw materials used in this study, a typical set of non-specific model viruses representing the four virus species categories were selected: enveloped and non-enveloped DNA viruses were represented by pseudorabies virus (PRV) and minute virus of mice (MVM); enveloped and non-enveloped RNA viruses were represented by xenotropic murine leukemia virus-related virus (XMulV) and reovirus type 3 (Reo-3). As a human-derived cell line was used, two human viruses – hepatitis A (HAV) and herpes simplex virus 1 (HSV-1) – were also added, based on risk assessment of possible sources of contamination by adventitious viruses that could originate from process operators and propagate in the producer cells.

To determine the extent of viral clearance across the affinity purification step, the viral titers in load and elution were measured, and elution were measured by infectivity assay for non-enveloped viruses. Since the AAVX column elutes at low pH, and low pH elution inactivates enveloped viruses, an infectivity assay was unsuitable for demonstrating the removal of these viruses by the chromatography step. For this reason, qPCR was used for enveloped virus detection. In addition to evaluating load and elution fractions, the rest of the column fractions were measured to perform the mass balance calculation.

To assess the robustness of the purification step, 'worst-case' conditions outside of typical manufacturing ranges were chosen to evaluate whether efficient viral clearance could still be achieved. The specific conditions chosen were:

- Higher virus load ratio: maximize amount of virus loaded at 145% of manufacturing target;
- High load residence time: allows for maximum virus–resin contact time during loading; at 170% of manufacturing target;
- High elution residence time: maximum virus-buffer contact time during elution; at 170% of manufacturing target;
- Combination (high load ratio + high load/elution residence time).

A further set of experiments were designed to evaluate product–virus interactions and non-specific interactions between viruses and resin bead as follows.

Product–virus interactions were evaluated using AAV-null load, which was generated by collecting the non-bound fraction from the affinity column run. The AAV-null load, or affinity non-bound fraction, was spiked with viruses and purified at the same conditions as the control run containing AAV8 capsids, and viral clearance was compared to the control.

Non-specific interactions between viruses and resin beads were also evaluated by using resin consisting of POROS™ beads without V_HH ligand and evaluating viral clearance across that column. Lastly, interactions between virus and camelid-derived V_HH ligand were assessed using a V_HH ligand with alternate CDR regions (not targeting AAV capsids).

RESULTS

Viral clearance

The results of the viral clearance runs evaluating performance of the AAVX affinity resin at both standard manufacturing process conditions and at worst case conditions are summarized in Table 1.

The POROS™ CaptureSelect™ AAVX affinity column showed robust clearance at manufacturing target parameters for most of the tested viruses: under target manufacturing process conditions, XMuLV, MVM, HAV, and PRV all demonstrated clearances of ≥4 LRV. Of these, MVM is the most problematic virus to clear due to its similar size to AAV and its

TABLE 1

POROS™ CaptureSelect™ AAVX Affinity resin viral clearance study results: process space.

Run description	RNA enveloped	DNA non-enveloped	RNA non-enveloped		DNA enveloped	
	XMuLV	MVM	Reo-3	HAV	PRV	HSV-1
Manufacturing process conditions	>6.4	4.4	2.7	>4.9	4.0	3.1
Higher residence time	5.9	4.6				
Higher load ratio	4.3	3.7				
Higher load ratio + residence time	4.6	3.6	2.5	5.0	3.8	3.6
Clearance level	Effective	Effective	Contributing	Effective	Effective	Contributing

The extent of viral clearance achieved for each tested virus is color coded as follows: **Green = effective (or higher than 4 LRV)**, **Yellow = contributing (1 to 3 LRV)**, and **Red = negligible clearance** (which was not observed for any of the viruses).

high resistance to inactivation. The POROS™ CaptureSelect™ AAVX affinity column also contributed to the clearance of Reo-3 and HSV-1 (≥2.5 LRV). However, as Reo-3 is larger than AAV, it can be separated through filtration, whilst HSV is enveloped and can be inactivated by detergent or low pH.

The run assessing higher residence time demonstrated a comparable level of viral clearance to that achieved at manufacturing process parameters. With an additional 45% viral load, viral clearance was reduced slightly; however, in conditions of combined higher load and reduced flow rate, similar viral clearance was observed that were within 1 LRV of the clearances achieved with manufacturing target conditions.

When studying interactions between viruses, the product, and resin ligand beads, only two test viruses were selected due to study capacity constraints. XMuLV and MVM were chosen as they are commonly used in viral clearance studies, with XMuLV representing large enveloped retroviruses, and MVM representing small, hardy, non-enveloped DNA parvoviruses.

The control run was performed at target manufacturing parameters. The AAV-null run, which was loaded with material containing the spiked virus but without AAV capsids, showed similar clearance to the control, indicating minor-to-no interactions between the capsid and the virus. One can conclude that neither the absence nor presence of AAV8 capsid in the load had a significant effect on the LRV achieved.

As shown in Table 2, further runs testing virus–V_HH ligand and virus–POROS™ bead non-specific interactions demonstrated clearances that were similar to the control run, indicating that the modifications implemented during those runs did not influence the extent of the observed viral clearance.

TABLE 2

POROS™ CaptureSelect™ AAVX Affinity resin viral clearance study results: interactions.

Evaluated interaction	Run description	Virus	LRV	ΔLRV to control
	Control run	XMuLV	>6.4	
		MVM	4.4	
Virus product	AAV-null load	XMuLV	5.1	>1.3
		MVM	4.0	<1
Virus alternative V_HH	Resin with alternate V_HH ligand	XMuLV	5.1	>1.3
		MVM	3.8	<1
Virus-POROS™ beads	Base matrix resin, no ligand	XMuLV	5.8	>0.6
		MVM	3.6	<1

Conclusion: no significant interactions between viruses and resin/ligand given minor difference in LRV results relative to control.

When observing the mass balance, the results were in line with expectations: the majority of viruses were in the non-bound fraction (Table 3). Due to assay variability, it is difficult to achieve results with enough accuracy for the mass balance to add up to 100%, but by observing the quantitative trends, non-bound fractions contained most of the virus, and the amount of virus binding and remaining on the resin in the strip fraction was significantly less than 1%.

CONCLUSIONS

The results indicate that the POROS™ CaptureSelect™ AAVX resin is an effective resin for viral clearance, with robust clearance of XMuLV, MVM, HAV, and PRV viruses achieved under manufacturing conditions. Of particular benefit is the effective clearance of MVM, for which there are currently limited clearance options (outside of chromatography). MVM is particularly problematic owing to its similar size to AAV and being highly resistant to inactivation steps such as incubation with detergents or at low pH conditions.

The two viruses that had clearances below 4 LRV (Reo-3 and HSV) still demonstrated significant reductions of more than 2.5 LRV at target manufacturing conditions. The industry-accepted safety target for processes without endogenous viruses is 6 LRV, which is likely to be achieved by a further chromatography step in the downstream process. If necessary, a viral filtration step could also be implemented, as these viruses are significantly larger than AAV.

In the case of HSV, this is an enveloped virus that should be effectively inactivated during low pH hold. The low pH hold can be added at the AAVX column elution stage as AAV capsids have been observed to be stable while held at lower pH conditions.

For XMulV and MVM viruses, minimal interactions were observed between AAV capsids and the viruses. This may present a case for bridging viral clearance results generated with one AAV8-based product to other AAV8-based products, providing that inserting a different transgene into the capsid does not affect these interactions.

TRANSLATIONAL INSIGHT

As demand for viral vectors in the gene therapy field increases and large-scale manufacturing capabilities grow ever more important, improved downstream purification solutions are a key consideration when aiming to produce high yield and high purity vector products.

The POROS™ CaptureSelect™ AAVX affinity resin has demonstrated robust viral clearance during vector purification across a range of those AAV serotypes that are core to current gene therapy approaches. The POROS™ CaptureSelect™ platform provides a simplified and highly effective means of optimizing this critical downstream bioprocessing step, which is central to ensuring the safety and efficacy of gene therapies.

The POROS™ CaptureSelect™ platform provides a simplified and highly effective approach to achieving effective viral clearance, and can help optimize this critical downstream bioprocessing step, central to ensuring the safety and efficacy of gene therapies.

TABLE 3

POROS™ CaptureSelect™ AAVX Affinity resin viral clearance study results: mass balance analysis.

Column fraction	XMuLV	MVM	Reo-3	PRV	HSV-1	HAV
Non-bound	165.8%	66.1%	83.7%	64.7%	79.3%	25.8%
High-salt wash	3.1%	1.0%	1.1%	1.4%	0.7%	1.2%
Low-salt wash	0.7%	0.2%	0.1%	0.3%	0.2%	0.01%
Product elution	<0.00004%	0.004%	0.2%	0.01%	0.1%	0.0004%
Low pH strip	<0.0002%	0.08&	0.03%	0.002%	0.02%	0.006%

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VIEW RESOURCES

AUTHORSHIP & CONFLICT OF INTEREST

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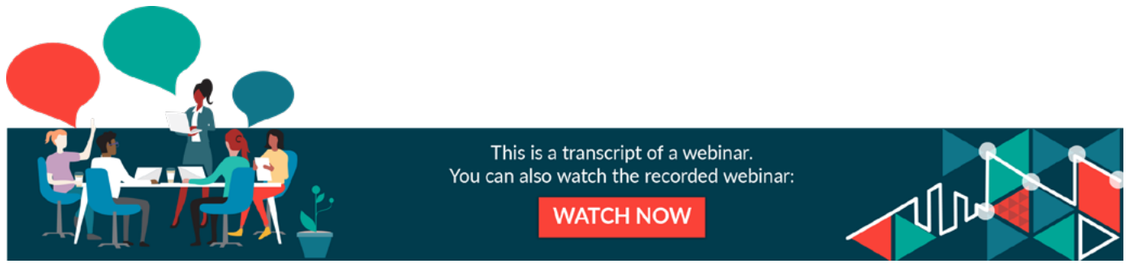

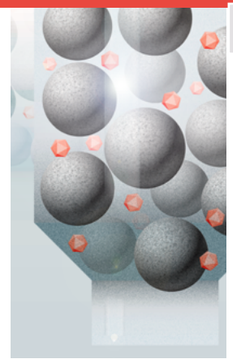

CELL & GENE THERAPY INSIGHTS

VECTOR Purification

CHAPTER 3

Platform optimization for efficient AAV purification: insights from a CDMO

Vincent Ravault & Nicolas Laroudie

Over the last decade, the number of clinical trials involving recombinant adeno-associated viral (AAV) vectors has dramatically increased, the diversity of serotypes has expanded, and the demand for larger quantities of highly purified material manufactured to cGMP standards has rocketed. For contract development and manufacturing organizations (CDMOs) like Yposkesi, the manufacturing challenges are centered around flexibility, robustness, and productivity, especially with regards to purification. Universal tools able to address any serotype with minimal process adjustments are critical. In this article, we describe how POROS™ CaptureSelect™ AAVX resin can be used as a pan-affinity tool for the universal capture of AAV vectors, and how Yposkesi optimized the operational parameters to make the resin an efficient, robust, and productive purification platform that fits within the constraints faced by CDMOs.

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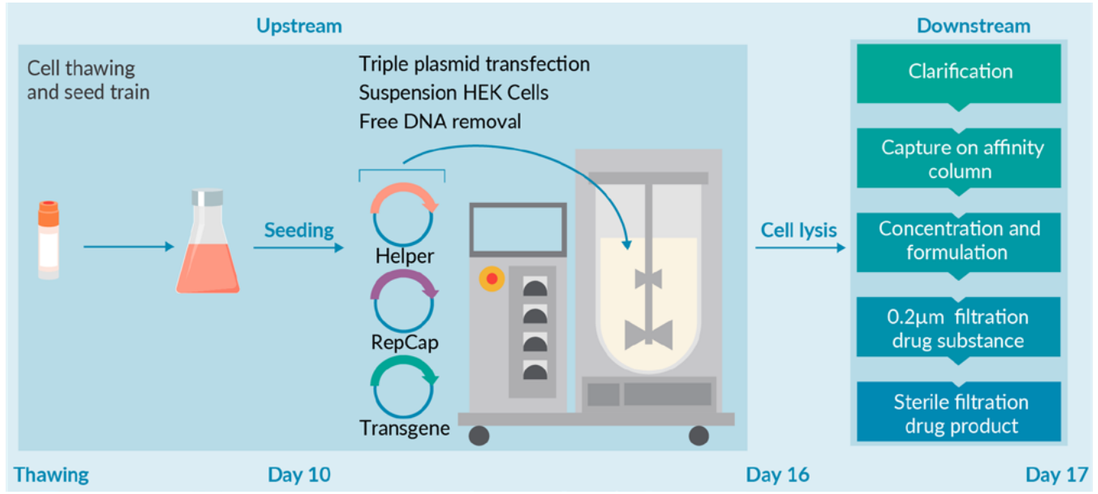
As a full-service CDMO for innovative gene therapy products, Yposkesi supports customers from early-stage development, including process and analytical development, through to large-scale production and commercial supply of gene therapy products.

Yposkesi produces recombinant adeno-associated virus (rAAV) and recombinant lentivirus (rLV) vectors using adherent- and suspension-adapted cell platforms. The manufacturing platform at Yposkesi currently includes four independent production suites equipped with 200 L single-use bioreactors, which will evolve to include a 1000 L single-use bioreactor from 2023. Yposkesi is currently building an additional 5,000 m^2 clinical/commercial

FIGURE 1

AAV manufacturing process at Yposkesi.



manufacturing plant to support the growing demand for viral vector supply.

This article describes how Yposkesi developed an AAV purification platform for a range of serotypes based on Thermo Fisher Scientific's POROS CaptureSelect AAVX Affinity Resin.

YPOSKESI'S AAV MANUFACTURING PROCESS

The established AAV manufacturing process at Yposkesi is shown in Figure 1. AAV vectors are produced by triple plasmid transfection in human embryonic kidney (HEK) cells. The lysate is clarified and then directly loaded onto an affinity column. The eluted vectors are concentrated and formulated, before being sterile filtered. The full process lasts 17 days, from cell thawing to drug product filling.

The current AAV purification process involves the use of different affinity sorbents according to the AAV serotype to be produced. The POROS CaptureSelect AAVX Affinity Resin leans on the use of a ligand derived from a heavy-chain antibody that can bind AAV serotypes 1–9 and synthetic or recombinant AAV vectors, offering a great opportunity to develop the next AAV purification platform at Yposkesi (Figure 2) [1].

EVALUATION OF DYNAMIC BINDING CAPACITY

As a first step to evaluate the AAVX resin as a platform purification solution, the dynamic binding capacity was evaluated using an AAV8 serotype. The binding capacity was assessed using 1 mL-prepacked columns, packed with either POROS CaptureSelect AAV8 or POROS CaptureSelect AAVX. The binding capacity was assessed at 1 and 3 mins residence time on two different feedstocks, each with different initial virus titers.

Clarified supernatant containing AAV8 vectors was directly loaded on the affinity columns until a 10% breakthrough in AAV8 was observed in the flowthrough.

Multiple fractions (column volumes [CV]) were collected at the outlet of the column during the loading phase, and the quantity of capsids was determined by ELISA assay in each collected fraction. The results for the 3 mins residence time are presented in Figure 3.

No breakthrough was observed on the AAVX resin at loading volume of up to 1,500 or 2,000 column volumes for the low viral titer and higher titer feedstock, respectively. Both resins showed higher binding capacity when feeds contained a higher vector titer, but overall, the AAVX resin showed a higher binding capacity for AAV8 than the Poros CaptureSelect AAV8 resin.

Figure 4 shows the binding capacity of AAV8 vectors measured at 1 min residence time on both resins, showing similar binding

FIGURE 2

Key features of camelid-derived, recombinant expressed ligands used in CaptureSelect™ Affinity Resins.

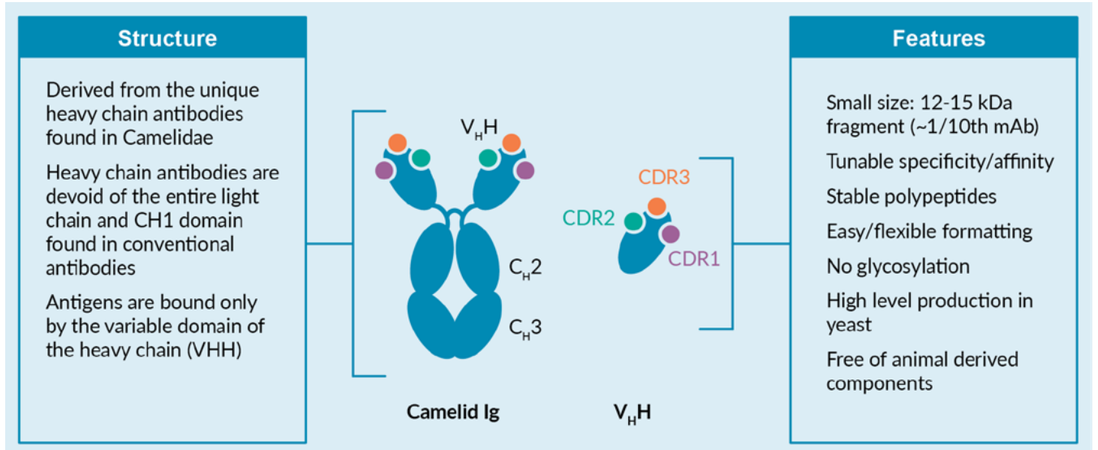
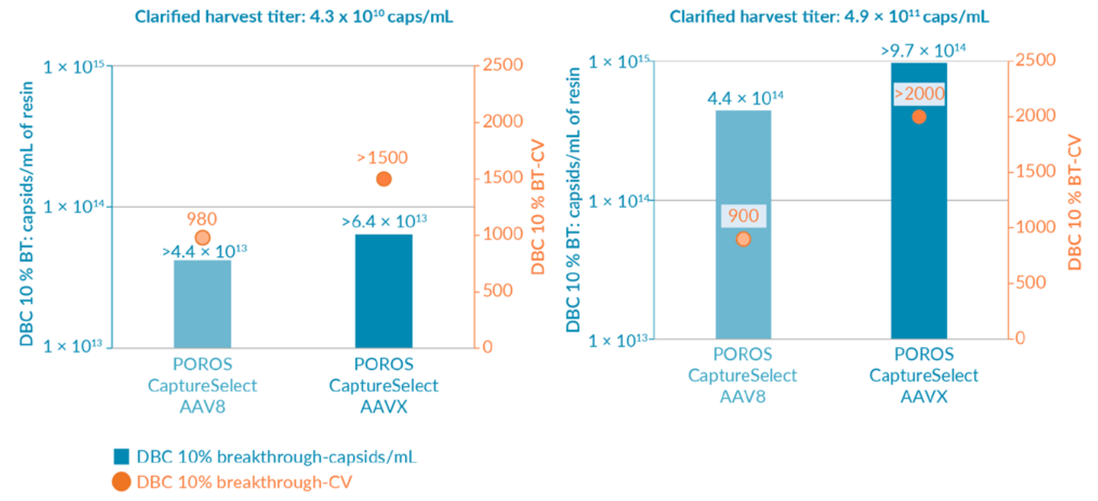


FIGURE 3

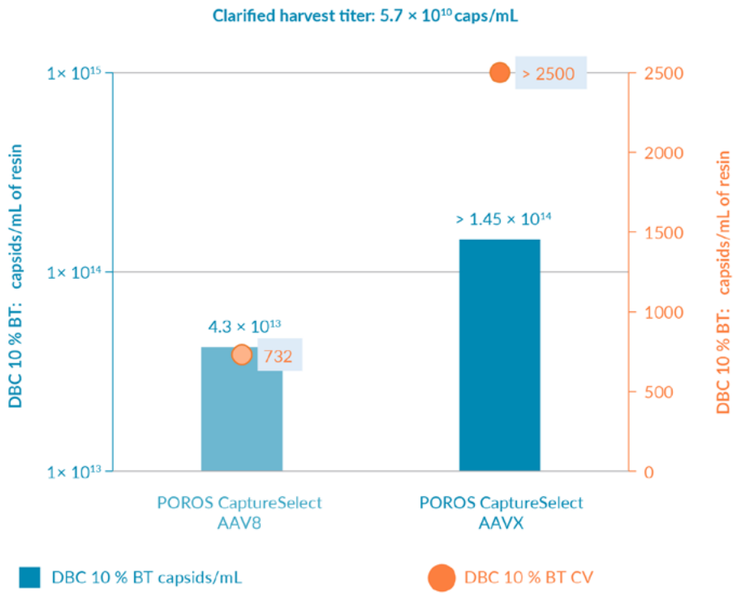
Binding capacity at 3 mins residence time and 10% breakthrough for POROS CaptureSelect AAV8 and POROS CaptureSelect AAVX at AAV titers of 4.3×10^{10} caps/mL (left) and 4.9×10^{11} caps/mL (right).



The blue bars represent the binding capacity in terms of capsids per mL of resin at 10% breakthrough. The orange dots represent the column volumes that lead to 10% breakthrough.

FIGURE 4

Binding capacity at 1 min residence time and 10% breakthrough for POROS CaptureSelect AAV8 and POROS CaptureSelect AAVX at an AAV titer of 5.7×10¹⁰ caps/mL.



The blue bars represent the binding capacity in terms of capsids per mL of resin at 10% breakthrough. The orange dots represent the column volumes that lead to 10% breakthrough.

capacities compared with the 3 mins residence time. At both residence times, there was no breakthrough on AAVX, with loading volumes up to 2,500 column volumes (CV).

The results from these binding capacity studies led to three main conclusions:

1. The AAVX resin has a better AAV8 binding capacity than the AAV8 resin
2. Binding capacity increases with harvest titer
3. Residence time has no significant effect on the binding capacity

DEFINING OPERATING CONDITIONS FOR PURIFICATION OF AAV8 & AAV2

The operating conditions for the capture of the AAV8 serotype were defined according to the DBC data obtained previously. Screening of capture conditions was performed on 1 mL-pre-packed columns with AAV8 or AAVX resin (Figure 5).

The material loaded onto the columns was a clarified supernatant containing AAV8 vectors. To align with our AAV manufacturing operating conditions, the maximum loading time selected was 18 hours – (overnight loading). Two residence times were evaluated: 3 mins and 1 min.

The loading volumes selected were 356 CV (for AAV8 and AAVX) with a 3 mins residence time, and 600 CV (AAV8) and 1080 CV (AAVX) with a 1 min residence time. These CVs are all below the resin binding capacities at 10% breakthrough defined earlier (Figures 3 & 4).

After loading and washing, purified product was recovered during the elution step at low pH and was immediately neutralized.

The clarified harvest and eluent were tested for viral genome (VG) titer.

Similar quantities of AAV vectors were loaded on the AAV8 and AAVX resins at 3 mins residence time. As shown in Figure 6, the quantity of AAV8 vector recovered after elution and the AAV8 yield was very similar for both resins. The resins showed no significant difference in performance when loading at 3 mins residence time or at 18 hours loading time.

Results at 1 min residence time are shown in Figure 7. As a consequence of the different binding capacities at 1 min residence time, the loading times were different for the two sorbents – 10 hours for the AAV8 resin and 18 hours for the AAVX resin. Thus, the total quantity of AAV8 capsid loaded on the resins was around 1.8 times higher for the AAVX resin compared with the AAV8 resin. As a result, the quantity of purified recovered product for AAVX was approximately 1.7 times higher. The step yields for both resins were also very similar and close to 90% which is higher than the yield of around 70% obtained with a residence time of 3 mins.

These results indicate that it will be possible to switch from POROS CaptureSelect AAV8 to AAVX for the purification of AAV8 serotype.

Based on these results with POROS CaptureSelect AAV8, the AAVX resin was also evaluated for the capture of another serotype of AAV: AAV2 (Figure 8). The aim was to compare POROS CaptureSelect AAVX with an affinity resin from another supplier, which is currently used at Yposkesi for AAV2 processes.

The screening of the capture conditions was performed on 1 mL prepacked columns. Two residence times were applied for the AAVX resin: 3 mins and 1 min. The residence time applied to the other affinity resin was 8 mins, according to supplier's recommendation.

Three purification conditions were screened for the capture of the AAV2 vector. At 3 mins residence time, the volume of clarified harvest loaded on the column was 455 CV, whereas at 1 min residence time the volume loaded on the column was 1,440 CV. The same starting material was used for all trials.

For the other resin, only 340 CV were loaded since the residence time applied was higher. After column washing, the product was eluted at low pH, and the loading and elution fractions were tested for VG titer.

Using the AAVX resin and decreasing the residence time from 3 min to 1 min resulted in an increase in VG yield from 57% to 89% (Figure 9). Using the affinity resin from another supplier with higher residence time (8 mins, imposed because of the compressibility of the

FIGURE 5

Experimental plan for the definition of operating conditions for purification of AAV8.

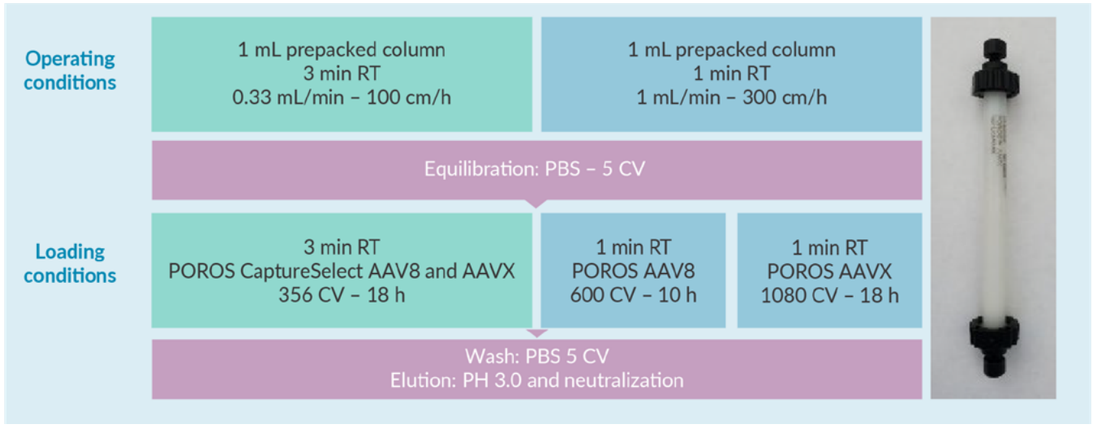
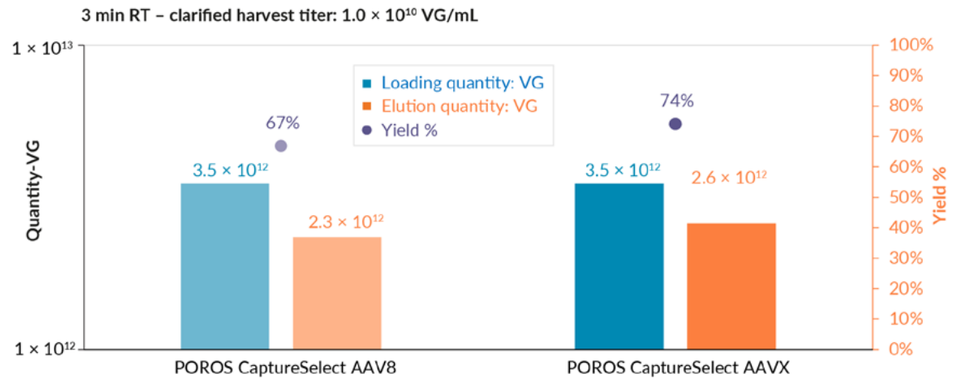


FIGURE 6

AAV8 capture conditions – results for 3 mins residence time.



The blue bars represent the product quantity loaded on each column. The orange bars represent the quantity of purified product recovered during the elution. The red dots represent yield.

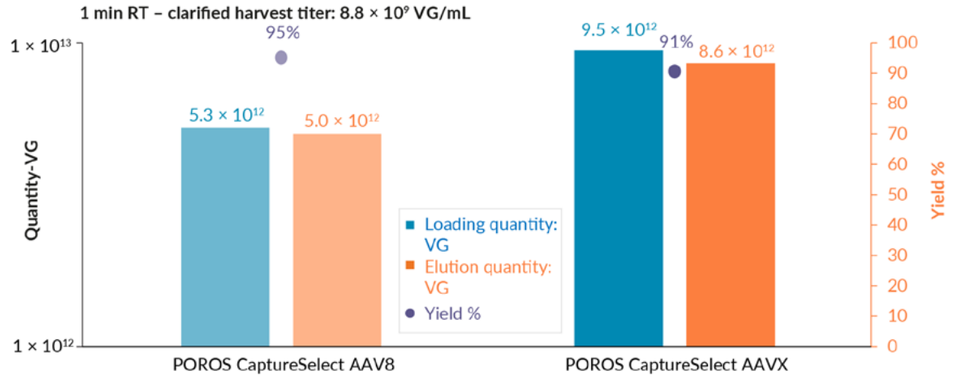
media, and as recommended by the supplier), resulted in a low volume loaded on this column. The AAV2 yield is significantly lower than the yield obtained with AAVX: 48% yield, versus 70–90% yield obtained with AAVX.

This part of the study demonstrated that using a lower residence time results in higher AAV binding capacities for both Thermo Fisher Scientific resins, and that the AAVX resin shows better results for the capture of AAV8 and AAV2 vectors. The volumes of clarified harvest that can be loaded on AAVX without any AAV breakthrough in the flowthrough are 1080 CV for AAV8, and 1440 CV for AAV2.

The promising results obtained with POROS CaptureSelect AAVX led us to select this resin for the next part of the study and to work with a residence time as close as possible to 1 min.

FIGURE 7

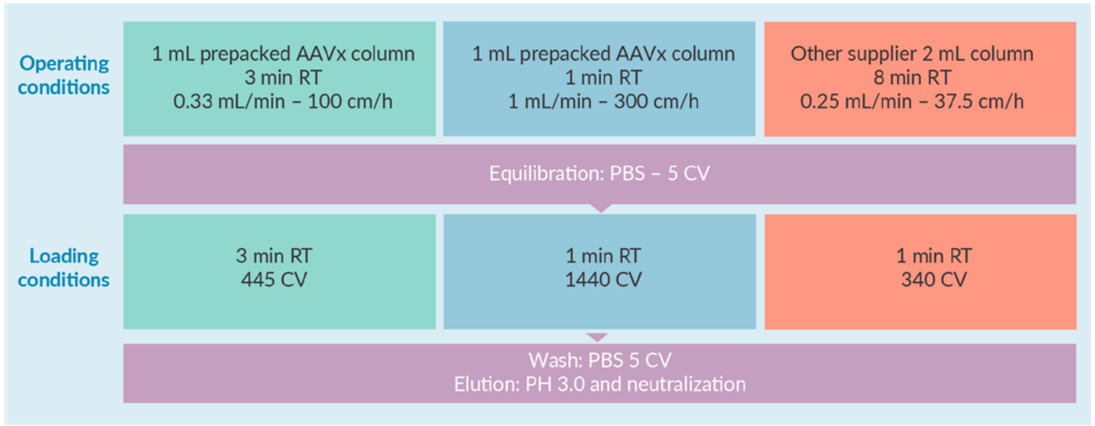
AAV8 capture conditions – results for 1 min residence time.



The blue bars represent the product quantity loaded on each column. The orange bars represent the quantity of purified product recovered during the elution. The red dots represent yield.

FIGURE 8

Experimental plan for the definition of operating conditions for purification of AAV2.



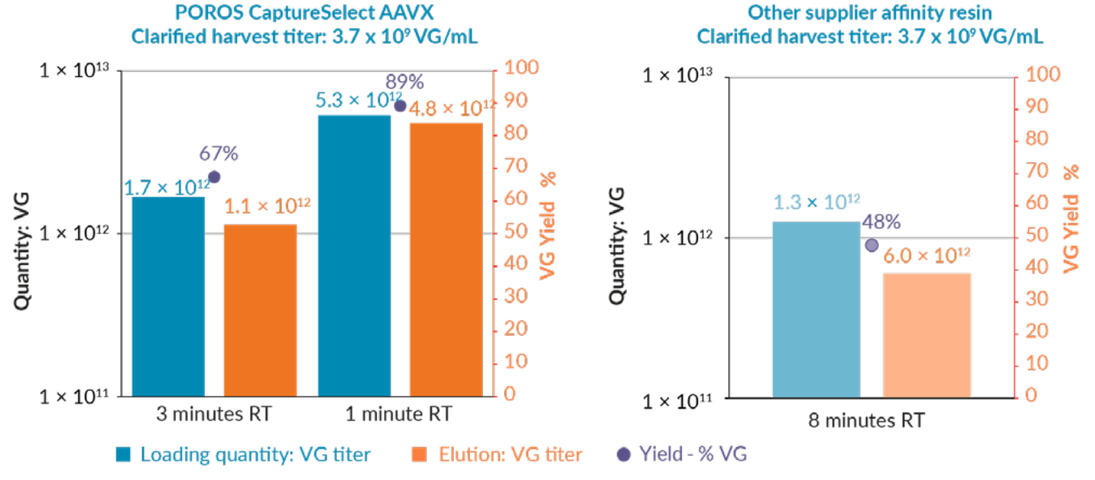
SCALE-UP OF THE CHROMATOGRAPHY STEP

The experimental conditions determined using AAVX for the capture of AAV2 and AAV8 vectors were adapted for the purification of clarified harvest from a 10-liter bioreactor (Figure 10).

The volume of resin necessary to purify a 10 L clarified harvest was calculated by applying the column loading capacity in terms of CV determined previously during the screening for AAV8 and AAV2 processes. This AAVX resin volume was found to be 14.3 mL for AAV8 capture and 11.3 mL for AAV2 purification process.

FIGURE 9

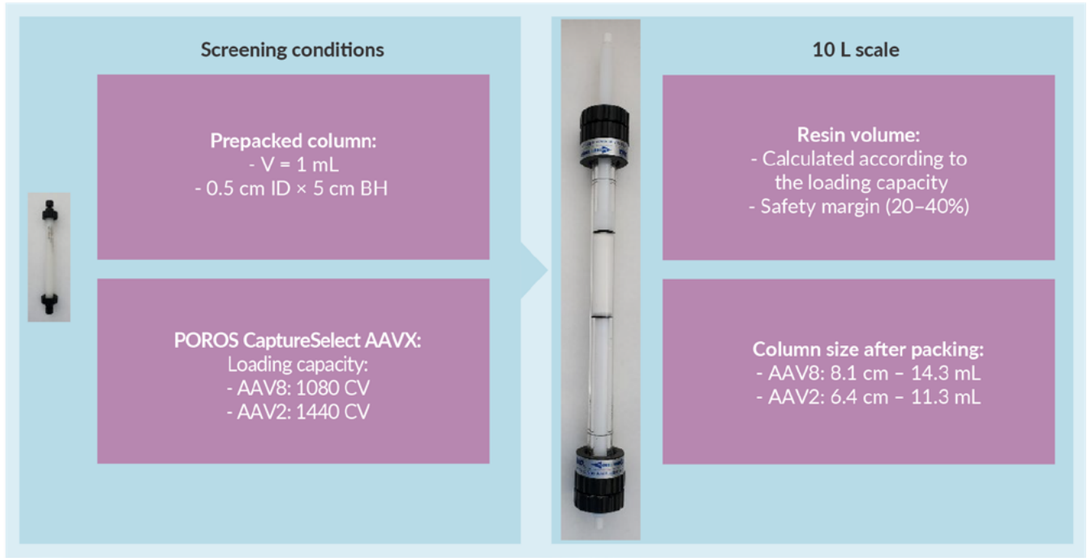
Definition of the operating conditions for the purification of AAV2 using POROS CaptureSelect AAVX (left) or alternative supplier's affinity resin (right).



The blue bars represent the product quantity loaded on each column. The orange bars represent the quantity of purified product recovered during the elution. The red dots represent yield.

FIGURE 10

POROS CaptureSelect AAVX chromatography scale up.



Conditions to be tested are listed for small-scale (left) and 10-liter scale (right).

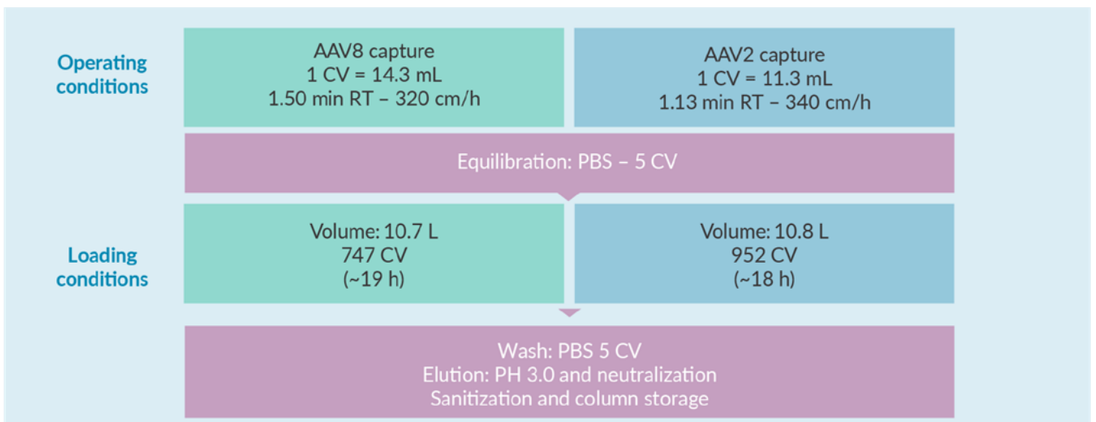
AAVX resin was packed in a 15 mm internal diameter glass column, which allowed for a resin bed height that would be easily transferrable to GMP scale. The column bed height was 8.1 cm for AAV8 purification and 6.4 cm for AAV2 purification.

In order to obtain the starting material for resin evaluation, two 10 L bioreactors were used to produce AAV2 and AAV8 vectors from HEK cells.

After AAV production, cells were lysed, and the lysate was clarified and filtered using a 0.22 µm filter. After lysate filtration, the pool titer was 1.10 x 10^{11} VG/mL for AAV8 vectors and 3.70 x 10^{9} VG/mL for AAV2 vectors.

FIGURE 11

Selected chromatography operating conditions at larger scale.



The selected operating conditions for the AAVX resin to purify AAV8 and AAV2 from a 10 L clarified harvest are shown in Figure 11.

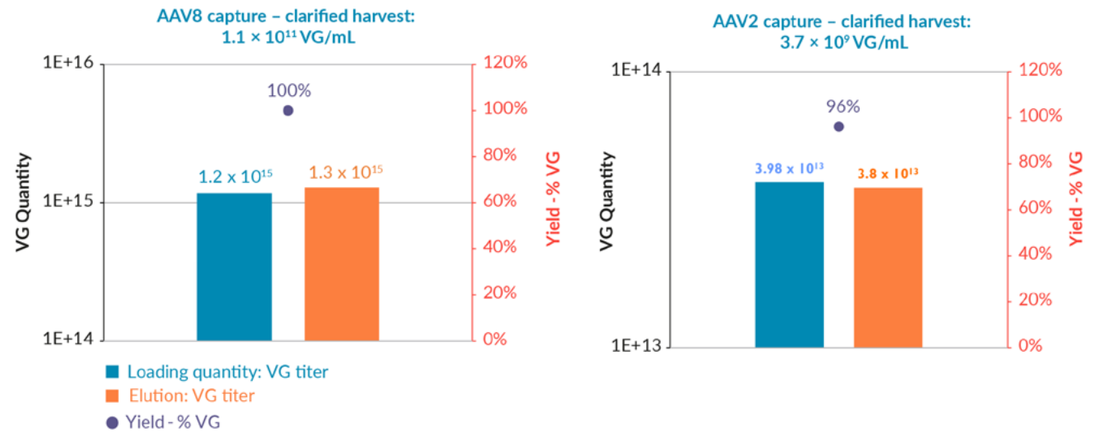
The residence time for both AAV processes was close to 1 min. 747 CV of clarified harvest were loaded on to the AAVX resin for AAV8, and 952 CV for AAV2, while the loading times were in the same range. The purified products were recovered during elution at low pH and then neutralized. VG titers, total protein content, and residual DNA levels were assayed in the clarified harvests (starting materials) and in the elution fractions.

The pressure was monitored at the inlet of the column during the loading step for the AAV8 and AAV2 capture process. The pressure slightly increased during the loading stage but stayed within an acceptable range. The pressure was around 1.5 bars at the end of the loading step, which helps to provide good conditions for a transfer to GMP scale.

Even though VG titers in the starting material were very different for the AAV2 and AAV8 serotypes, the final yields of the capture step are close to 100% for both serotypes and there was good scalability from lab scale development to the 10 L scale (Figure 12).

FIGURE 12

AAV capture on POROS CaptureSelect AAVX – yields for AAV8 (left) and AAV2 (right).



The blue bars represent the product quantity loaded on each column. The orange bars represent the quantity of purified product recovered during the elution. The red dots represent yield.

Additional experiments revealed that the purity of AAV vectors captured with AAVX resins appears to be very high. There was an impurity reduction of over 99% in the purified product after capture on AAVX for each serotype. This clearance rate could be even further optimized by adding an intermediate washing step or implementing a polishing column after the AAV capture step.

CONCLUSIONS

This long-term study with POROS CaptureSelect AAVX resin has highlighted several advantages of AAV capture using this resin compared to other affinity resins commercially available:

- Flexibility in terms of serotypes: capture of AAV1 to AAV9 serotypes and synthetic and recombinant serotypes

- Possibility to standardize a purification platform for several AAV serotypes with only a few adjustments
- Cost reduction due to shorter residence times and very high loading volumes.
- Low level of impurities captured on the resin. This could be further optimized for each serotype if needed (wash conditions screening or addition of a polishing step)
- Good scalability of the downstream platform. It is compliant for a large-scale GMP AAV manufacturing process

Overall, Yposkesi concluded that the POROS CaptureSelect AAVX resin appears to be a great tool to improve purification processes in terms of quality, cost, and standardization. Yposkesi plan to implement this resin for the purification of other AAV serotypes.

ASK THE EXPERTS

Elisa Manzotti, Founder, *Cell and Gene Therapy Insights,* speaks to **Nicolas Laroudie** (Thermo Fisher Scientific) and **Vincent Ravault** (Yposkesi) to answer readers' questions on implementing POROS CaptureSelect technology into viral vector production.

Q Can the POROS CaptureSelect resin be cleaned and re-used?

NL: Yes, the resin can be cleaned and reused. Many customers use the resin once, particularly CDMOs that deal with multiple serotypes and multiple transgenes and want to avoid spending a lot of time validating cleaning. But the resin can absolutely be cleaned and reused, and many customers are doing that.

Notably, the resin is not very caustic stable, and so for cleaning, we do recommend using acidic solutions such as phosphoric or citric acid. In case of very dirty resin, we advise additional cleaning with chaotropic agents, such as guanidine hydrochloride or urea.

I would encourage people who want to clean and re-use the resin to reach out to their local application specialist, who can help them develop a process for this.

Q Apart from AAV2 and AAV8, do you have experience with other AAV serotypes and POROS CaptureSelect AAVX?

VR: The goal for Yposkesi now is to expand this platform to a broad range of AAV serotypes. With our experience of AAV2 and AAV8 serotypes, we know that the AAVX resin is a good solution to use as a purification platform.

Currently, we are working with AAV5, 6, and 9, and the results so far are promising. We also know that we can work with modified capsids.

Q Is the resin available in a pre-packed format?

NL: Yes, we do have pre-packed formats. We have 1 and 5 mL pre-packed formats available that are compliant with standard benchtop chromatographic systems. We also have robocolumns available, at 200 µL and 600 µL, for high-throughput screening.

Of course, the resin can be purchased as bulk material and our local Field Application Specialists are happy to support customers in packing the resins in their own columns, whatever the scale.

Q Which additional washing conditions would be suitable for host cell protein and host cell DNA reduction?

VR: Several washing conditions are interesting to assess. For example, you can add an extra washing step using high salt concentration. You can also wash your column with a low pH buffer in order to remove impurities from the column before recovery of AAV in the eluate.

If you decide to implement the second washing step you have to be careful that your washing condition won't affect the integrity of your capsid. Moreover, if the washing step pH is too close to the pH of the elution buffer, a significant quantity of capsids could be eluted during your washing step, and as a result, lower your AAV yield during the elution.

Q Is the resin GMP compliant?

NL: The resin is used in GMP manufacturing by many of our customers. While not manufactured in a cGMP process, the resin is produced under an ISO 13485 environment.

When you purchase the resin, you can request the regulatory support package, including documents regarding quality, stability, production, control method, and so on. Those documents are useful when you make a product and submit a dossier to a regulatory agency.

For each of our commercially available CaptureSelect resins, we developed an ELISA assay to monitor the level of ligand leakage over the purification process.

Q Vincent, why did you use two different analytical methods during this study – ELISA for dynamic binding capacity and viral genome titers at termination during your screening?

VR: During our DBC study, a lot of fractions were collected in the flowthrough at the outlet of the column in order to calculate 10% breakthrough for AAV vectors, so we needed to use a high throughput assay for the analysis of the first full fractions. The ELISA assay allowed us to test several samples in parallel and to get the results quickly, in around half a day.

In the screening study, the number of samples was much lower – only two samples for each set of conditions screened were produced – so here we used an internal assay for the quantification of the viral genome titer in the product. The viral genome titer was determined by qPCR for each serotype.

Q Which resin can be implemented for a polishing step?

VR: Several different resins can be implemented for this step. Commonly, an anion exchanger is implemented in order to reduce host cell protein and host cell DNA. Anion exchange also has the capability to separate empty and full AAV capsids, and some suppliers have developed resins specifically for the polishing step. For more information, you can contact chromatography resin suppliers.

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1. Sierkstra L. Overcoming purification challenges in antibody therapeutics manufacturing with subdomain-specific affinity solutions. *Immuno-Oncology Insights* 2021; 2: 229–39.

BIOGRAPHIES

VINCENT RAVAULT is a technician within the Process Development and Industrialization department at YposKesi since 2018. He focuses on upstream and downstream process development and optimization for AAVs and Lentiviruses vectors to support and advance their large-scale production for gene therapies. He is also involved in the technical transfer of processes for viral vectors from pilot scale to manufacturing scale. Graduated of a Biochemistry degree, Vincent Ravault has over 15 years of experience at Pall Life Sciences where he was in charge of the technical support for chromatography resins. His main role at Pall was to provide purification solutions and strategies to customers. He was also involved in the promotion and evaluation of new products for downstream.

NICOLAS LAROUDIE, a biochemist by education, used to work for Généthon, France, between 2001 and 2011 as Head of Downstream Development. He was leading a team in charge of developing and scaling-up purification processes for AAV, retroviral and lentiviral vectors used in gene therapy treatments. He then joined Merck Millipore as a BioManufacturing Engineer where he used to technically support European customers for all DSP technologies - from clarification to sterile filtration, including TFF and systems - with a strong focus on chromatography. In particular, he took an active role in the establishment of a fully continuous, large-scale disposable DSP process for the purification of a monoclonal antibody, within the framework of a large multi-company European consortium. He eventually joined ThermoFisher Scientific in 2019 as Field Application Specialist for purification, technically supporting the implementation of POROS and CaptureSelect chromatography products for south-western European customers.

AUTHORSHIP & CONFLICT OF INTEREST

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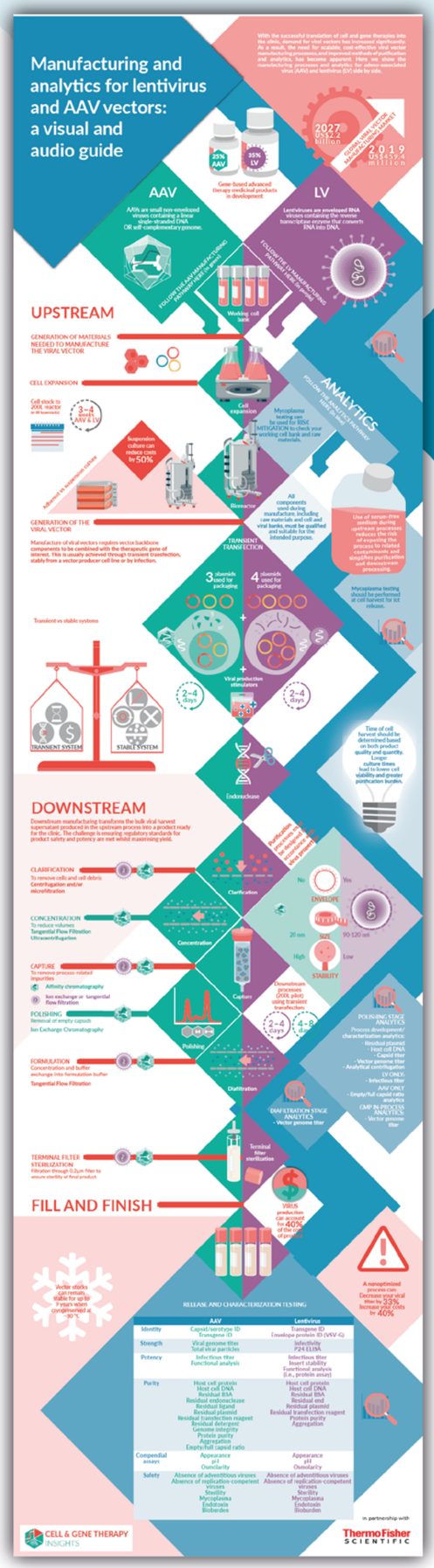

INTERACTIVE INFOGRAPHIC

Manufacturing and analytics for lentivirus and AAV vectors

Interactive

Highlight the key pathways:
- Analytic
- Lentiviral
- Adenoviral

With audio narration

VIEW INFOGRAPHIC

CELL & GENE THERAPY INSIGHTS

VECTOR Purification

CHAPTER 4

Optimizing downstream purification of high-quality plasmid DNA for gene therapy and vaccine production

Alejandro Becerra & Johannes F Buyel

The demand for plasmid DNA (pDNA) has increased in recent years, in part due to its utilization in both cell and gene therapies and mRNA therapeutics. Due to the physical properties of these molecules, plasmid production and purification pose some distinct challenges. A design of experiment (DoE) study was conducted in order to evaluate POROS AEX resins for pDNA capture, with the goals of optimizing process conditions to maximize purity and recovery, determine the dynamic binding capacity (DBC) of POROS AEX resins for pDNA, and confirm optimal operating parameters.

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CONSIDERATIONS & CHALLENGES FOR PLASMID DNA PURIFICATION

Plasmid DNA has multiple uses, ranging from basic cloning in research to therapeutic applications, and in recent years, the demand for pDNA has increased. This is partly due to the growth of the gene and cell therapy industry, as plasmid is one of the key raw materials required for commonly used viral vectors such as adeno-associated virus (AAV) and lentivirus. Plasmids are also one of the key components in the production of mRNA therapeutics, as they are used as a template during *in vitro* transcription.

In the context of plasmid production and purification, there are some important physical properties to consider. Firstly, plasmids are generally much larger than proteins in terms of mass and hydrodynamic radius, which is important for chromatography.

For gene therapy applications, typical sizes of these plasmids are in the range of 5–10 kilobase pairs. More recently, there has been a trend towards larger constructs, for example when two plasmids used for AAV transfection are combined into one, or in the context of mRNA when working on self-amplifying mRNA.

Another key characteristic of these molecules is that they are very highly charged, and maintain a high negative charge over a wide range of pH levels. They are also sensitive to degradation, both by nucleases and shear, which can modify their topology.

pDNA can be found in various forms, including supercoiled, open circular, and linear. Supercoiled plasmid is the most relevant form for therapeutic applications, and in that context, a high purity is generally desired from the purification process.

There are some inherent challenges to the purification of these molecules, including:

- Product and contaminants (gDNA, Endotoxin, RNA, plasmid isoforms) are similar in charge and size
- Shear sensitivity and high viscosity limit operational flow rates
- Plasmid generally represents <1% dry cell mass
- Conventional chromatography resins exhibit low binding capacities for pDNA

A typical downstream process for plasmids normally has multiple steps after fermentation, and anion exchange followed by hydrophobic interaction chromatography are commonly utilized. Thermo Fisher Scientific has developed a variety of resins well-suited for these steps, designed to simplify workflows and increase purity and yield.

ADVANTAGES OF POROS ANION EXCHANGE RESINS

POROS™ Chromatography Resins from Thermo Fisher Scientific have a number of unique features (Figure 1), and different base beads are available for different resins, allowing for control of pore size, surface area and overall porosity.

Thermo Fisher Scientific offers four different POROS™ AEX resins (Figure 1), and each offers unique surface chemistries, and therefore unique selectivity, as compared to other commercially available AEX resins. This offers a potential solution to unique purification challenges, as a protein of interest or an impurity may bind to a POROS AEX resin differently than it does to other AEX resins.

FIGURE 1

Unique features of POROS resin technology.

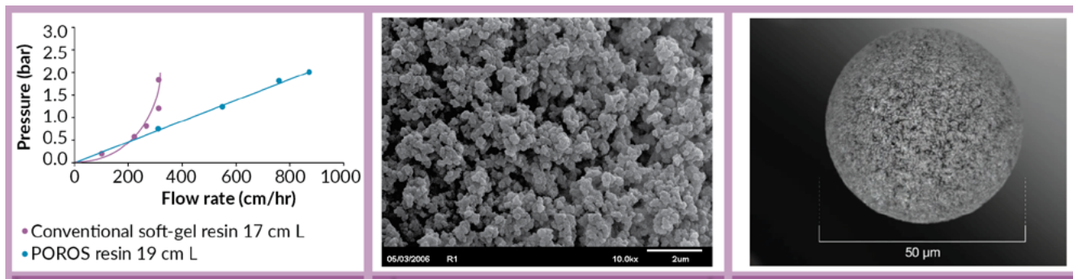


TABLE 1

POROS anion exchange resins.

POROS resin	Type of AEX resin	Surface chemistry	Pore size (angstrom)	BSA binding capacity (mg/mL)	AEX applications
D50	Weak	Dimethylaminopropyl	1100	>100	**Bind/elute:** Protein, virus, plasmid DNA purification **Flow through:** Trace impurity removal by binding impurities (DNA, viruses, HCP, aggregates, endotoxin)
PI	Weak	Polyethyleneimine (mixed amine)	2000	80	
HQ50	Strong	60% quaternized poly-ethyleneimine (mixed amine)	2000	75	
XQ	Strong	Fully quaternized amine	1100	>140	

A full range of weak and strong anion exchange resins with unique surface chemistries, that provide unique selectivity.

In this work, we focused on three out of the four resins in Table 1; POROS™ D50 has a dimethyaminopropyl functional group and is a weak AEX resin, and its chemistry is slightly different than traditional DEAE (Di-EthylAminoEthyl) resins. POROS™ PI (not tested in this study) is also a weak AEX resin with a polyethyleneimine functional group. The functional groups are primary, secondary, and tertiary amines, and are ionizable over a shorter pH range as compared to a strong ion exchanger.

POROS™ HQ is a legacy strong AEX resin. It is unique because it has both weak and strong AEX capabilities. There is a mixture of primary, secondary, tertiary, and quaternary amines on the bead, and about 60% of the tertiary amines are converted to quaternary amines, yielding a strong anion exchanger. This unique PEI-based chemistry and distribution of amines makes POROS HQ50 unlike any other commercially available AEX resin.

The pore size of these resins is also relatively larger compared to other products, which facilitates the diffusion of large molecules such as plasmids.

With this background in mind, the POROS resins were studied for plasmid capture applications in collaboration with the Fraunhofer Institute for Molecular Biology, Germany. The study had two objectives:

- Produce pDNA containing lysate using representative fermentation and primary recovery steps
- Evaluate POROS AEX resins for pDNA capture
  - Optimize process conditions to maximize purity and recovery using a DoE approach
  - Determine DBCof POROS AEX resins for pDNA
  - Confirm optimal operating parameters

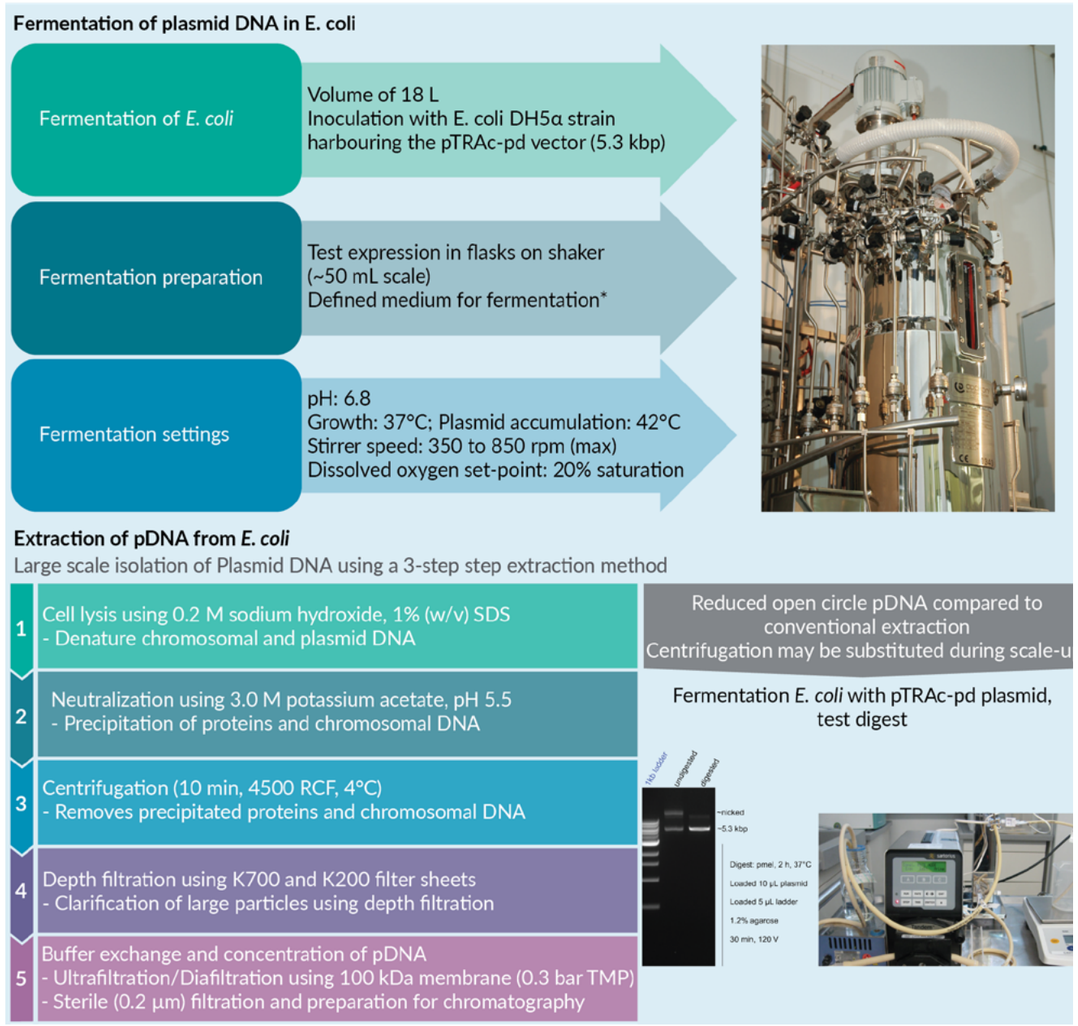
AEX DESIGN OF EXPERIMENT (DOE) OPTIMIZATION

The first step of the study was pre-processing. i.e., generating the materials to be tested for chromatographic separation. *E. coli* was selected as a representative system; the specific fermentation and extraction processes are shown in Figure 2. This preparation procedure provided a starting material with a higher closed circle/supercoiled DNA content than an extraction process that does not use ultrafiltration/diafiltration.

The ion exchange resins discussed above were then investigated, focusing on several parameters: loading buffer pH, loading conductivity, and quantity of plasmid loaded per mL of resin. The design quality was assessed before beginning the experiments, as seen on the right of Figure 3. The flat surface indicates that the model has a good and even predictive power throughout the entire design space.

FIGURE 2

Fermentation and extraction processes used to prepare pDNA for chromatographic separation.



*[1]. TMP: Transmembrane pressure.

pH & purity

Recovery at pH 7.0 was investigated first. Looking at all of the chromatography resins, the initial finding was that overall recovery was fairly high (Figure 4). Notably, for the POROS HQ50 resin, the different parameters had little effect; in this case the load conductivity and load concentration. In contrast, for POROS D50, we found that with an increasing load conductivity the relative recovery of products increased. For POROS™ XQ, the recovery decreased with an increasing load concentration, i.e., with a higher quantity of plasmid loaded per volume of resin.

Using a pH of 6, this initial behavior was amplified (Figure 5). In the case of POROS XQ, the reduced recovery with increasing load concentration was more pronounced. Similarly, for the POROS D50, the effect of load conductivity was more pronounced, and for POROS D50 we also see an effect of the load concentration. In contrast, the POROS HQ50 again showed relatively stable behavior throughout the design space. Interestingly, most pDNA was lost in the elution fractions.

The effect of pH was then compared in more detail for the D50 resin, which showed a dependence on load conductivity and concentration: as can be seen in Figure 6, with an increasing pH from right to left, the recovery increases overall and becomes more robust. In this case, a high pH was favorable to ensure a good recovery throughout the entire design space.

Purity for all three resins was in a good range – between 60 to 75% of total nucleic acid was supercoiled pDNA, and conditions were identified that gave close to 100% recovery for all resins.

FIGURE 3

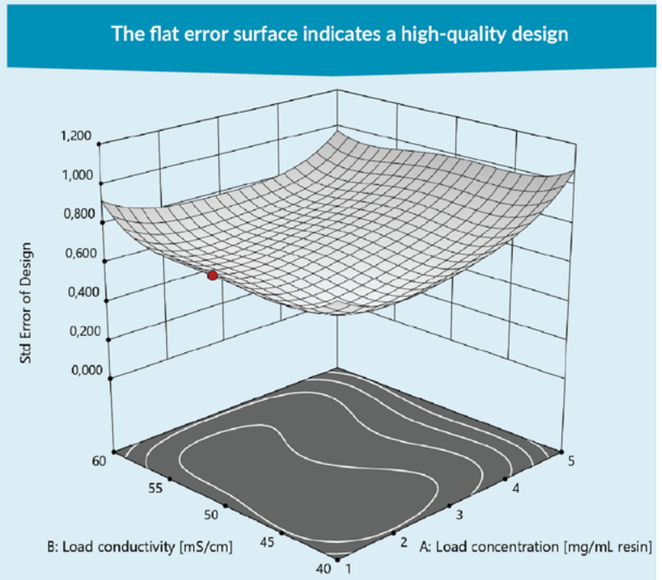
AEX Design of Experiments.

AEX Design of Experiments (DoE)

Design of Experiments Parameters

Parameter	Values tested
Loading buffer	pH 6, 6.5, 7
POROS™ resins	D50, HQ, XQ (RoboColumn® 200 µL)
Load density	1, 3, 5 mg/mL resin
Load conductivity	40, 50, 60 mS/com

RoboColumn® is s registered trademark of ATOLL GmbH



Dynamic binding capacity

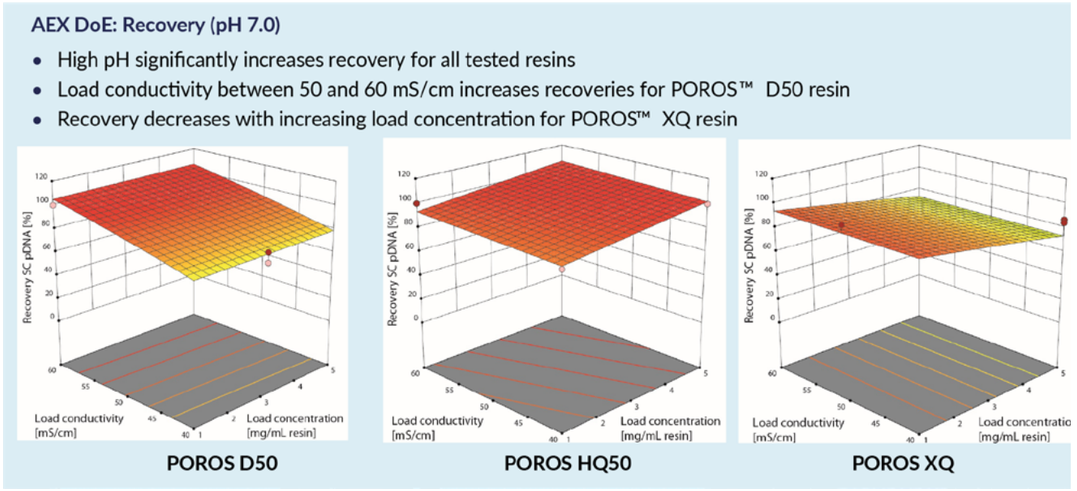
The DBC of the different resins is an important question to address, as this will ultimately dictate the process economics.

For the XQ resin, based on a UV trace, we calculated a DBC of 5.5 milligrams of pDNA per mL resin (Figure 7). This is in the high range of what is typically reported. Looking at the chromatogram to the top left of Figure 7, a double breakthrough curve can be seen – a steep increase at around 10 mL, and a second increase after 32 mL.

DNA concentration of individual samples was then checked, and it was observed that this second breakthrough is associated with a breakthrough of the relevant plasmid DNA. The initial phase can likely be disregarded as it is likely that other compounds such as proteins are breaking through the column at this point. Based on gel analysis, a substantially higher DBC of approximately 9 milligram per mL resin was achieved.

FIGURE 4

Resin recovery at pH 7.0.



A similar double breakthrough curve was seen for POROS HQ50. However, an inverse behavior was seen, where the DBC based on the UV trace is similar but when looking at the elution fraction and detecting the pDNA concentration, we found that the DBC is lower, at around 3 milligrams per mL.

With the last resin, POROS D50, we found that there is some breakthrough, and also some breakthrough regarding nucleic acid (Figure 8). Looking at the gel, we found that the breakthrough is up to a very late point – around 50 mL – and consisting of small nucleic acids, likely RNA or some fragments of genomic DNA. In this case, we

FIGURE 5

Resin recovery at pH 6.0.

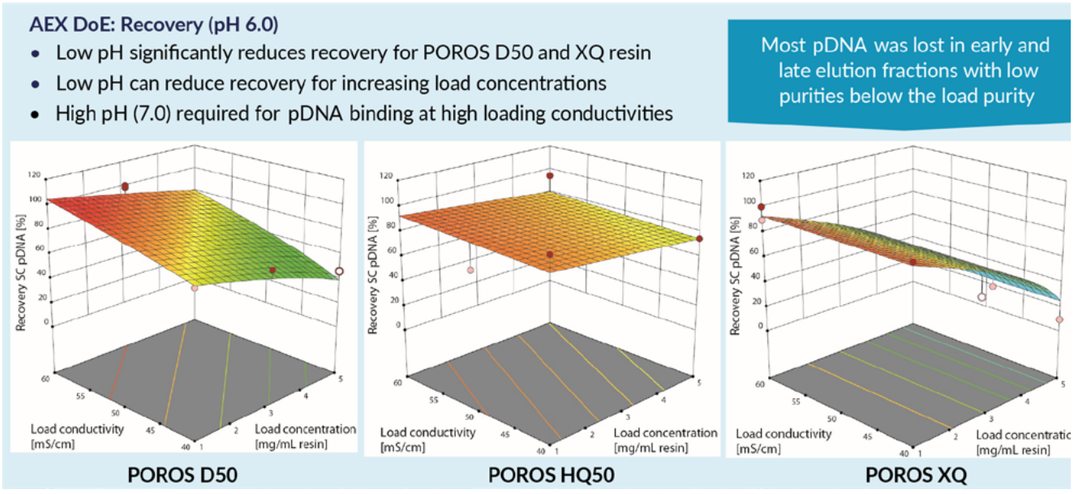


FIGURE 6

pH effect on POROS D50 recovery.

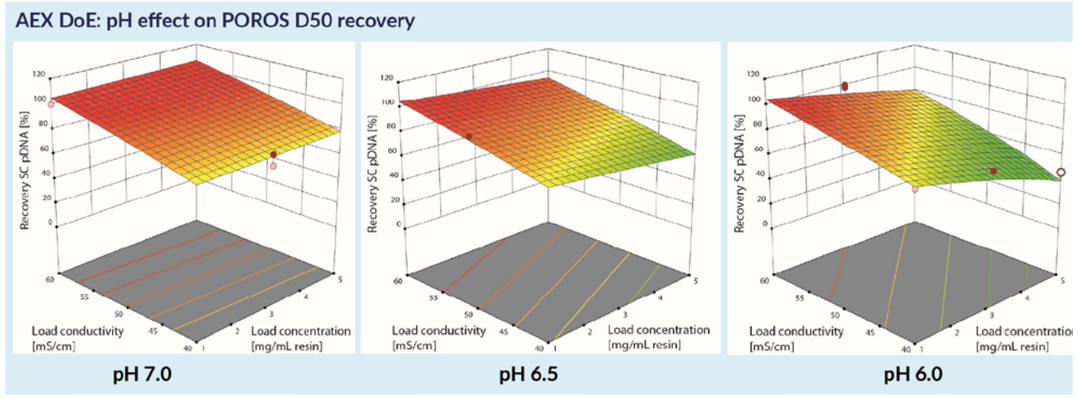


FIGURE 7

POROS XQ dynamic binding capacity.

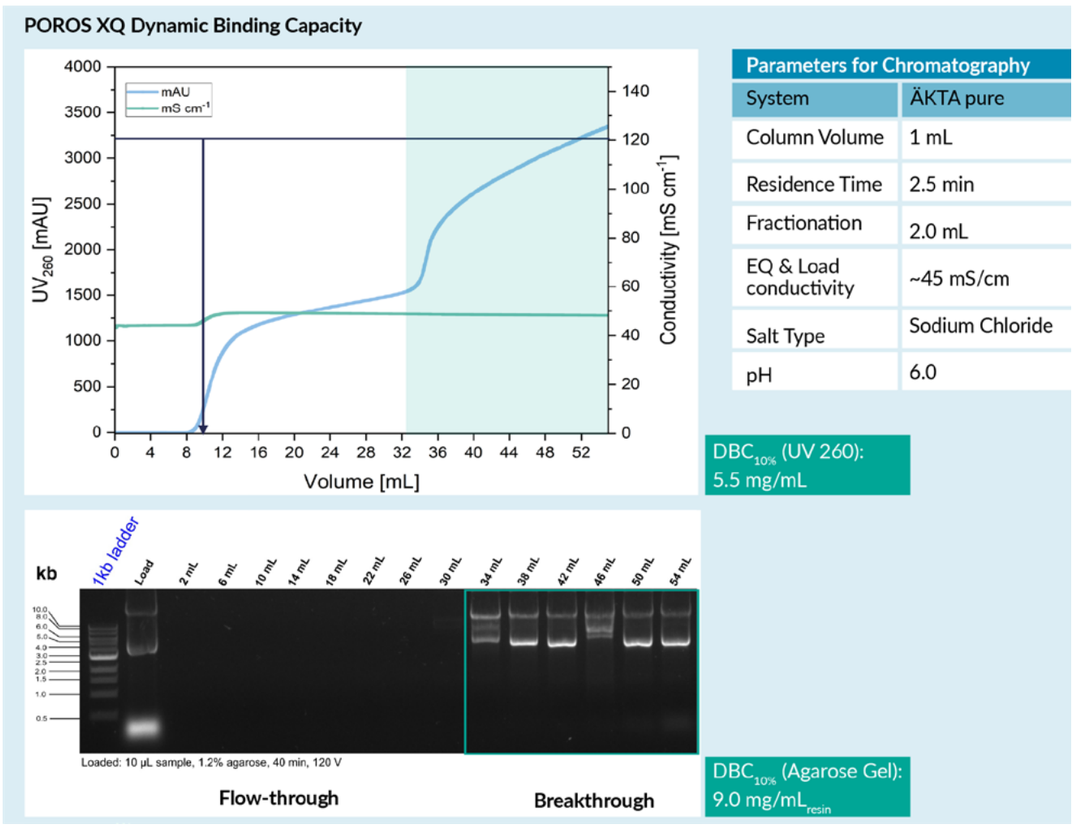
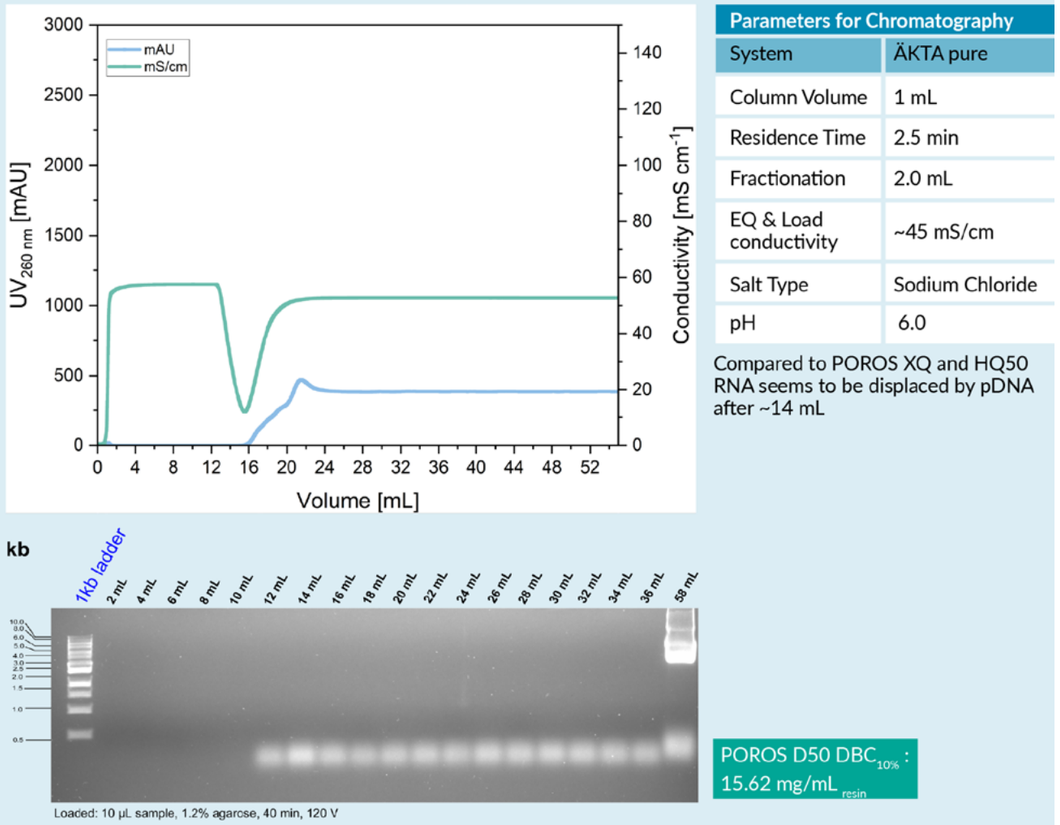


FIGURE 8

POROS D50 dynamic binding capacity.



assume that the supercoiled pDNA is replacing previously-bound RNA or smaller DNA molecules, resulting in an overall DBC of more than 15 milligram per mL.

While the XQ resin has a very high relative surface area, the D50 resin had the highest DBC. Speculatively, this may be because it is not the relative surface area of the bead, but in fact the accessible surface area to a given molecule, that is relevant when it comes to the DBC of the resins.

As the D50 resin provides the highest dynamic binding capacity, it was therefore the best suited to our next step, which was to verify these results using a scaled-up version of the experiment.

POROS D50 scaled up verification

The scaled-up experimental procedure remained essentially the same, but instead of the small-scale 0.2 milliliter columns used initially, a 5 mL column was used for verification.

We verified that the binding capacity was more than 10 milligrams per mL – this loading is less than what was observed previously, but still relatively high (Figure 9). In the gel at the bottom of Figure 9, it can be observed that in addition to the plasmid in the different salt elution steps – which can be seen here as staircase-like bands – there is a fraction of product that is eluting only once the cleaning procedure is applied (seen on the right side of the gel, in the lane labeled with CIP). Therefore, it is likely that optimizing the current elution conditions can increase the recovery.

- Preliminary work suggests Benzyl and Benzyl Ultra as potential candidates
- Potential assessment of other chromatography types for isoform separations (AEX)
- Evaluation of larger pDNA constructs

FUTURE WORK

Planned future directions include:

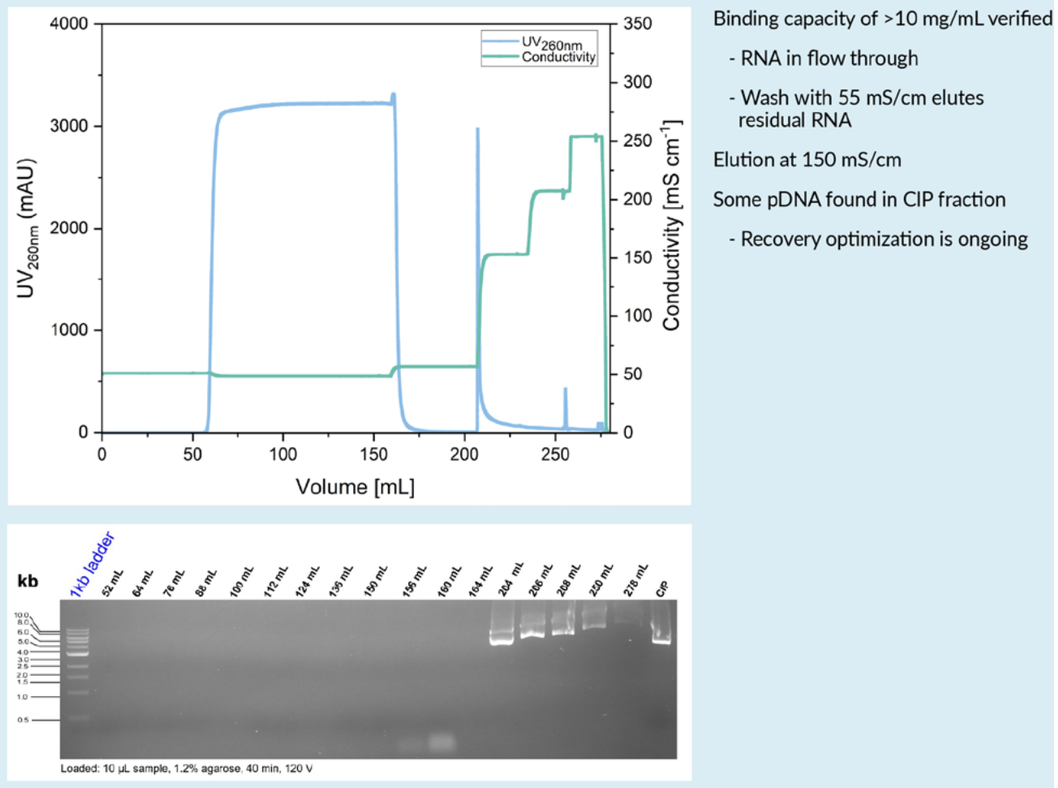
- Further optimization of the capture step with POROS D50
- Separation of pDNA isoforms with POROS hydrophobic interaction chromatography (HIC) resins

INSIGHT

High binding capacity was obtained for all three resins, with POROS D50 demonstrating the best binding capacity during this work. It is important to note that residence time was at the lower end

FIGURE 9

POROS D50 scaled up verification.



at 2.5 minutes, and increasing this may increase the binding capacity observed. Initial scale-up verification confirmed the high capacity, purity, and recovery for POROS D50, and work is ongoing to optimize the D50 capture step.

These DoE results provide a good guide towards optimal purity and recovery conditions for POROS D50, HQ, and XQ, and demonstrate how optimizing process conditions using a DoE approach can maximize purity and recovery of pDNA.

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ASK THE EXPERTS

Charlotte Barker, Editor, *Cell and Gene Therapy Insights,* speaks to **Alejandro Becerra** Principal Applications Scientist and Global Purification Technical Lead, Thermo Fisher Scientific and **Johannes F Buyel** Head of Bioprocess Engineering Department Fraunhofer IME

Q What is the benefit of using ultrafiltration/diafiltration for preconditioning?

JFB: Even though it is not part of the actual ion exchange step, we chose it for preconditioning because we think it has two benefits. On one hand, it allows us to concentrate the product, so that all the subsequent steps can be operated faster, using smaller equipment. On the other hand, it allows us to bring the plasmid DNA into conditions that are compatible with the ion exchange capture step.

Q What are the major impurities that remain after anion exchange chromatography?

JFB: This is very relevant in terms of what comes next after this project. At the moment it is mostly nicked plasmid DNA that is not really the target of the production.

We also need to look in more detail at the endotoxin content, and maybe genomic DNA. We will use the samples that we obtained from the scaled-up verification run to analyze them, and build an impurity profile which will then be used to guide the second purification step.

Q Was RNase used in the process, and can this step remove RNA?

JFB: No RNase was used, and the data showed the removal of RNA in the flowthrough by the agarose gel.

Q Which second purification step would you suggest, and why? Which have you tested so far?

JFB: As mentioned earlier, it's most likely going to be HIC as the next purification step as it is an orthogonal method, and that is what typical process development would use as a different mode of interaction to purify.

Multimodal chromatography could also be used, and other ion exchange resins could be an option depending on how the other resins perform. So far, we have done some preliminary testing with HIC, but that is next on the list.

Q How would you design a new resin specifically designed for pDNA purification?

AB: As I mentioned earlier, we are able to control the different characteristics of the beads, as well as the functional group. Based on this work, we could potentially try to further understand how each of those parameters such as pore size, surface area, and ligand density, may influence binding capacity and selectivity. By manipulating those, I think we could further optimize a resin for these applications.

Q How does the binding capacity presented in this work compare to other resins or absorbents?

AB: In the literature there aren't many actual breakthrough curves – at least that I am aware of. Even in the information that is available, those binding capacities are generally in the area of 1–3 milligrams of plasmid per mL of resin. Even for more convective adsorbents,

some of the recommendations for operation are still below 5 mgs per mL. We were pleasantly surprised with the higher binding capacity of these resins, particularly with D50.

Q Would you expect the dynamic binding capacity to be similar with larger plasmids?

AB: We think it will likely be lower. It all depends on the accessible surface area, but generally speaking, the binding capacities tend to be lower with larger molecules.

We are aware that with larger molecules sometimes the recovery suffers more, and that may be related to some potential physical entrapment within that pore network, whether it is a resin or a different adsorbent.

BIOGRAPHIES

Dr Alejandro Becerra

Principal Applications Scientist and Global Purification Technical Lead, Thermo Fisher Scientific

Dr Alejandro Becerra is a Principal Applications Scientist and Global Purification Technical Lead. Alejandro has over 14 years of experience in downstream processing and customer support having worked as Purification Team Manager and other bioprocess engineering roles prior to joining Thermo Fisher Scientific in 2018. Dr Becerra is a subject matter expert in preparative chromatography with expertise in the development, optimization and scale-up of antibody, recombinant protein and viral vector purification processes. Alejandro holds a PhD in Chemical Engineering from Cornell University.

Associate Prof. Dr Johannes F Buyel

Head of Bioprocess Engineering Department Fraunhofer IME

Associate Prof. Dr Johannes Buyel is a Biotechnologist by training and received his Bachelor of Science from the RWTH Aachen University in Aachen (one of Germany's Excellence Universities) in 2006. He continued his master studies in Molecular Biotechnology with stays abroad in Sweden (Lund University, ERASMUS schoolarship) and the USA (Fraunhofer Center for Molecular Biotechnology, Newark, Delaware). Finishing his Masters with honours in 2009 he was awarded the Springoroum Coin. Johannes conducted his PhD at RWTH Aachen University during which he was a visiting scientist at the Rensselaer Polytechnic Institute (Troy, NY, USA) and Karlsruhe Institute of Technology (KIT) (Germany) for several months. He received his PhD with honours and was awarded the Borchers Medal. In 2014 he joined Fraunhofer IME as a group leader and was promoted head of department the year after. In parallel, Johannes started a second PhD in Bioprocess Engineering at the KIT, which he finished in 2017. Between 2018 and 2020 he conducted his habilitation and is an Associate Professor at the RWTH since 2020. Johannes is an active member of the German Biotechnology Association (DECHEMA), and member of the Editorial Boards of Frontiers in Bioengineering and Biotechnology, Frontiers in Plant Science as well as Transgenic Research, where he is Editor-in-Chief since 2021. Since 2021, Johannes is heading the Bioprocess Engineering Department at Fraunhofer IME (~20 employees plus ~10 students, gender balance 1:1). He has 15 years of experience in the development of tailor-made upstream production and downstream processing for 50+ recombinant proteins, small molecules and DNA in different expression systems such as E. coli, yeast, plant cell cultures and whole plants both under GMP and non-GMP conditions. He is also focussing on integrated processing, i.e. the use of residual biomass for cascading use. Johannes was involved in the two international projects PHARMAPLANT and FUTURE-PHARMA and received a €2.5 million Fraunhofer Attract grant for a project to establish a model-driven high-throughput expression and purification platform for recombinant proteins (FAST-PEP). Johannes is a principal investigator (PI) in the tumor-targeted drug delivery graduate school of the German Research Foundation (DFG) as well as the International Center for Networked Adaptive Production (ICNAP) for which he has so far received funding of €0.9 million and €1.8 million respectively. His work is strongly inter- and cross-disciplinary, combining research in, for example, biotechnology, bioprocess engineering, informatics, mathematics, data science, oncology, cosmetics and nutrition. He has currently more than 50 publications (h-index 19, i10-index 36, 1321 citations as of June 2021).

AUTHORSHIP & CONFLICT OF INTEREST

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

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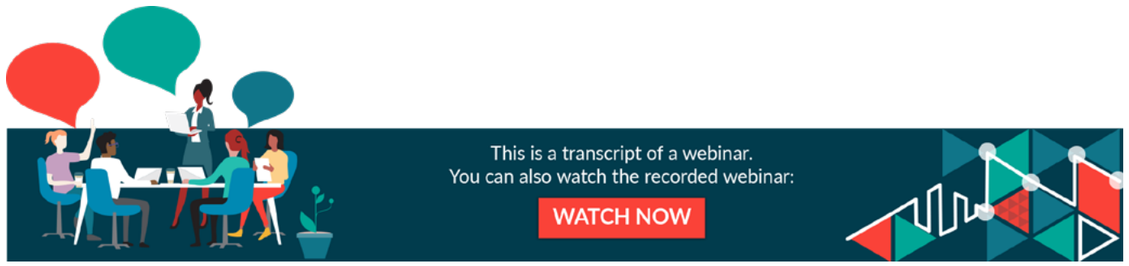
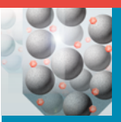




CHAPTER 5

Optimizing downstream purification of high-quality plasmid DNA with POROS Chromatography Resins

Alejandro Becerra, Principal Applications Scientist and Global Purification Technical Lead, Thermo Fisher Scientific, and
Johannes F Buyel, Head of Bioprocess Engineering Department Fraunhofer IME Scientific Project Coordinator, Rubhu Biologics

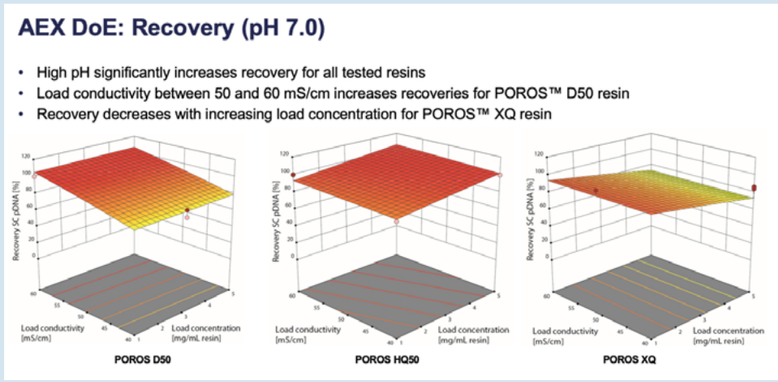
The demand for plasmid DNA (pDNA) has increased in recent years, but due to their physical properties there are some inherent challenges to the purification of these molecules. A typical downstream process for plasmids normally has multiple steps after fermentation, and anion exchange followed by hydrophobic interaction chromatography are commonly utilized. Thermo Fisher Scientific has developed a variety of resins well-suited for these steps, designed to simplify workflows and increase purity and yield. A series of experiments were conducted in order to evaluate POROS™ AEX resins for pDNA capture, with the goals of optimizing process conditions to maximize purity and recovery, determining the dynamic binding capacity (DBC) of POROS AEX resins for pDNA, and confirming optimal operating parameters. Some highlights of these studies, performed in collaboration with the Fraunhofer Institute for Molecular Biology, Germany, are presented here. POROS™ D50, HQ50 and XQ were selected and evaluated for plasmid capture applications.

PH AND PURITY

Overall recovery at pH 7.0 was fairly high (Figure 1). Notably, for the POROS™ HQ50 resin, the different parameters had little effect; in this case the load conductivity and load concentration. In contrast, for POROS™ D50, we found that with an increasing load conductivity the relative recovery of products increased. For POROS™ XQ, the recovery decreased with an increasing load concentration, i.e., with a higher quantity of plasmid loaded per volume of resin. Using a pH of 6, this initial behavior was amplified. POROS HQ50 again showed relatively stable behavior throughout the design space.

Purity for all three resins was in a good range – between 60 and 75% of total nucleic acid was supercoiled pDNA, and conditions were identified that gave close to 100% recovery for all resins.

Figure 1. Resin recovery at pH 7.0.



DYNAMIC BINDING CAPACITY

The DBC of the different resins is an important question to address, as this will ultimately dictate the process economics. The D50 resin provided the highest dynamic binding capacity (Figure 2), and was therefore the best suited resin to verify our results using a scaled-up version of the experiment.

POROS D50 SCALED UP VERIFICATION

Using a scaled-up experimental procedure we verified that the binding capacity was more than 10 mg/mL (Figure 3). In the gel at the bottom of Figure 3, it can be observed that in addition to the plasmid in the different salt elution steps there is a fraction of product that is eluting only once the cleaning procedure is applied (seen on the right side of the gel, in the lane labeled with CIP). Therefore, it is likely that optimizing the current elution conditions can increase the recovery.

Figure 2. POROS™ D50 dynamic binding capacity.

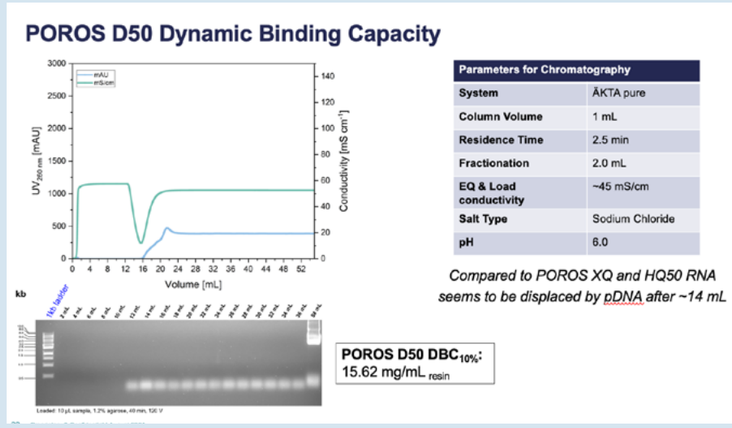
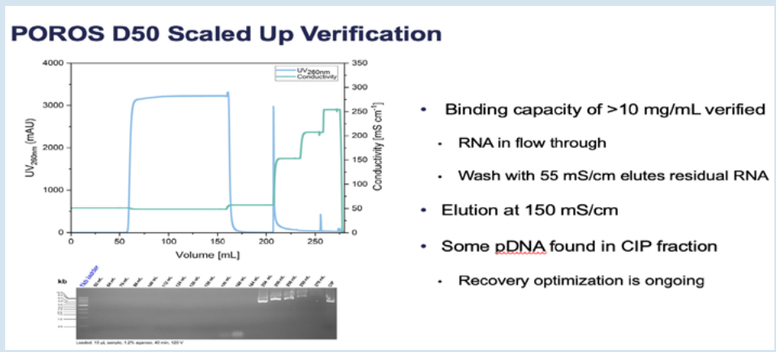


Figure 3. POROS™ D50 scaled up verification.



INSIGHTS & FUTURE DIRECTIONS

High binding capacity was obtained for all three resins, with POROS D50 demonstrating the best binding capacity. Residence time was 2.5 min, and increasing this may increase the binding capacity observed. Initial scale-up verification confirmed the high capacity, purity, and recovery for POROS D50, and work is ongoing to optimize the D50 capture step.

To explore the full study design & results, along with an author Q&A, watch the webinar or read the article

Read the full article here

CELL & GENE THERAPY INSIGHTS

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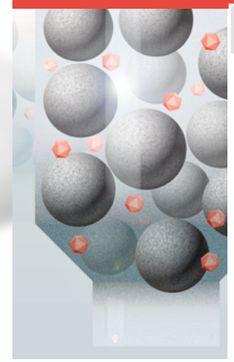

CELL & GENE THERAPY INSIGHTS

VECTOR Purification

CHAPTER 6

Supporting development of mRNA-based therapies by addressing large-scale purification challenges

Kelly Flook

The field of mRNA-based therapies is a rapidly emerging area with increasing real-world applications. The potential of these therapies is being demonstrated in various fields. Although the potential of mRNA in therapies is seemingly endless, obtaining the quantities of synthetic mRNA needed for clinical treatment remains a challenging obstacle, and current methods for mRNA purification are creating a bottleneck in large-scale manufacturing. Particularly for vaccine development, obtaining the quantities of synthetic mRNA needed for clinical treatment remains an obstacle. As a result, a robust, scalable and easy-to-use platform to support all mRNA therapies is needed. To support the development of mRNA-based therapies, Thermo Fisher Scientific has developed an affinity resin for the purification and isolation of mRNA from *in vitro* transcription (IVT) manufacturing processes. The following article and case studies will highlight how the Thermo Scientific POROS™ Oligo (dT)25 affinity resin can enable efficient and simplified mRNA purification.

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THE RISE OF mRNA THERAPEUTICS

Whilst mRNA now offers a new therapeutic paradigm, mRNA itself is not a new modality. The first concept proposal and successful study was published over 30 years ago, and the first clinical trial began nearly 20 years ago – and today, the growing applications of mRNA as a therapeutic have been greatly spurred on by the success of novel mRNA-based vaccines

being made available for emergency use against the novel coronavirus.

The rapid growth of mRNA as a therapeutic can also be attributed to the fact that the action of mRNA is relatively simple and well understood, making it a promising candidate for the development of platform technology. Synthetic mRNA has many applications – it can be used to create induced pluripotent stem cells, or induce cell differentiation into desired cell types by introducing proteins that stimulate these processes. It can be used to create secreted proteins such as antibodies, and to express a homing receptor to improve cell migration to specific areas in the body. Additional uses include vaccination of rare and common diseases, and synthetic mRNA can also be used for gene editing using TALENs or CRISPR.

THE PURIFICATION CHALLENGE

For a platform technology to fully succeed, a corresponding purification platform is key. Traditionally, purification of mRNA is achieved by a variety of methods (Table 1), but each option brings disadvantages. Many scientists try to scale up tried and tested methods from the research laboratory – but when moving from micrograms to grams, and potentially even kilograms of mRNA, this may not be the most successful, or optimal approach. Scalability is not the only challenge to tackle – other important considerations include purification efficiency, ease of use, recovery, selectivity, and the option to integrate an affinity resin as a platform solution for various mRNA molecules.

Reverse phase purification

Reversed phase purification is highly effective and achieves high resolution. It offers some selectivity for product related impurities, but when considering this approach from a scale up perspective, there is limited column capacity. An additional challenge is the need for flammable and toxic solvents that pose safety concerns for operators and necessitate intrinsically safe suites which are not commonplace in biotherapeutic manufacturing. These suites are costly to set up, and bring additional cost implications related to disposal of organic solvents. In addition, ion pair reagents add a toxic component that then requires additional purification steps to remove.

Without very stringent cleaning protocols, fouling from smaller proteins and enzymes can impact the selectivity and separation efficiency of the column over time.

Ion exchange chromatography

Ion exchange chromatography is a common approach when working with smaller nucleic acids, and is effective for native purification. When working with increasingly larger constructs, capacity and recovery issues arise – due to the multiple charges on the mRNA, it binds very effectively to ion exchange resins, and in some instances eluting the mRNA molecule from the column with good recovery can prove difficult.

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is a common chromatography technique that is also being used for the purification of mRNA. It allows for native purification, and the resins are scalable. Similar to reversed phase, HIC takes advantage of the difference in hydrophobicity of mRNA and its impurities, and is commonly used by the industry as an orthogonal purification method. It has the potential to replace the traditional reversed phase method as no toxic chemicals are needed. But as with reversed phase, selectivity can be a challenge to remove specific product impurities.

Now that mRNA therapies and vaccines are making their way to the clinic, the need for a robust purification platform becomes apparent – and affinity chromatography can overcome the challenges the field is currently facing. The method allows for native purification, is scalable and highly selective as it uses the poly-A tail to purify the mRNA molecules. Any impurity lacking a poly-A tail will not bind the column and is easily flushed away, allowing all impurities without a poly-A tail to be removed in a single step. Product related impurities containing a poly-A tail such as double stranded RNA can be removed with a second polishing step. Alternatively, it is possible to engineer out the formation of double stranded RNA during upstream synthesis. This approach allows the use of affinity chromatography as a single step purification solution that can be scaled up as manufacturers move through the clinic.

THE POWER OF AFFINITY CHROMATOGRAPHY

Affinity chromatography offers many benefits beyond a selective approach, and is applicable regardless of which modality is being used. It has earned credit in therapeutic antibody development and more recently also in viral vector manufacturing. Depending on the molecule, as well as the process and product related impurities, multiple purification steps may be needed to reach the desired purity. This means that each purification step added to the process will result in lower overall yield.

The graph in Figure 1 demonstrates the number of process steps against product yield. Even with a high step yield, for example 85%, after four process steps the overall product yield is reduced to 50%. Affinity chromatography can address this challenge. Due to high affinity for the target molecule, a higher purity and yield is achieved in the first step alone. This helps to reduce the number of purification steps needed in the overall process, increasing the overall product yield. A simplified purification process also reduces bioprocessing development time, allowing manufacturers to get to the

TABLE 1

Methods of RNA purification.

Method	Advantages	Disadvantages
Reversed phase	▶ High resolution ▶ Some selectivity for product impurities	▶ Limited column capacity ▶ Use of expensive/flammable/toxic chemicals ▶ Column fouling impacts resolution
Ion exchange chromatography	▶ Native purification possible ▶ Scalable	▶ Column capacity and recovery (HPLC) ▶ May need toxic chemicals for denaturation ▶ Purified product can contain traces of elution salts
Size exclusion chromatography	▶ Native purification possible	▶ Separation efficiency affected by alternative folding ▶ Flow limited
HIC	▶ Native purification possible ▶ Scalable ▶ Replacement for reversed phase	▶ Non-selective
Affinity chromatography	▶ Native purification possible ▶ Scalable ▶ Platform solution for wide range mRNA molecule sizes – selective to polyA	▶ Requires additional polishing step to remove product-related impurities

Affinity chromatography can be used as a scalable platform solution for mRNA purification.

market faster, and decreasing the overall cost of goods.

THE THERMO SCIENTIFIC POROS™ OLIGO (dT)25 AFFINITY RESIN

In 2020, Thermo Fisher Scientific launched a new affinity chromatography resin specifically designed for the purification and isolation of mRNA from IVT manufacturing processes in order to address the challenges associated with the purification of mRNA for therapeutic use. Figure 2 shows a schematic of the POROS™ Oligo(dT)25 resin. The resin is comprised of a 50µm porous poly(styrene-co-divinylbenzene) base bead with a polydeoxythymidine (poly-T) 25-mer (dT-25) conjugated to the surface using a proprietary linker.

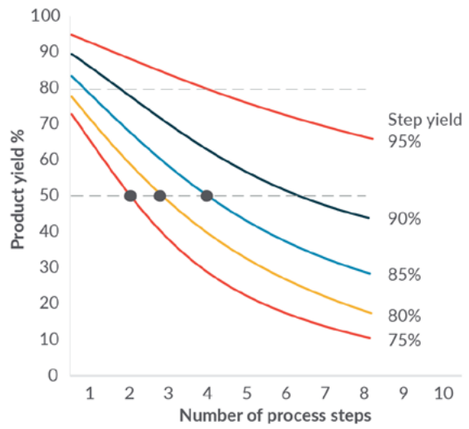
A poly-T ligand on the surface of the resin allows for simple mRNA capture through AT base pairing. To load the mRNA IVT mixture on the column, salt is added. Once the mRNA is bound to the resin, the column can be flushed to remove process related impurities. To elute the mRNA from the column a low concentration of buffer, or simply water, is used.

The resin has a high binding capacity in comparison to the laboratory-based techniques discussed above, with a dynamic binding capacity of up to 5 mg/mL for 4,000 nucleotides (nt) RNA. Across a wide range of mRNA construct sizes, the recovery in the first step yield has demonstrated to be greater than 90%, and in most cases, greater than 96–98%.

As the POROS™ Oligo (dT)25 Affinity Resin is a chromatography resin, it is easily scaled, with the ability to pack columns anywhere from a few milliliters or liters, up to hundreds of liters. Like other bioprocess resins offered by Thermo Fisher Scientific, it is a 100% non-animal derived, pharmaceutical-grade reagent, suitable for the manufacturing and purification of clinical therapeutics. The POROS™ Oligo (dT)25 Affinity Resin provides a simple solution to maximize workflow efficiency and reduce the complexity of any subsequent polish steps required.

FIGURE 1

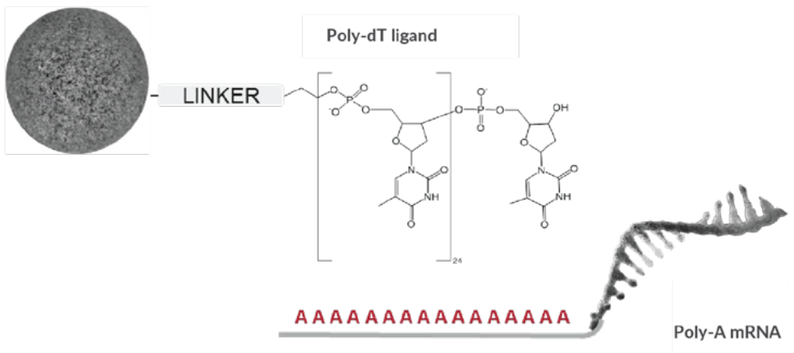
Product yield declines with increasing number of process steps.



Affinity capture can reduce the required number of purification steps, thereby increasing yield.

FIGURE 2

Mechanism of action of the Thermo Scientific POROS™ Oligo (dT)25 affinity resin.



The resin is comprised of a 50µm porous poly(styrene-co-divinylbenzene) base bead with a polydeoxythymidine (poly-T) 25-mer (dT-25) conjugated to the surface using a proprietary linker.

The poly-dT ligand will bind to the poly-A tail of the mRNA through simple AT base pairing.

THE POROS™ BEAD

There are three main attributes that differentiate POROS™ from other chromatography resins (Figure 3).

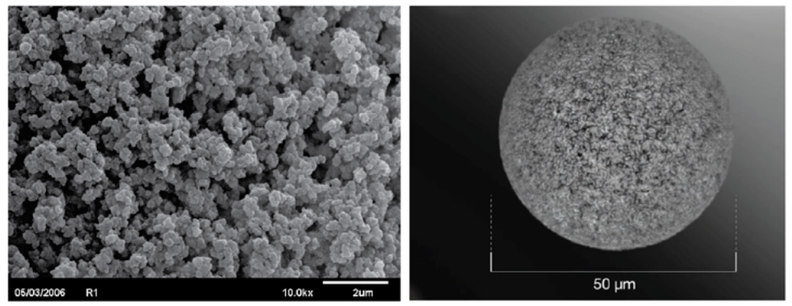
1. **Poly(styrene-co-divinylbenzene) backbone.** The beads are rigid and incompressible compared to agarose type resin. This results in stable column beds as well as linear pressure-flow profile over a wide range of column dimensions, allowing the user to maintain high operational flow rates with a modest pressure drop.
2. **Large pore structure.** The open pore structure of the beads makes POROS™ resins ideal for the purification of larger molecules such as mRNA or viral vectors. The large pores effectively increase the surface area available for interaction between the target molecule and the resin increasing both capacity and resolution. In addition, the larger pores result in reduced mass transfer resistance, which helps to improve process efficiency and productivity.
3. **50-micron bead size.** The average particle size is 50 µm, and this small particle size allows for less band broadening in packed beds, improving the ability to separate proteins and obtain effective impurity removal. Due to the reduced mass transfer resistance mentioned above, this superior resolution is well maintained and independent of linear velocity. In practice, this results in narrower peaks and smaller elution pool volumes which overcomes tank size limitations at large scale.

POSITIONING THE POROS™ OLIGO (dT)25 RESIN IN THE mRNA PURIFICATION WORKFLOW

Ideally, having just one purification step can fully maximize the productivity of the

FIGURE 3

Scanning electron microscope images showing a POROS™ bead (left) and the large throughpores of the bead surface (right).



workflow. Purification with the POROS™ Oligo (dT)25 affinity resin will remove process related impurities, such as DNA template, nucleotides, enzymes, and unwanted buffer components. If some product related impurities remain such as double stranded RNA or uncapped mRNA, an additional polishing step can be used.

Affinity purification can also be used in a polish step. Some users may want to retain an initial non-affinity first step, then implement a second affinity polishing step to remove any unwanted components that are left over from the IVT reaction. One advantage of this approach is that it can also be used as a buffer exchange step, as the mRNA can be eluted directly into water.

PROCESS DEVELOPMENT & RESIN PERFORMANCE STUDIES

The goal of process development was to first understand how a range of mRNA molecules behaved, in order to more effectively optimize binding capacity without impacting the mRNA. Utilization of a high throughput screening approach allowed rapid optimization over a range of conditions. Once favorable conditions were found, methods were transferred to column format for further optimization.

SALT TYPE & CONCENTRATION EFFECT ON mRNA BINDING

To better understand the stability of the mRNA, and to determine favorable initial loading conditions, various conditions were examined using a 96-well plate design (Figure 4). Three different mRNA construct sizes were studied ranging from 1,000 to 3,000 nucleotides using increasing salt concentrations and various salt types. Since the overall structure of these mRNAs is different, different behaviors are expected.

When increasing the sodium chloride concentration up to 1.4 M, precipitation began to occur for the 2,000 nt mRNA. Interestingly, this effect was not seen with the 1,000 or the 3,000 nt mRNAs, which demonstrates that the effect is not related purely to size, but to construct design. When switching from sodium chloride to potassium chloride, the 2,000 nt mRNA was not affected in the same way. Depending on the mRNA sequence being used, it may be necessary to optimize not only the loading salt concentration, but also the salt type used to neutralize the backbone.

Using the information from the 96-well plate precipitation experiment, salt concentration was then studied to determine optimal binding capacity in relation to salt concentration. A decrease of mRNA was seen in the elution pool as salt concentration was increased, demonstrating the promotion of binding – whereas at low salt concentrations, the backbone is not fully neutralized in order to promote annealing. The profile of binding capacity was again different across the three different constructs, indicating that this is another tool that can be used to optimize binding conditions.

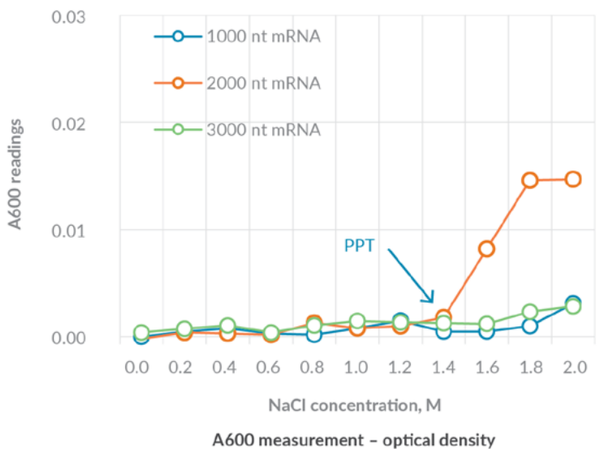
When considering buffer choice, the impact of binding across a range of pH in Tris buffer was studied. Again, optimal binding conditions were not consistent across the range of mRNA sizes used. These differences can be used to further optimize later column experiments, which will in turn assist in optimizing load concentration and flow rate.

DYNAMIC BINDING CAPACITY

The binding capacity of a capture step is an important parameter to determine how much product can be loaded on the column. In a study of binding capacity compared to flow rate, it was observed that increasing residence time resulted in increased binding capacity (Figure 5). This is due to the diffusional effects of the large mRNA molecule, and is common for larger biomolecules. In addition, higher concentrations of mRNA in the load pool better enabled the mRNA to reach the surface of the resin due to improved binding kinetics at higher concentrations at lower flow rates. However, when considering productivity gains, benefits began to diminish beyond a 2-minute residence time. As a result of this study, a 2-minute residence time was selected for further experiments.

FIGURE 4

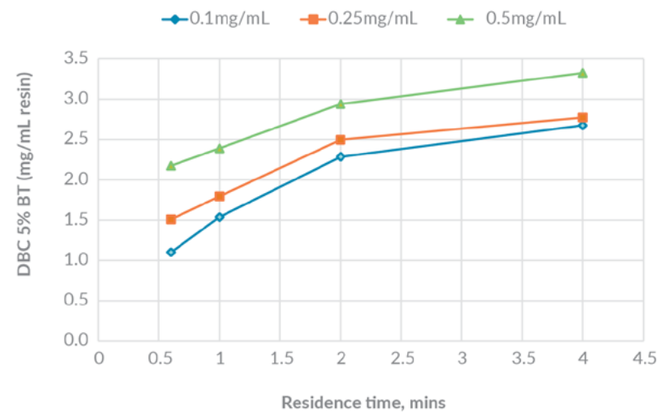
Effect of salt concentration on mRNA stability.



To determine the mRNA precipitation point (PPT) for three sizes of mRNA construct, the optical density (A600) was measured at increasing salt concentrations. Precipitation of 2000 nt mRNAs occurred at lower salt concentrations than 1000 or 3000 nt mRNAs, suggesting that structure, as well as size, plays a role in stability.

FIGURE 5

Dynamic binding capacity (DBC) of 3000 nt mRNA at three different feed concentrations.

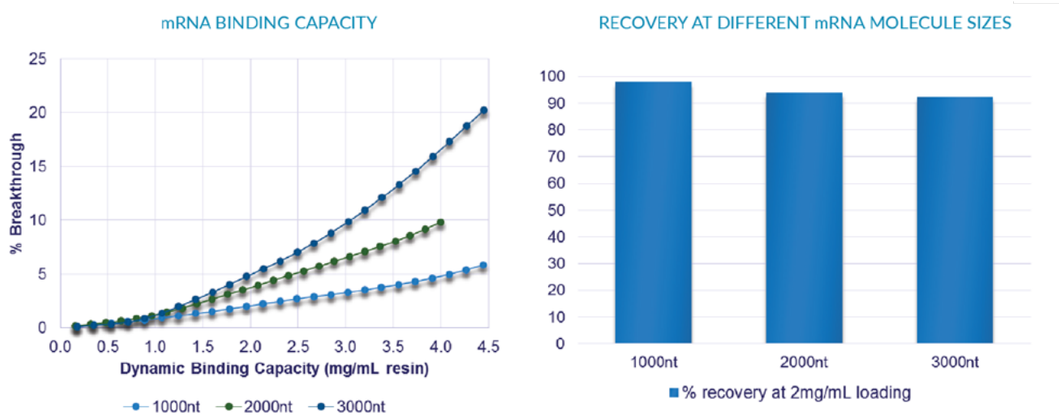


Residence time for mRNA load (Flow rate)
0.6 min (300cm/hr)
1.0 min (180cm/hr)
2.0 mins (90cm/hr)
4.0 mins (45cm/hr)

DBC increases with higher mRNA concentration and longer residence time.

FIGURE 6

Binding capacity and recovery of three sizes of mRNA construct (1000, 2000, and 3000 nt).



Smaller mRNA has a higher binding capacity (left) but size does not impact final recovery (right).

INFLUENCE OF MOLECULE SIZE ON BINDING CAPACITY & RECOVERY

Next, the effect of mRNA size on binding capacity was studied. To study comparative differences this experiment was not optimized for each individual mRNA size – load concentration, flow rate, and column dimensions were all kept constant in order to observe the direct effects of mRNA size. As expected, the size of the mRNA has an impact on the binding capacity and the smaller the mRNA, the higher the binding capacity achieved (Figure 6). As the mRNA constructs gets larger, steric hindrance becomes an issue, and the mRNA lacks the physical room to reach the surface of the resin.

Looking at recovery of the different construct sizes, consistent recovery well above 95% is shown, and is independent of the size of the mRNA.

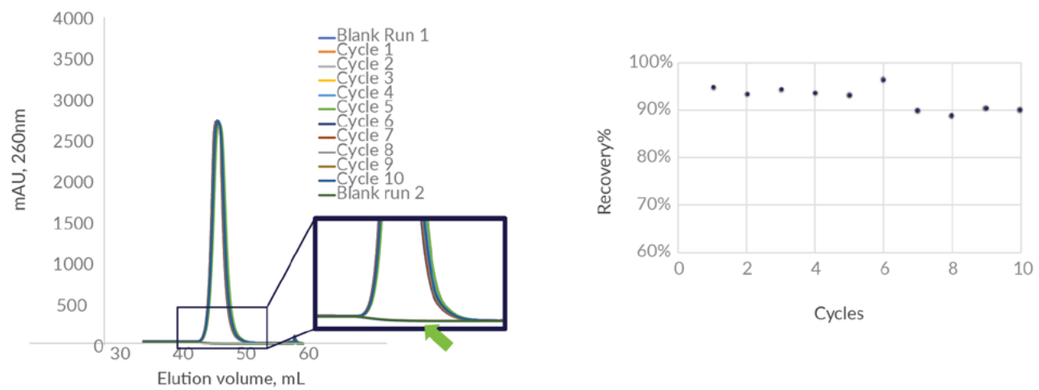
REUSE, CLEANING & STABILITY OF THE OLIGO (DT)25 AFFINITY RESIN

A 2,000 nt mRNA was used to assess the ability to reuse the resin (Figure 7). Multiple purification cycles were performed. The mRNA was bound and eluted over 10 cycles, with a cleaning step at the end of each cycle. Before the first cycle and after the 10th cycle, a blank buffer run was performed to monitor if any mRNA was eluted in the final blank run. The overlays of the blank runs appeared identical, demonstrating no carry over of mRNA from subsequent runs. In addition, this experiment demonstrated that the recovery, measured based on peak area, was consistent over the 10 cycles.

To study the effects of cleaning and sanitization with NaOH, incubation with different concentrations of NaOH was studied. Constant incubation was studied up to a total of 48 hours, which is equivalent, depending on the residence time of the NaOH, to potentially hundreds of cleaning cycles. The experiment demonstrated that the resin can withstand up to 0.5N NaOH, allowing for stringent cleaning and sanitization. In addition, the resin demonstrates good stability over a wide range of pH conditions (1–13).

FIGURE 7

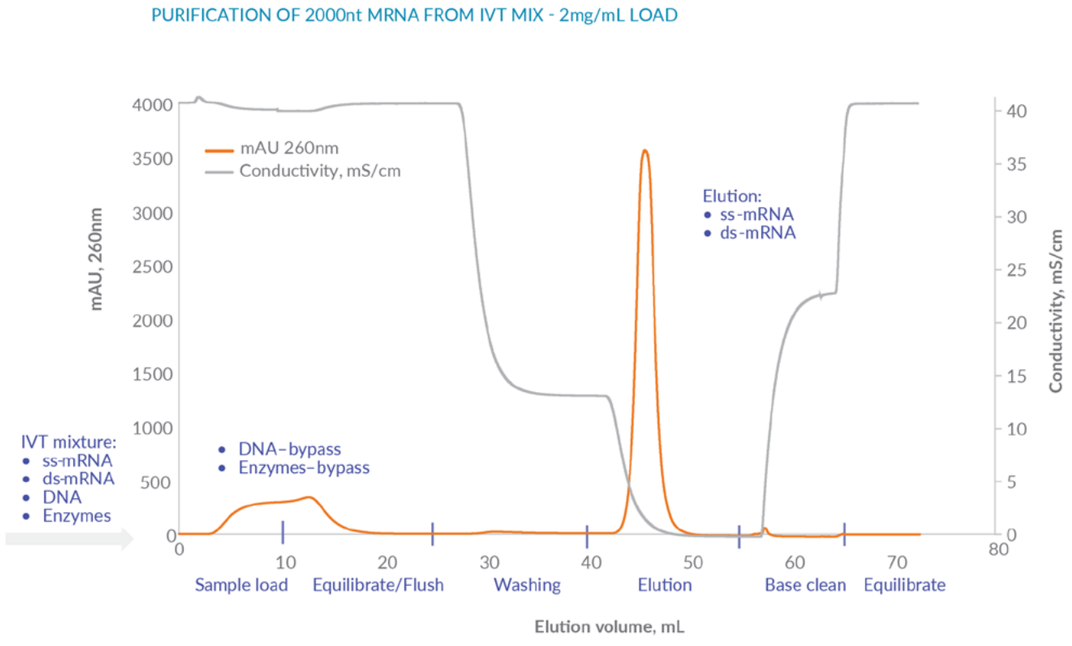
Effect of resin reuse and cleaning on mRNA purification.



Recovery is not impacted by resin reuse and cleaning. Left: Multiple cycles of mRNA (1809 nt + polyA 120 nt) purification from IVT mixture. Chromatograms from blank buffer runs carried out before cycle 1 and after cycle 10 were identical (green arrow), showing that there was no carry over of mRNA. Right: Recovery rates for each cycle, showing consistency between cycles.

FIGURE 8

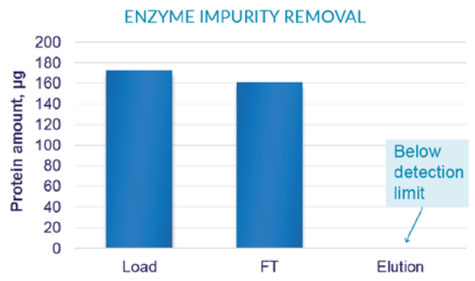
Output of a chromatographic purification run.



Conductivity (representing salt concentration) is shown in gray, while the chromatogram is shown in orange.

FIGURE 9

Enzyme impurity in load, flowthrough fraction, and elution pool.



The amount of enzyme (protein) is high in loading (load) and flowthrough (FT) fraction, but undetectable in the elution pool.

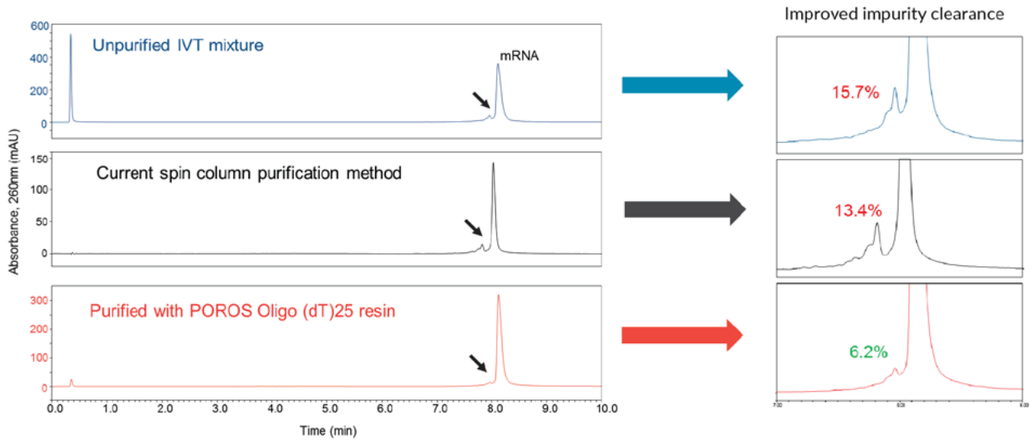
PURIFICATION VERIFICATION

Shown in Figure 8 is the output of a chromatographic purification run. The conductivity trace across the run, salt concentration measurement during the load, a step wash, and then elution and subsequent cleaning is shown in grey. The orange line is the UV 260 nm absorbance measurement and shows the chromatographic profile. At the beginning, an increase in absorbance is seen, which is indicative of DNA and other components flushing through the column. The step elution down to 150 mM NaCl helps to elute smaller truncated poly-A components that bind weakly to the column, as well as components bound to the mRNA itself, and the subsequent transition into water gives a sharp, narrow mRNA elution peak. A small peak is seen in the base cleaning step using NaOH, indicating some residual components were still on the column and are removed by this cleaning step.

The purification run was performed twice – first with already purified mRNA, where excellent recoveries of about 96% were seen. When run again with an unpurified portion of the IVT mixture, the same recovery was achieved. This was a key finding, as it demonstrates that the concentration of components present in the IVT mixture does not impact mRNA binding. This is important when considering resin reuse.

FIGURE 10

HPLC of IVT mixture after no purification (top), spin column purification (middle) and POROS™ Oligo (dT)25 affinity resin purification (bottom).



Purification with POROS Oligo (dT)25 leads to a significant reduction in impurities.

IMPURITY REMOVAL

Enzyme impurity removal was also studied using the IVT mixture (Figure 9). A relatively high concentration of protein was initially present in the loading pool, as measured by a BCA assay, and again a large amount of enzyme was present in the flowthrough fraction. When protein was measured in the elution pool, any enzyme present was below the limit of detection.

In addition, a comparison was done between a silica-based spin column method known for efficient removal of IVT components and the POROS™ Oligo (dT)25 resin. The results are shown in Figure 10.

The top trace shows the unpurified IVT mixture, and the peak on the far left represents enzyme, DNA, and smaller components. The impurities eluting the left (before) the main mRNA peak account for almost 16% of the main peak group. As shown in the middle trace, using the current spin column method, smaller enzymes are eliminated, but over 13% of the impurities remain in the main peak.

Applying an affinity resin (bottom trace) significantly decreased the amount of impurity to close to 6%, giving a significant reduction in impurities compared to the spin column method. Further study to identify the remaining components is ongoing, initial data (not shown) suggests the remaining impurities are polyadenylated. Earlier retention also suggests a smaller size than the full-length mRNA.

CONCLUSION/INSIGHT

Affinity chromatography offers a highly efficient and scalable method that has already proven its worth in the development of biologics, and it offers a powerful tool to help address the current bottlenecks in commercial manufacturing of mRNA therapeutics. With high affinity for the target molecule, it can deliver higher yield and purity in the first purification step, helping to reduce the number of purification steps in the overall process, and increasing total product yield. By reducing bioprocess development time, it can result in a decrease in overall cost of goods, and ultimately, a faster time to market for innovative mRNA-based therapeutics.

ASK THE EXPERTS

Elisa Manzotti, Founder, *Cell and Gene Therapy Insights*, speaks to **Kelly Flook,** Senior Product Manager, Thermo Fisher Scientific

Q Do you need to use heat to elute the RNA?

KF: For purification, we developed this resin so you wouldn't need to use heat. With more traditional, R&D types of mRNA extraction from cells, heat is typically used because the mix in the cell extract is a lot more complex, so it is used to break down a lot of the higher order

structures that can bind to those resins and therefore heat aids elution. But in the case of purification, and with this resin, we see a lot of customers using it successfully at room temperature.

Q Does temperature have a negative effect on the stability of mRNA in the chromatography step – and what do you recommend to try and stabilize mRNA?

KF: If there is a stability effect with temperature, it is more related to the construct sequence versus the chromatography. We see people adding EDTA to their buffers in order to help with that stabilization.

Q What sizes of RNA can be purified, and is there a construct size limit?

KF: When we developed this resin, we had relatively small mRNA sizes in mind, typically anywhere from a 1,000 up to about 5,000 nucleotides. We were not really focusing on those larger, self-amplifying RNA up to the 10,000-12,000 range.

What we do see is an impact on binding capacity, as I discussed earlier. With smaller mRNA, you will see a larger binder capacity than you will with something that is significant bigger.

Additionally, the amount of salt you need to neutralize those charges will also be slightly different, because the larger the RNA, the more charges you need to neutralize. You would expect more salt to be needed to achieve that and maximize your binding.

Q How many cycles can you typically get out of the resin?

KF: In this case we looked at cycling just up to 10 cycles. However, we have seen some customers using this resin that are getting 30, 40, 50 cycles, so it is robust. They have a cleaning step in between those cycles as well, this is also a quick sanitization step between cycles.

Q What would you advise for salt concentration to get optimal binding?

KF: We have seen good success starting at about 0.5M sodium chloride in the initial instance. Then either increasing that slightly to increase binding, or simply decreasing that down to the minimum level you need to achieve binding.

Q What is the maximum operating pressure for the resin?

KF: The resin has a robust poly(styrene-*co*-divinylbenzene) core, so the resin itself can withstand pressures over 100 bar. As far as operating and packing for a purification set up, your pressure limitations are really going to be limited by the hardware, and not necessarily the resin.

Q How can you separate single stranded mRNA from double stranded, and do you have any particular products that fit this goal?

KF: As I mentioned earlier, one of the great things about the dT is that it will bind poly-A well. This also includes double stranded RNA. We recommend our HIC resin range – we have a POROS™ Ethyl, Benzyl and Benzyl Ultra, that can be used to separate the double stranded RNA from single stranded.

BIOGRAPHY

Kelly Flook, Senior Product Manager, Thermo Fisher Scientific

Kelly Flook is Senior Product Manager for Purification products within the Bioproduction Division at Thermo Fisher Scientific. Kelly has a Ph.D in Polymer and Analytical Chemistry from the University of Durham, UK. During her 15 years at Thermo Fisher, Kelly has gained extensive experience in product development across all scales of chromatography and related biological workflows. Kelly has a strong expertise in bead technology and bio-separations. Drawing from a diverse technical background, in her current role Kelly is responsible for new product development and commercialization of solutions across the downstream workflow.

AUTHORSHIP & CONFLICT OF INTEREST

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

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Regulatory statement: *Pharmaceutical Grade Reagent. For Manufacturing and Laboratory Use Only.*

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FASTFACTS

Efficient and scalable purification of mRNA using affinity **chromatography**

Synthetic mRNA is a versatile modality with a wide array of applications, from stem cells and cell differentiation to vaccines. What all these platforms have in common is the need for a stable, reliable and scalable production and purification platform to enable production of high quantities of mRNA with the required purity and quality attributes. To support the development of mRNA-based therapies, Thermo Fisher Scientific developed the POROS™ Oligo (dT)25 affinity resin to enable efficient and simplified mRNA purification.

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SELECTIVE, SCALABLE AND CGMP COMPLIANT PURIFICATION

The POROS™ Oligo (dT)25 Affinity Resin was specifically designed for the purification and isolation of mRNA from *in vitro* transcription manufacturing processes, and offers a scalable and highly selective purification platform for any mRNA with a PolyA tail. The resin is fully cGMP compliant and animal-origin free, and Thermo Fisher Scientific can provide users with regulatory support.

RECOVERY AND IMPURITY REMOVAL

High recovery is seen independently of construct size and sample type. Figure 1 shows percentage recovery for three different mRNA constructs. Consistent recovery well above 90% was seen in all cases.

PURIFICATION RUN

Figure 2 shows the typical output of a chromatographic purification run when using the Oligo (dT)25 resin. The sample was loaded at high conductivity, and upon loading the absorbance increases as the impurities flow through the column. An intermediate wash step was used to remove any loosely and non-specifically bound components, and elution was performed using pure water. A minor peak was seen during the alkaline cleaning step, showing that some residual components were still present and can be removed by alkaline conditions.

RESIN REUSE & CLEANING

The effect of incubation of the resin in sodium hydroxide and hydrogen chloride was studied (Figure 3). The ionic capacity, which has a direct correlation with ligand density, was well maintained after 48 hours of constant exposure to cleaning agents. A cycling study demonstrated consistent performance, with overlapping elution peaks, and no carry over seen in a blank run performed after 10 cycles.

HIGHER YIELDS WITH LOWER TIME AND COST

The POROS Oligo (dT)25 Affinity Resin offers a highly stable and reusable method for mRNA purification. With high affinity for the target molecule, it can deliver high yield and purity, and help to reduce the number of purification steps in the overall process. By reducing bioprocess development time, it can result in a decrease in overall cost of goods, and ultimately, a faster time to market for innovative mRNA-based therapeutics.

Pharmaceutical Grade Reagent. For Manufacturing and Laboratory Use Only.

Figure 1. High recovery and purity is achieved with the Oligo (dT)25 resin, independent of construct size.

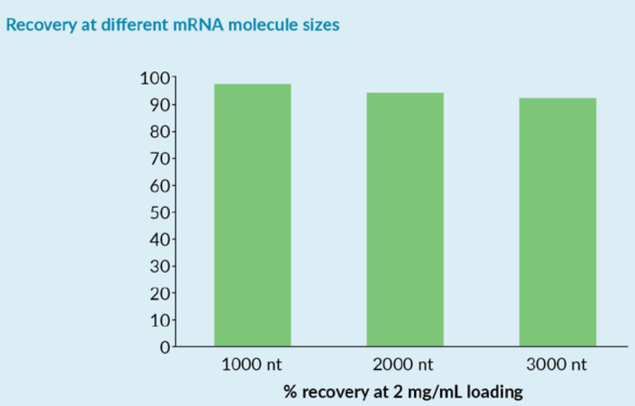


Figure 2. Output of a chromatographic purification run using the Oligo (dT)25 Affinity Resin.

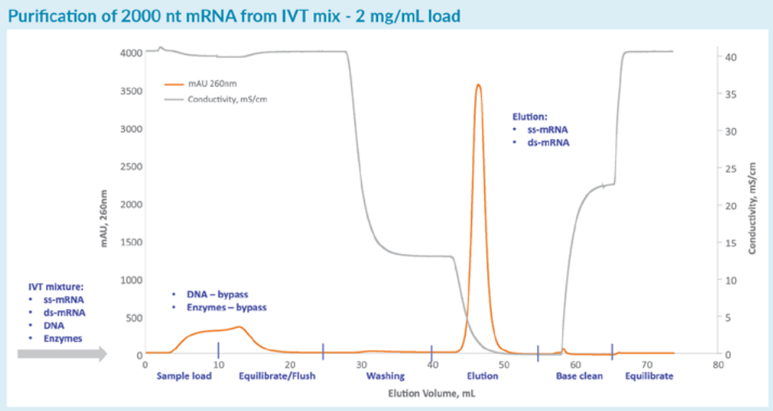
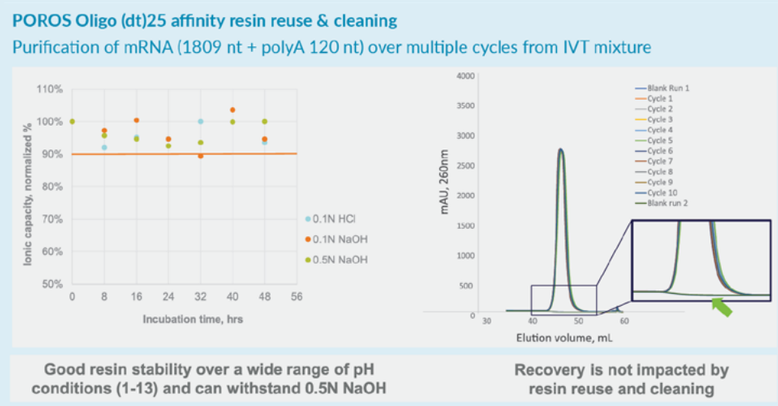


Figure 3. Resin stability over a range of pH conditions (right) and overlay of elution peaks over 10 cycles (left).



CELL & GENE THERAPY INSIGHTS

In partnership with: ThermoFisher SCIENTIFIC