

## INNOVATOR INSIGHT

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

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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.

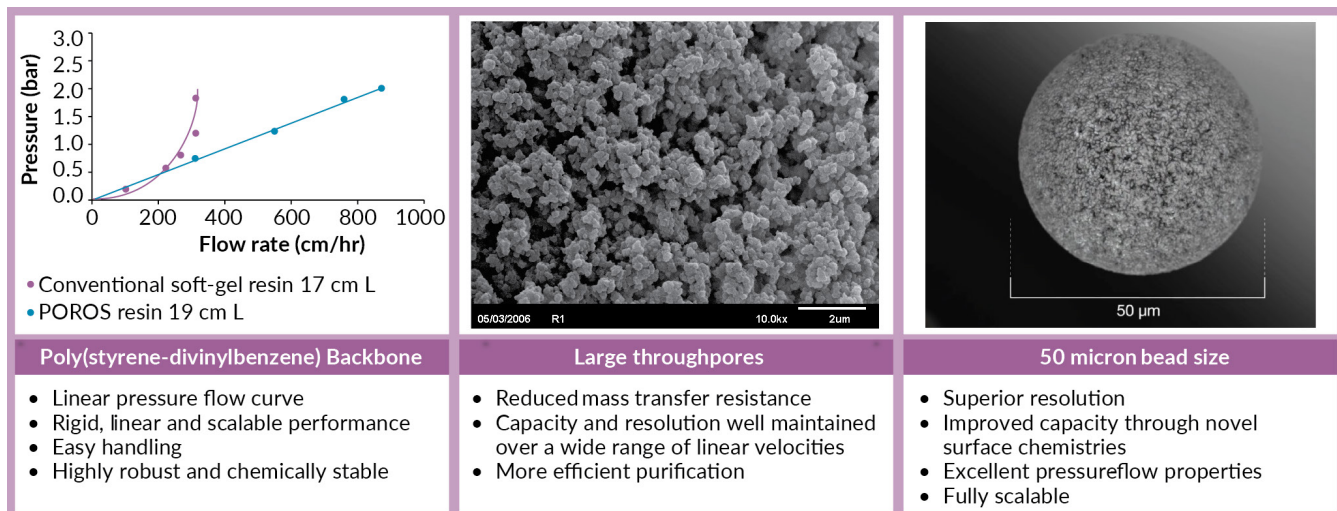
### ADVANTAGES OF POROS ANION EXCHANGE RESINS

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

▶ **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	<b>Bind/elute:</b> Protein, virus, plasmid DNA purification <b>Flow through:</b> Trace impurity removal by binding impurities (DNA, viruses, HCP, aggregates, endotoxin)
PI	Weak	Polyethyleneimine (mixed amine)	2000	80	
HQ50	Strong	60% quaternized polyethyleneimine (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.

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.

In this work, we focused on three out of the four resins in **Table 1**; POROS™ D50 has a dimethylaminopropyl 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

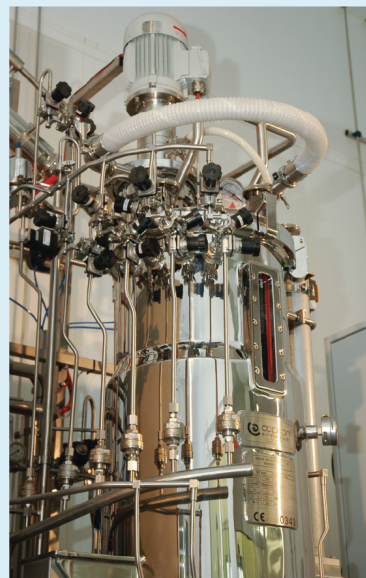
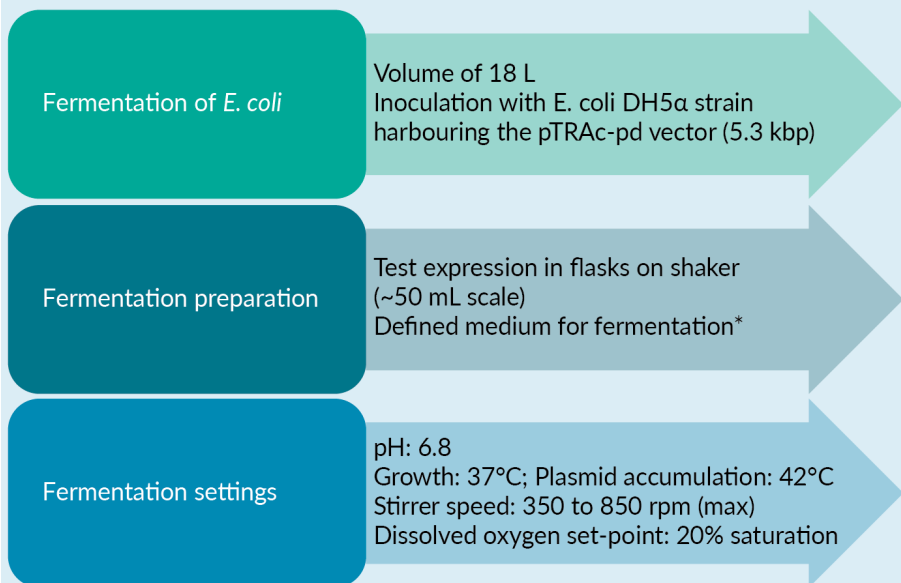
The first step of the study was pre-processing, i.e., generating the materials to be tested for chromatographic separation. *E. coli* was selected as a representative system; the specific fermentation and extraction processes are shown in **Figure 2**. This preparation procedure provided a starting material 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

► **FIGURE 2**

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

**Fermentation of plasmid DNA in E. coli**



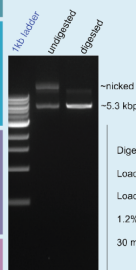
**Extraction of pDNA from E. coli**

Large scale isolation of Plasmid DNA using a 3-step step extraction method

- 1 Cell lysis using 0.2 M sodium hydroxide, 1% (w/v) SDS  
- Denature chromosomal and plasmid DNA
- 2 Neutralization using 3.0 M potassium acetate, pH 5.5  
- Precipitation of proteins and chromosomal DNA
- 3 Centrifugation (10 min, 4500 RCF, 4°C)  
- Removes precipitated proteins and chromosomal DNA
- 4 Depth filtration using K700 and K200 filter sheets  
- Clarification of large particles using depth filtration
- 5 Buffer exchange and concentration of pDNA  
- Ultrafiltration/Diafiltration using 100 kDa membrane (0.3 bar TMP)  
- Sterile (0.2 µm) filtration and preparation for chromatography

Reduced open circle pDNA compared to conventional extraction  
Centrifugation may be substituted during scale-up

Fermentation *E. coli* with pTRAc-pd plasmid, test digest



~nicked  
~5.3 kbp  
Digest: pmet, 2 h, 37°C  
Loaded 10 µL plasmid  
Loaded 5 µL ladder  
1.2% agarose  
30 min, 120 V



\*[1]. TMP: Transmembrane pressure.

that the model has a good and even predictive power throughout the entire design space.

**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,

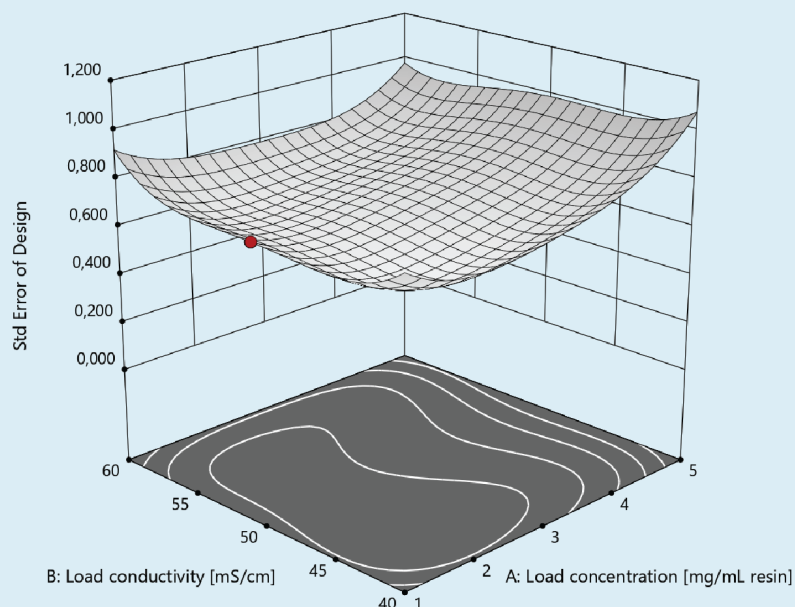
for the POROS HQ50 resin, the different parameters had little effect; in this case the load conductivity and load concentration. In contrast, for POROS D50, we found that with an increasing load conductivity the relative recovery of products increased. For POROS™ XQ, the recovery decreased with an increasing load concentration, i.e., with a higher quantity of plasmid loaded per volume of resin.

**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/cm

RoboColumn® is a registered trademark of ATOLL GmbH

The flat error surface indicates a high-quality design



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.

### 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.

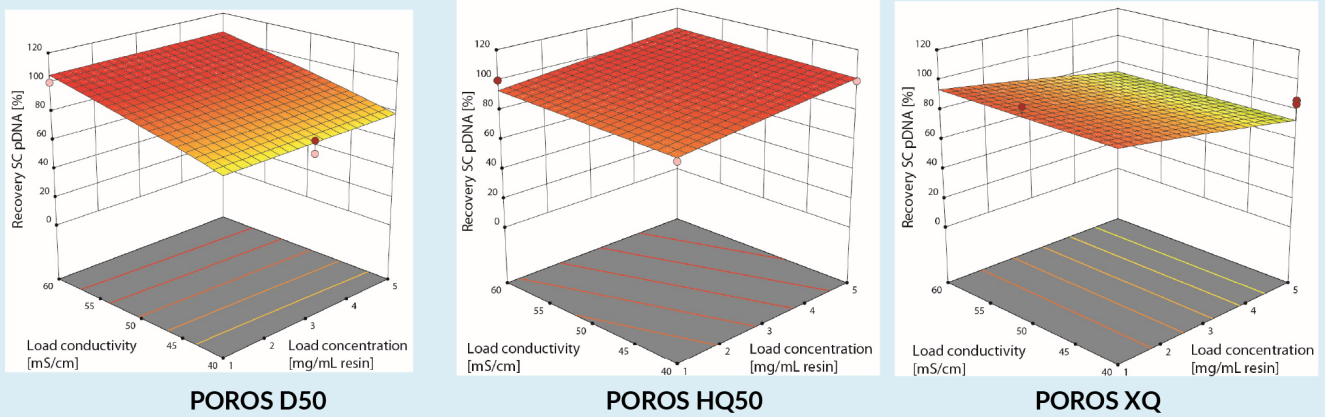
A similar double breakthrough curve was seen for POROS HQ50. However, an

► **FIGURE 4**

Resin recovery at pH 7.0.

**AEX DoE: Recovery (pH 7.0)**

- High pH significantly increases recovery for all tested resins
- Load conductivity between 50 and 60 mS/cm increases recoveries for POROS™ D50 resin
- Recovery decreases with increasing load concentration for POROS™ XQ resin



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 assume that the supercoiled pDNA is replacing previously-bound RNA or smaller DNA

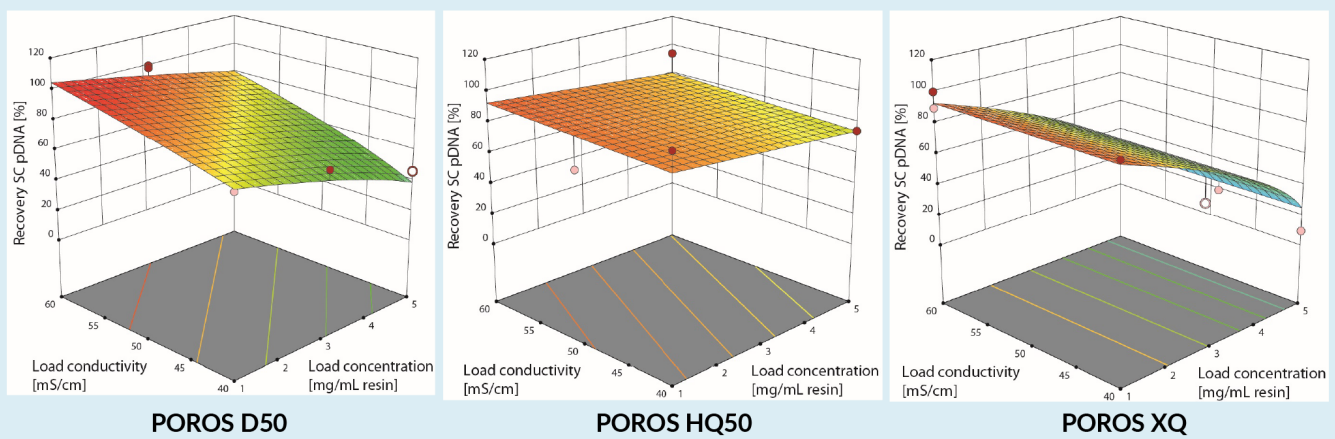
► **FIGURE 5**

Resin recovery at pH 6.0.

**AEX DoE: Recovery (pH 6.0)**

- Low pH significantly reduces recovery for POROS D50 and XQ resin
- Low pH can reduce recovery for increasing load concentrations
- High pH (7.0) required for pDNA binding at high loading conductivities

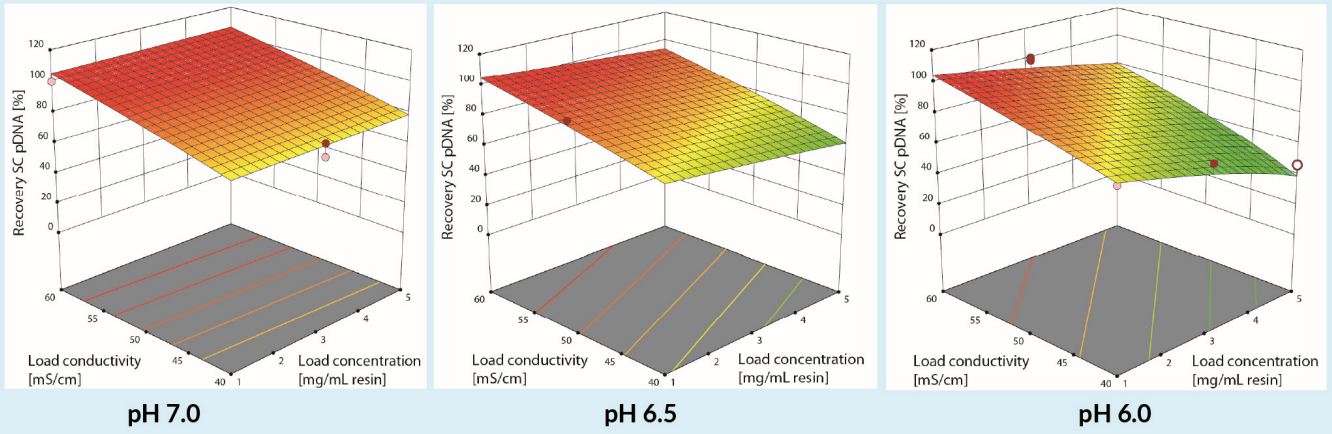
Most pDNA was lost in early and late elution fractions with low purities below the load purity



▶ **FIGURE 6**

pH effect on POROS D50 recovery.

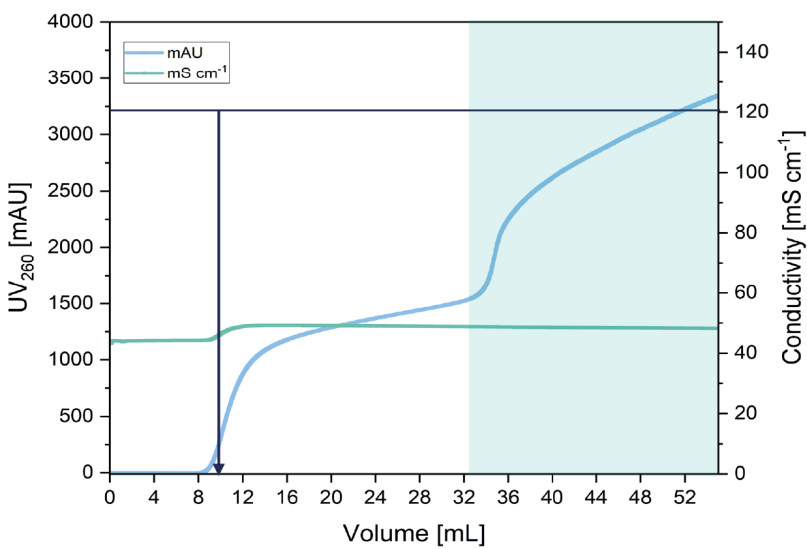
AEX DoE: pH effect on POROS D50 recovery



▶ **FIGURE 7**

POROS XQ dynamic binding capacity.

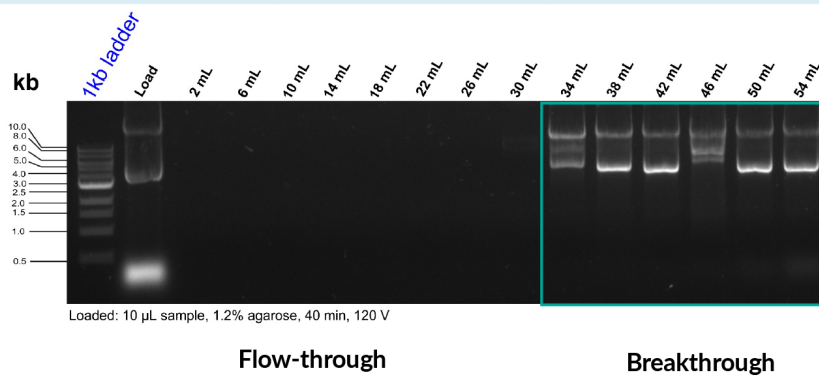
POROS XQ Dynamic Binding Capacity



Parameters for Chromatography

System	ÄKTA pure
Column Volume	1 mL
Residence Time	2.5 min
Fractionation	2.0 mL
EQ & Load conductivity	~45 mS/cm
Salt Type	Sodium Chloride
pH	6.0

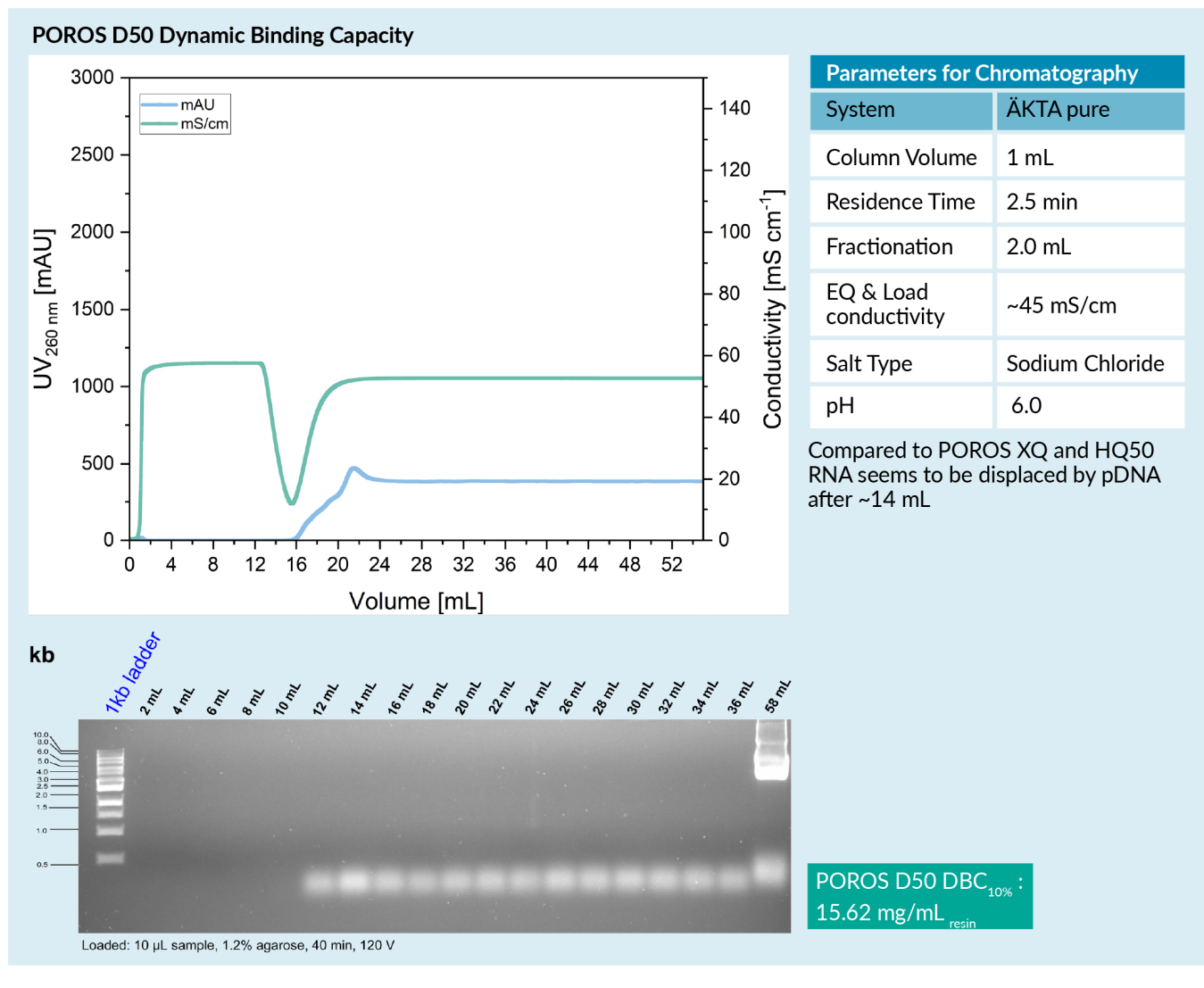
DBC<sub>10%</sub> (UV 260):  
5.5 mg/mL



DBC<sub>10%</sub> (Agarose Gel):  
9.0 mg/mL<sub>resin</sub>

► **FIGURE 8**

**POROS D50 dynamic binding capacity.**



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:

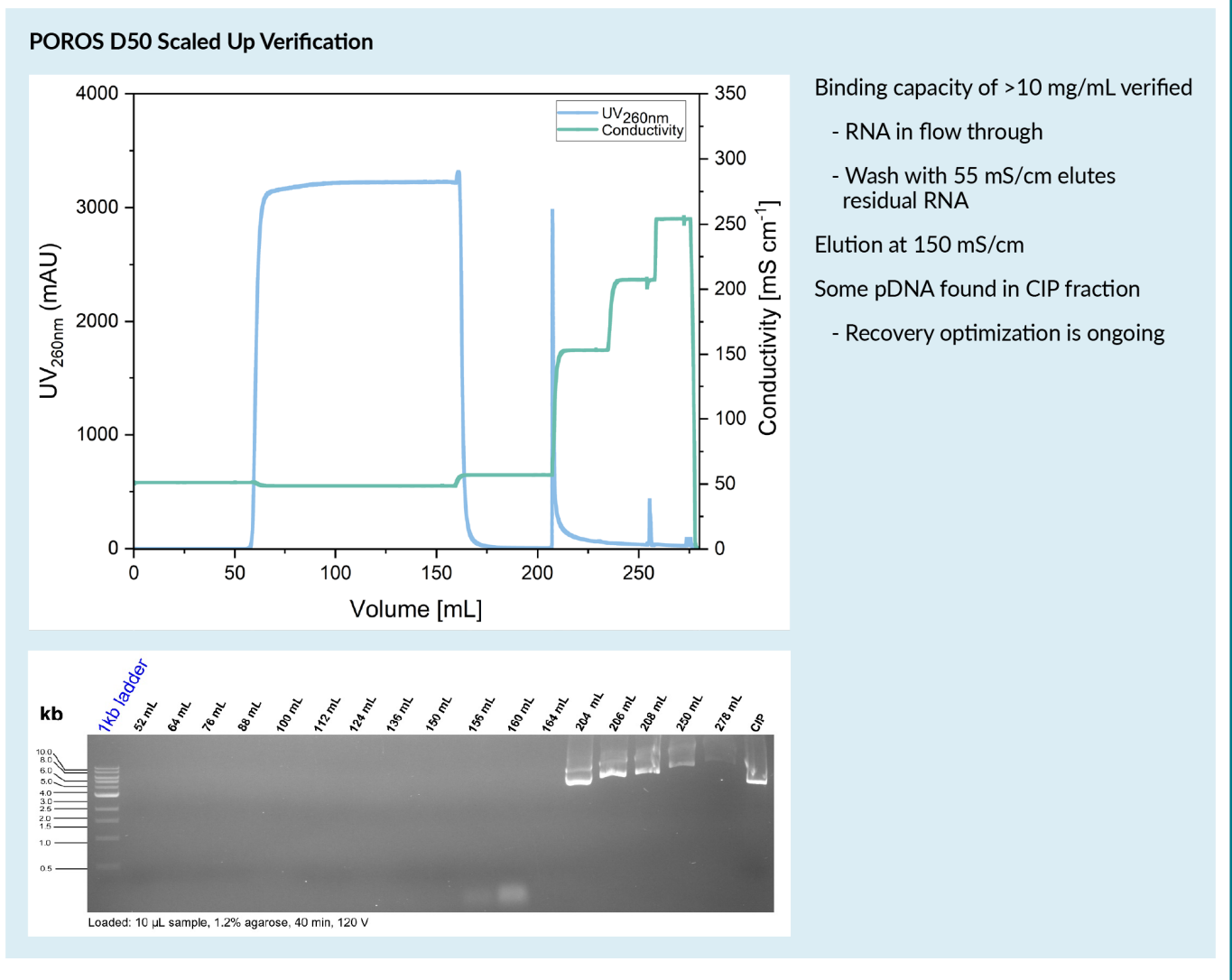
- ▶ Further optimization of the capture step with POROS D50
- ▶ Separation of pDNA isoforms with POROS hydrophobic interaction chromatography (HIC) resins

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 at 2.5 minutes,

▶ **FIGURE 9**

POROS D50 scaled up verification.



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.

### REFERENCES

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1. Carnes AE, Hodgson CP, Williams JA. Inducible Escherichia coli fermentation for increased plasmid DNA production. *Biotechnol. Appl. Biochem.* 2006; 45: 155–66.



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# ASK THE AUTHORS

**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 adsorbents?

**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 scholarship) and the USA (Fraunhofer Center for Molecular Biotechnology, Newark, Delaware). Finishing his Masters with honours in 2009 he was awarded the Springorum 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 focusing 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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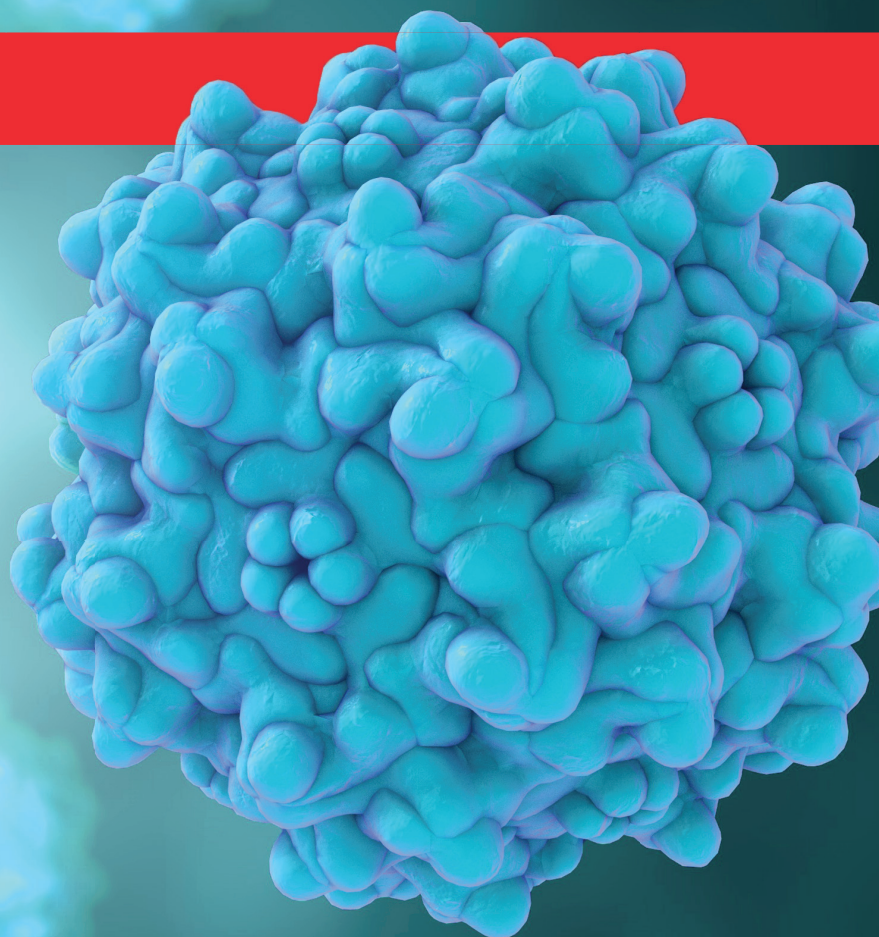
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