NUCLEIC ACID INSIGHTS

INNOVATOR INSIGHT

Process optimization of mRNA purification for vaccines and therapeutic applications

Chantelle Gaskin

Critical to bringing mRNA therapeutics to patients is an efficient, effective, and scalable downstream production process. This article explores the development, characterization, and optimization of an mRNA capture step using POROS™ Oligo (dT)25 Affinity Resin. This resin provides an affinity solution that leverages the rigid POROS backbone with its linear pressure-flow relationship, enabling a consistent purification platform from process development through to clinical manufacturing.

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INTRODUCTION

Synthetic mRNA has multiple applications, from enhancing allergen tolerance to treating serious diseases including cancer, genetic disorders, and infectious diseases. The current main methods of mRNA therapy delivery include direct injection, *ex vivo* injection of transfected cells, and transfection of genome editing enzymes. These diverse applications have led to the industrialization of mRNA therapy.

Novel biotherapeutics typically follow four stages of growth **(Figure 1)**. The COVID-19 pandemic has quickly moved mRNA past the 'nascent' and 'growth' phases and into the 'emerging modality' phase. However, in order to reach the 'mature technology' phase, considerable investment is needed into optimizing mRNA production. More specifically, a robust and easy-to-use purification platform is a prerequisite to drive the future success of mRNA's diverse applications and methods of delivery.

IMPROVING THE mRNA THERAPEUTICS PROCESS WORKFLOW

A typical mRNA process workflow includes DNA template preparation (including plasmid production and purification, which can be completed in-house or outsourced), mRNA synthesis, and downstream purification (Figure 2). Thermo Fisher Scientific's POROS™ products can be used for both the plasmid and mRNA purification steps.

FIGURE 1

Stages of biotherapeutic development.

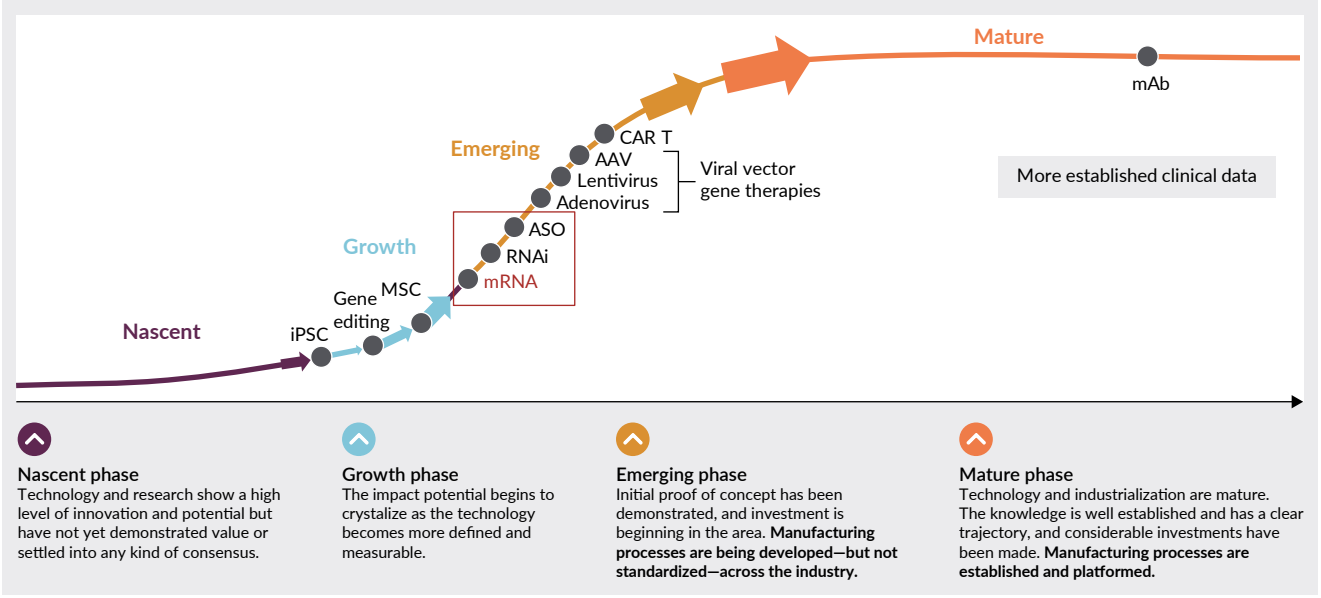


FIGURE 2

mRNA therapeutics process workflow overview.

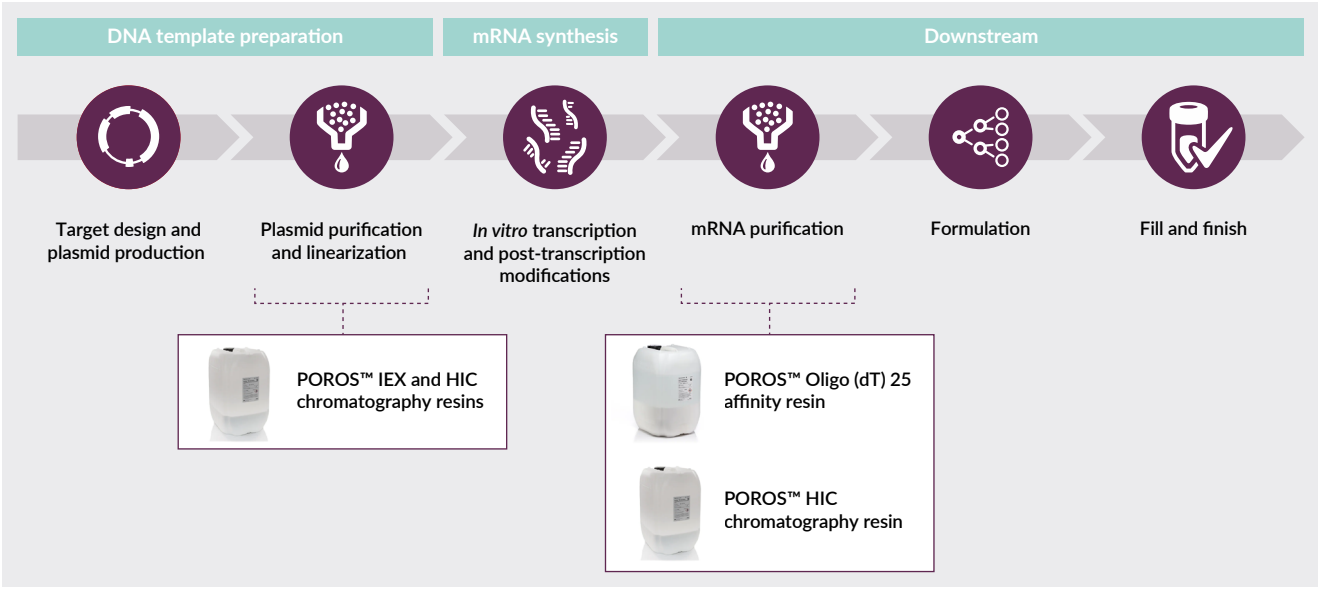


FIGURE 3

mRNA purification workflow overview.

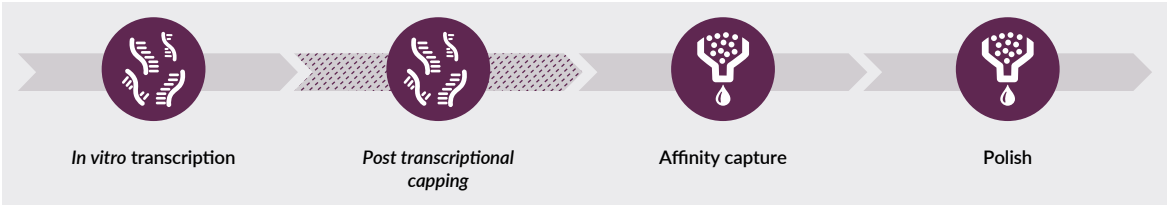


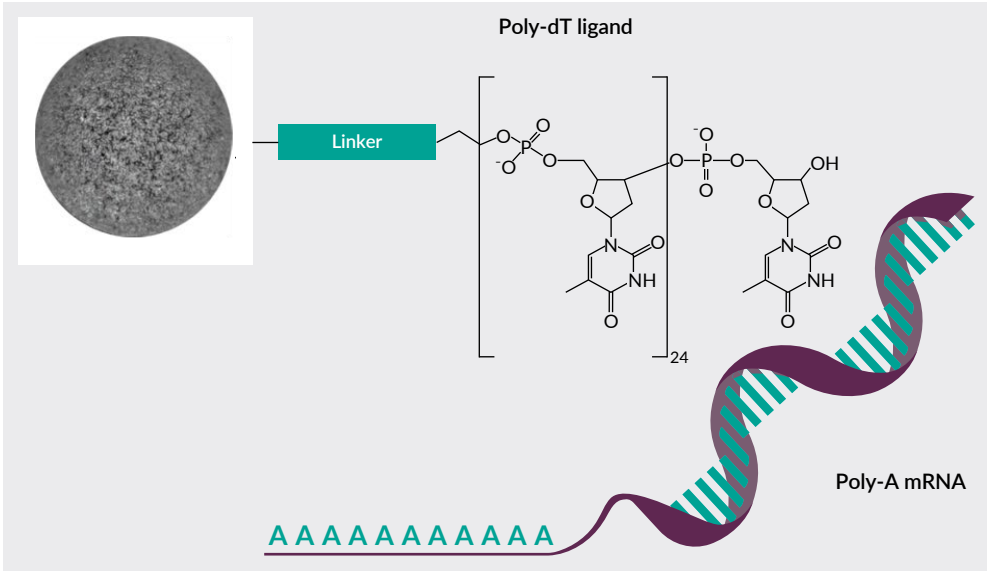
Figure 3 zooms in on the mRNA purification workflow, specifically, where the POROS™ Oligo (dt)25 Affinity Resin can be used for affinity purification. After the *in vitro* transcription (IVT) reaction (or following post-translational capping, if it is performed as a separate, subsequent upstream step), the full-length mRNA is selectively captured by the Poly(A) tail with the POROS Oligo (dT)25 Affinity Resin. Because of the specificity of the resin to the Poly(A) tail, only the full-length mRNA remains following the affinity capture step. Furthermore, this step enables the removal of process-related impurities such as DNA template, nucleotides, enzymes, and buffer components. An additional polish step can be conducted using POROS™ Benzyl Hydrophobic Interaction Chromatography (HIC) Resin, aiding in the removal of double-stranded (ds)RNA, uncapped RNA, and secondary RNA structures from the final product.

POROS OLIGO (DT)25 AFFINITY RESIN AND THE POROS BEAD

The POROS Oligo (dT) resin has been specifically designed for the purification and isolation of mRNA from the IVT reaction. This is enabled by A-T base pair hybridization. As illustrated in Figure 4, a Poly-dT ligand with a proprietary linker is attached to a 50 µm POROS bead. The resin has a dynamic binding capacity of up to 5 mg/mL

FIGURE 4

dT-25 (poly-deoxythymidine) ligand with proprietary linker.



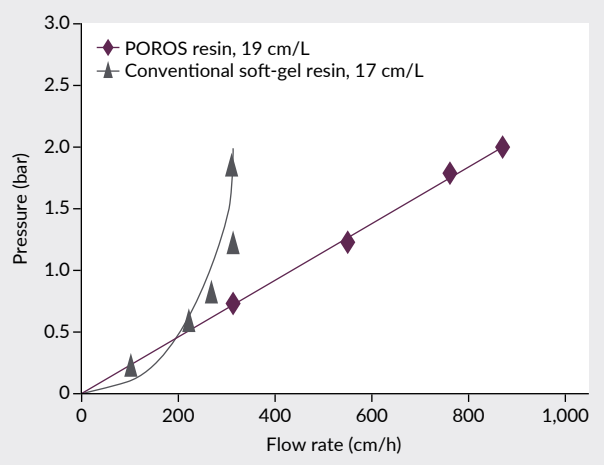
for 4,000 nucleotides (nt) of mRNA. With >90% recovery, the resin has excellent scalability and is also non-animal derived.

Additionally, the Oligo (dt)25 resin is offered in a variety of formats as a tool for early development, which can then be easily scaled for manufacturing. Conditions can be optimized with a 96-well plate, or with a POROS RoboColumn™ for a specific mRNA construct to maximize mRNA purification. Once the ideal conditions are determined, they can be verified with pre-packed columns at bench-scale. The optimal conditions thus established at small-scale can then be applied when scaling up mRNA purification to the liter+ scale.

The Oligo(dT) resin on the unique POROS bead has three main attributes that differentiate it from other chromatography resins. The first of these is that the bead is made of Polystyrene-divinylbenzene, which results in stable column beds with linear and scalable pressure. This enables the operation of high linear flow rates with modest pressure drops. For example, an operation at 800 cm/hour in a 20 cm column will result in a 2-bar pressure drop across the column **(Figure 5)**. This is a small change in comparison to a classic soft gel resin that shows a typical exponential increase in back pressure as linear velocity increases.

FIGURE 5

The POROS bead allows for high linear flow rates with modest pressure drops.



Additionally, due to the polymeric nature of the backbone and the robust covalent chemistries of the beads themselves, POROS beads exhibit strong physical and chemical stability. They are chemically stable at pH 1–14, in high-salt concentrations, and in the presence of detergents and denaturants, which enables the use of aggressive cleaning solutions and improves resin lifetime and reuse.

The second key attribute is the large pore structure, which results in reduced mass transfer resistance in comparison to other resins. This large pore structure enables linear velocity increases with minimal loss of capacity and resolution, as well as improved process productivity.

Finally, the third attribute is the small (50 µm) average particle size. This allows for reduced band broadening in packed beds, which improves the resolution of molecule separation as well as impurity removal.

mRNA PRECIPITATION POINT DETERMINATION

One of the challenges with mRNA purification is understanding and achieving good binding and recovery. This is largely dependent on the solubility of the molecule, which is dictated by the structure and size of the construct.

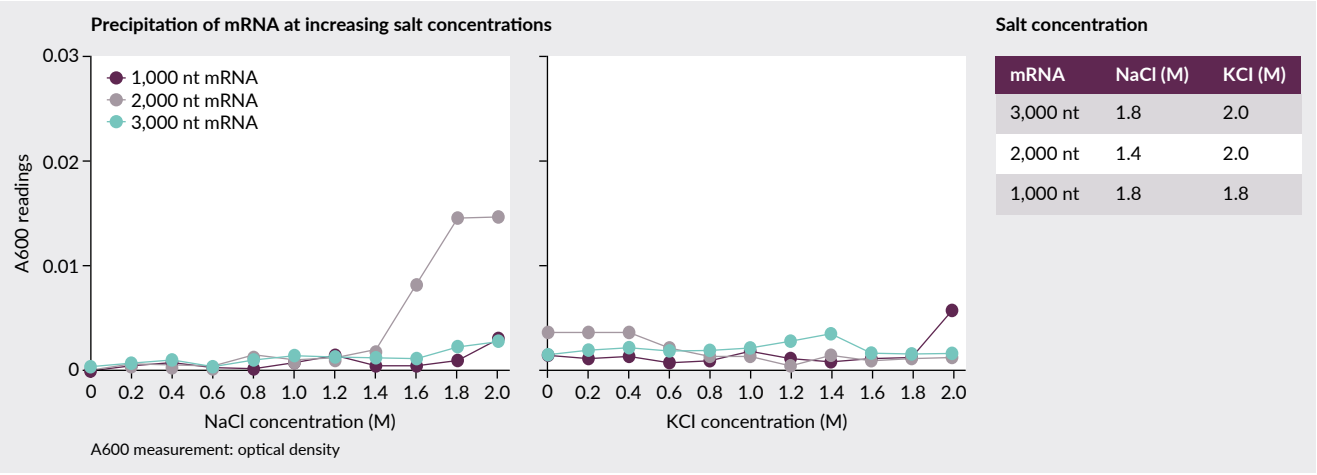
In the case study illustrated in **Figure 6**, constructs of 1,000, 2,000, and 3,000 nt were examined with the following experimental conditions:

- NaCl and KCl as salt additives
- Various salt concentrations were prepared in 10 mM Tris, 1 mM EDTA, pH 7.4
- 0.2 mg/mL mRNA solutions were prepared from the above
- 20 µL resin, 2 mg/mL mRNA load (40 µg)

Buffers containing both NaCl and KCl were tested at various concentrations. The

FIGURE 6

mRNA precipitation point determination: case study data.



Salt concentration

mRNA	NaCl (M)	KCl (M)
3,000 nt	1.8	2.0
2,000 nt	1.4	2.0
1,000 nt	1.8	1.8

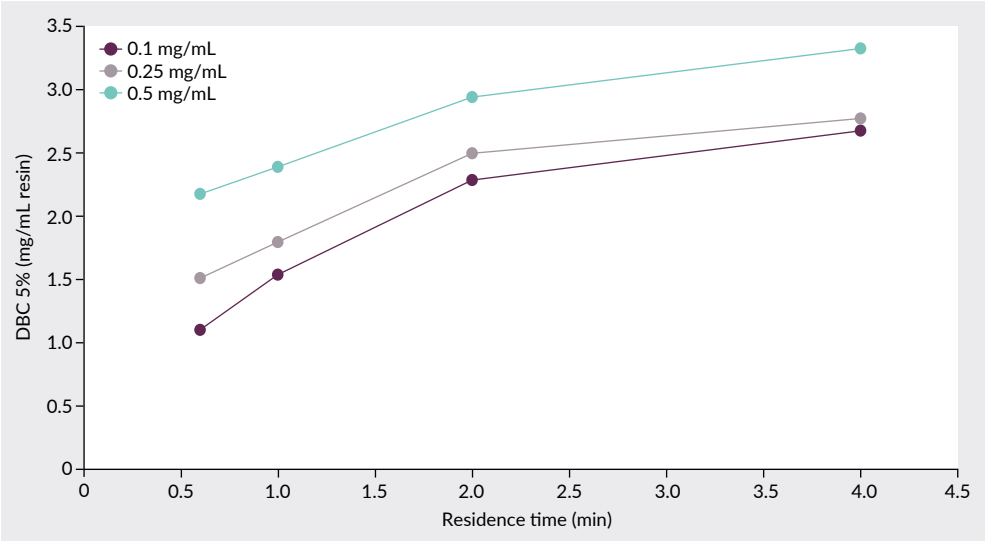
table on the right of **Figure 6** shows the salt concentrations at which precipitation began to increase. For a 2,000 nt construct, there was marked precipitation beginning at 1.4 M NaCl. These concentrations represent upper limits for further process development and optimization. The common operating window, however, is below the precipitation point (i.e., between 0.5–1 M concentration).

DYNAMIC BINDING CAPACITY DETERMINATION

The binding capacity of a capture step is an important parameter in determining how much product to load on the column. The concentration of mRNA on a mass basis is relatively low compared to monoclonal antibody or protein applications. This load concentration can impact the dynamic binding

FIGURE 7

Dynamic binding capacity determination: case study data.



capacity (DBC) because the binding is diffusion-limited. Diffusion is driven by a concentration gradient or difference between the load material and the resin. If the concentration in the load material is lower, there is a lower driving force to get the mRNA onto the resin. As an alternative explanation, if there is a lower concentration in the load material, then there is a reduced opportunity for these molecules to find a ligand to which to bind.

To determine the DBC, a breakthrough curve was generated for each of the three different load concentrations at four residence times in each case (Figure 7). The process conditions for this DBC study are based on Tris EDTA or TE saline buffers. For all three mRNA load concentrations, there was only a small increase in DBC at 5% breakthrough (BT) between 2–4 minutes' residence time, compared to that seen at ≤1 minute. In practice, this means that a process can be run at a 2-minute residence time without sacrificing capacity, which increases productivity.

The other important point to note in this data is that a higher DBC is obtained with a higher concentration of the load material, since diffusion is driven by the concentration gradient.

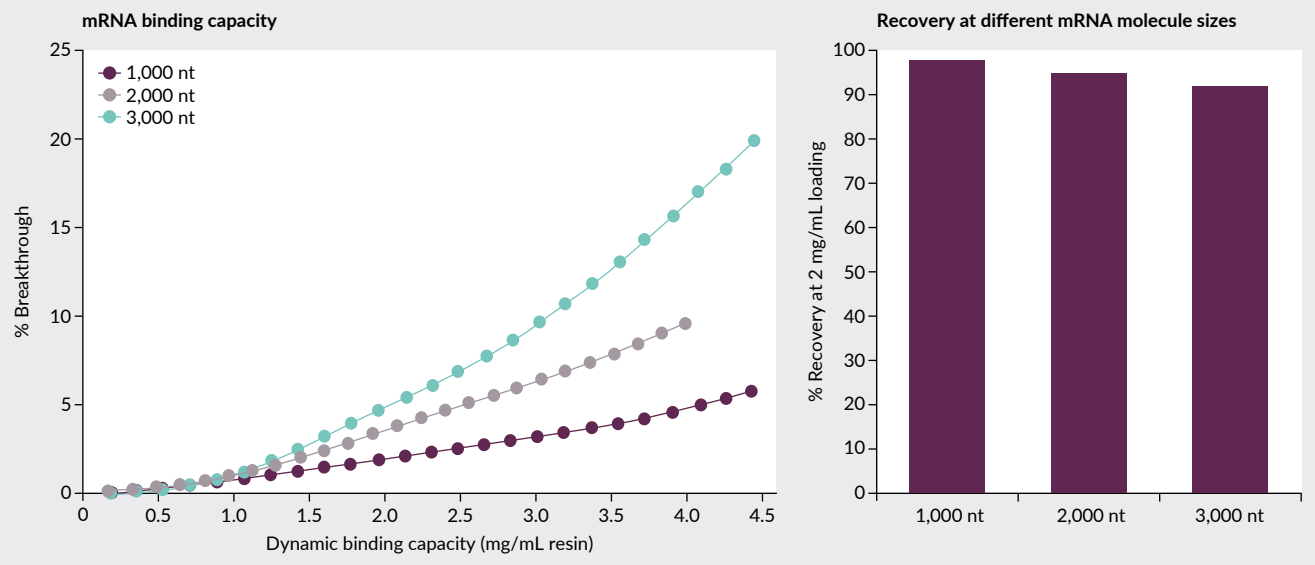
IMPACT OF MOLECULE SIZE ON BINDING CAPACITY AND RECOVERY

It is important to optimize an mRNA process for a specific molecule, especially considering binding capacity and recovery. Figure 8 examines binding capacity as a function of construct size and the corresponding recoveries. Again, 1,000, 2,000, and 3,000 nt constructs were used. For 1,000 nt mRNA, a binding capacity of 4.5 mg/mL was observed at 5% BT. However, a 4 mg/mL resin binding capacity was observed at 10% BT for a 2,000 nt mRNA. For a 3,000 nt mRNA molecule, a 3 mg/mL resin binding capacity can be obtained at 10% BT. These binding capacities were all determined at a 2-minute residence time and with 90 cm/hour column load.

Given these data, it is recommended to either reduce load density or increase residence time for larger constructs. While a >90% recovery is observed for all three mRNA molecule sizes, the key is to optimize loading densities depending on the size of the construct, and to optimize buffer conditions depending on the salt tolerance of the construct.

FIGURE 8

Impact of molecule size: case study data.



TYPICAL CHROMATOGRAM FROM A POROS OLIGO (dT)25 PRODUCTION RUN

An example chromatogram is shown in Figure 9 for the purification of a 2,000 nt mRNA from an IVT mixture with the POROS Oligo(dT) resin. The sample was loaded at 2 mg/mL resin with a final sample concentration of 0.2 mg/mL. The sample was diluted in 10 mM Tris, 0.8 M NaCl, and 1 mM EDTA at pH 7.4 binding buffer. During the sample loading, only the mRNA with the Poly(A) tail was bound to the resin, while the DNA, enzymes, and nucleotides flowed through the column. Both single-stranded mRNA and some dsRNA species bind to and elute from the POROS Oligo (dt)25 resin.

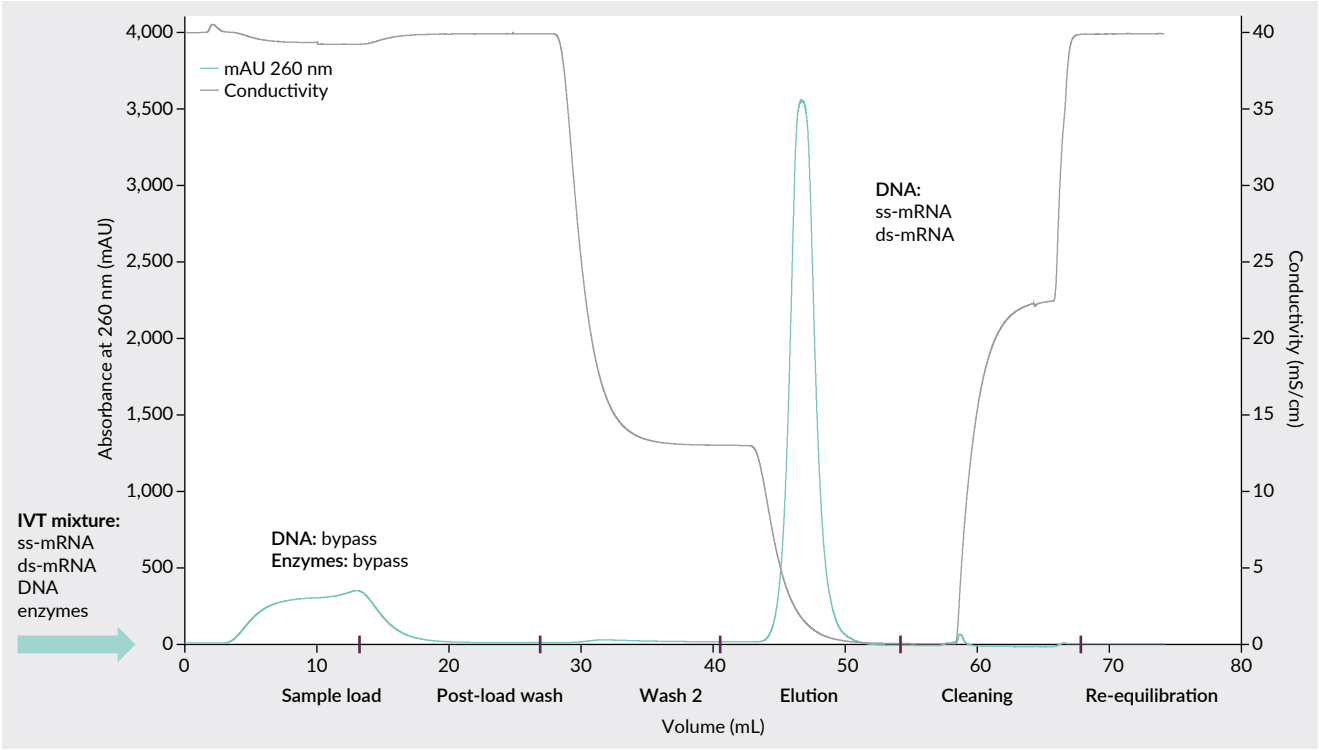
After sample loading, the column was cleaned with two washes—an equilibration buffer and a low-salt wash. Although impurities from the IVT reaction were present in the flowthrough as expected, a two-step wash is still recommended. The mRNA was eluted with RNase-free water and the column was cleaned with 0.1 M NaOH.

RECOVERY AND IMPURITY REMOVAL

High recoveries from Oligo (dT)25 are expected, reaching 95% or higher, regardless of the purity of the starting material. Figure 10 demonstrates a 95% recovery obtained using the same load density and the same residence times for both purified and crude mRNA feed streams. This is further evidenced by the protein content in the flowthrough versus that in the elution. Despite there being a difference in the protein content of the load versus the flowthrough, the remaining protein was actually present in the cleaning in place (CIP) step, which is not shown in Figure 10.

FIGURE 9

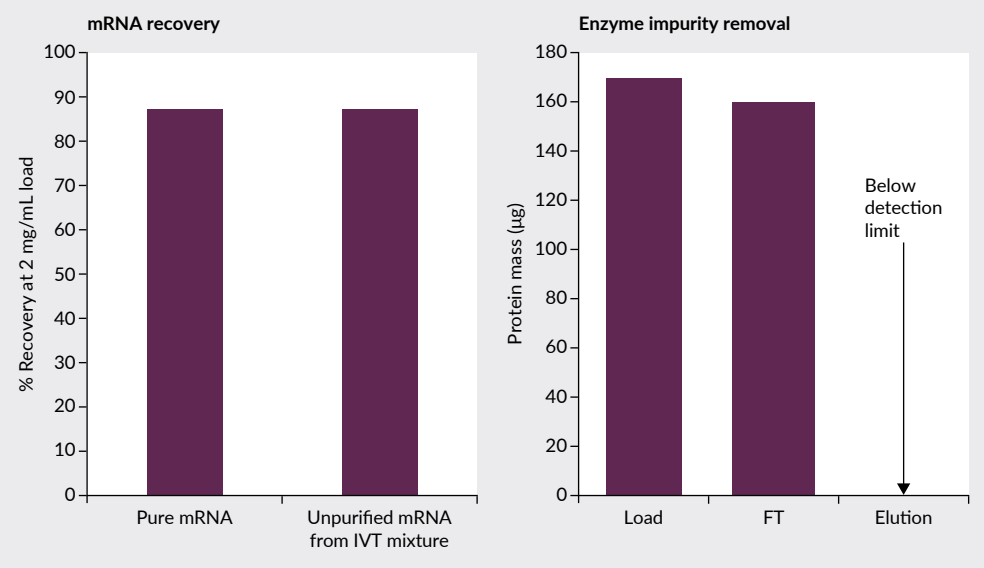
Purification of 2,000 nt mRNA from IVT mix.



IVT: *in vitro* transcription.

FIGURE 10

Recovery and impurity removal: case study data.

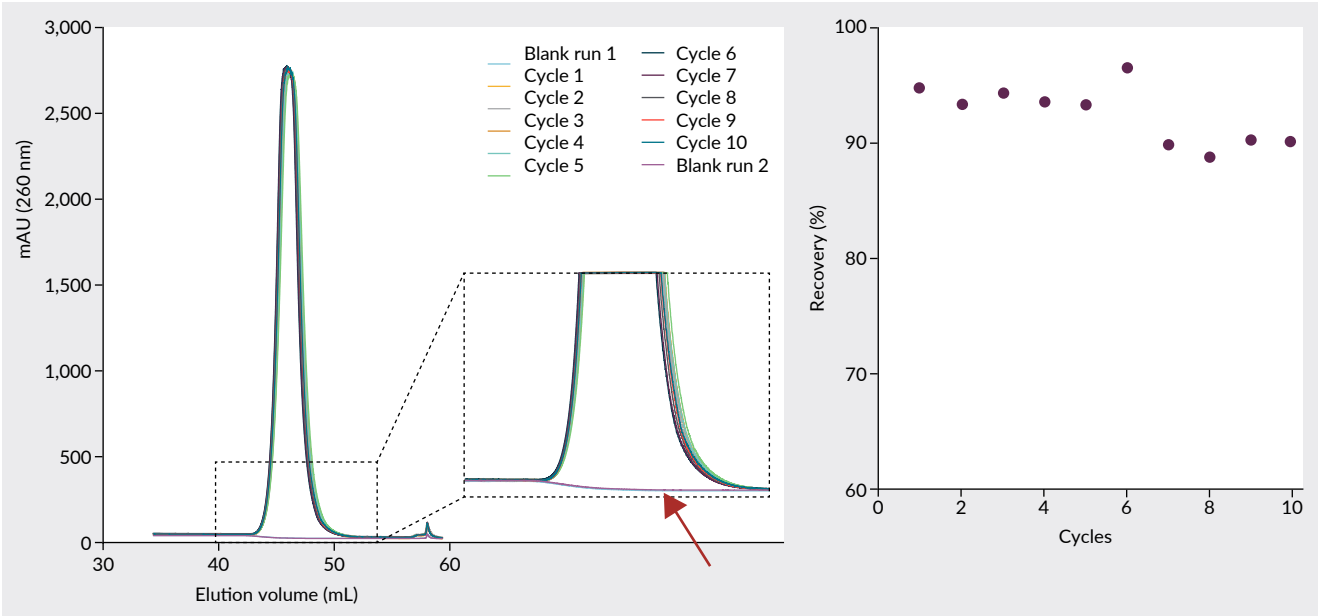


REUSE AND STABILITY

Before deciding to implement a resin in a process, it is important to determine that it is stable and functional for multiple cycles and following periods of storage in different conditions, thus ensuring cost–effectiveness. The following studies demonstrate the continued

FIGURE 11

Purification of mRNA (2,000 nt) over multiple cycles from IVT mixture.



IVT: *in vitro* transcription.

high-quality performance of the POROS Oligo(dT)25 resin in multiple cycles and various storage conditions.

Figure 11 shows the elution peak over 10 cycles for the purification of an IVT mixture. A blank buffer run was performed before and after the 10 cycles to monitor any mRNA that eluted in the final blank run. The overlay is identical to the initial run, showing that no mRNA is eluting from subsequent runs. Additionally, a >90% recovery is observed for each of the 10 cycles.

In order to supplement this reuse data, a further study was conducted. The objective of this additional study was to show that repeated uses of the resin with a molecule that binds to the Oligo (dT)25 ligand does not impact the performance. Due to limitations in the mRNA sample size, the study was designed as shown at the top of Figure 12. Specifically, the dynamic binding capacity of a 2,500 nt construct was determined on the first cycle. Then, 9 cycles were run using a dA40-mer instead of mRNA. At cycle 10, the DBC was measured again. This process was continued by loading a dA40-mer for the majority of the cycles, and every 10 cycles, the DBC was determined using the 2,500 nt mRNA construct. The box at the bottom of Figure 12 includes additional details about the two different samples used in the study.

The results of this second reuse study are presented in Figure 13. The data demonstrates consistent performance, meaning that there was no change in binding capacity for 70 cycles. All DBC values are within experimental error and assay variability limits.

Next, the cleaning stability of the POROS Oligo (dT)25 resin was tested with a variety of cleaning solutions (Figure 14). Resin samples were incubated in the following three solutions for up to 48 hours:

FIGURE 12

POROS Oligo (dT)25 reuse study: experimental summary.

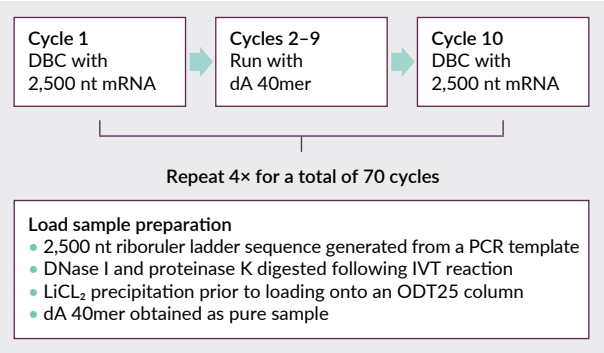
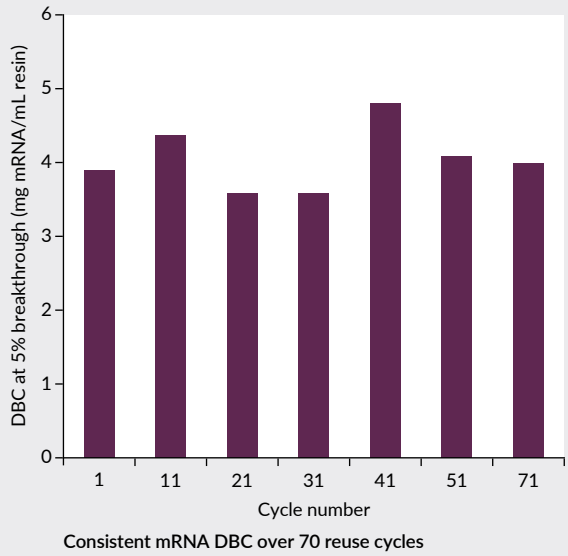


FIGURE 13

POROS Oligo (dT)25 reuse study results.



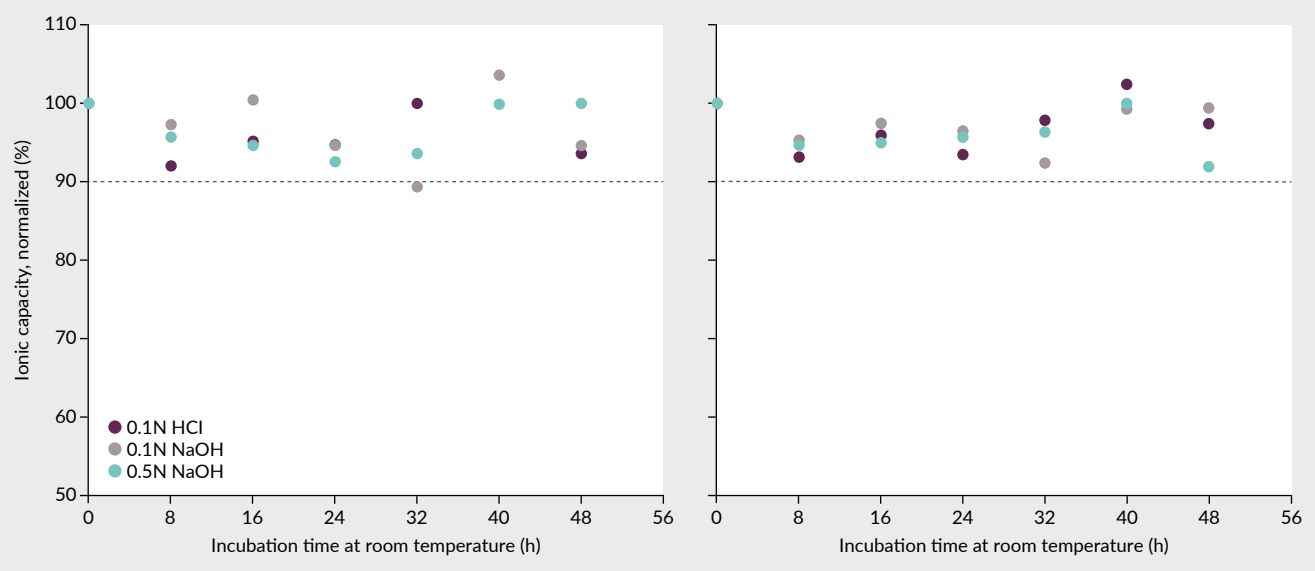
Consistent mRNA DBC over 70 reuse cycles

Column dimensions	0.5 cm D × 0.5 cm L
Resin	POROS Oligo (dT)25
Load sample A (DBC runs)	2,500 nt purified mRNA (0.25 mg/mL)
Load sample B (cycling runs)	dA40mer (0.1 mg/mL)

Step	Buffer	Res time (min)	CV
Equilibration	10 nM Tris, 1 mM EDTA, 0.8 M NaCl pH 7.4	1.0	5
Load A	2,500 nt purified mRNA	5.0	20
Load B	dA40mer	5.0	4
Wash 1	10 nM Tris, 1 mM EDTA, 0.8 M NaCl pH 7.4	1.0	5
Wash 2	10 nM Tris, 1 mM EDTA, 0.1 M NaCl pH 7.4	1.0	5
Elution	Water	1.0	5
CIP	0.1 mM NaOH	1.0	5
Re-equilibrate	10 nM Tris, 1 mM EDTA, 0.8 M NaCl pH 7.4	1.0	5

FIGURE 14

Cleaning and stability of POROS Oligo (dT)25 affinity resin utilizing a variety of cleaning solutions.



0.1 N HCl (pH at 1.1); 0.1 N NaOH (pH at 12.9); and 0.5 N NaOH (pH at 13.4). Both the resin's ionic capacity and the DBC using a 40 mer were tested after the treatments. The graphs in **Figure 14** are normalized to the time point 0 and show essentially no change in ionic capacity or dA40-mer binding capacity for the time period and conditions tested.

In most cases, end users utilize 0.–0.5 M NaOH for cleaning after every cycle and typically, the cleaning step takes 30–60 minutes. These data demonstrate the stability of the resin under such typical cleaning conditions,

FIGURE 15

Cleaning and stability of POROS Oligo (dT)25 affinity resin over 8 days.

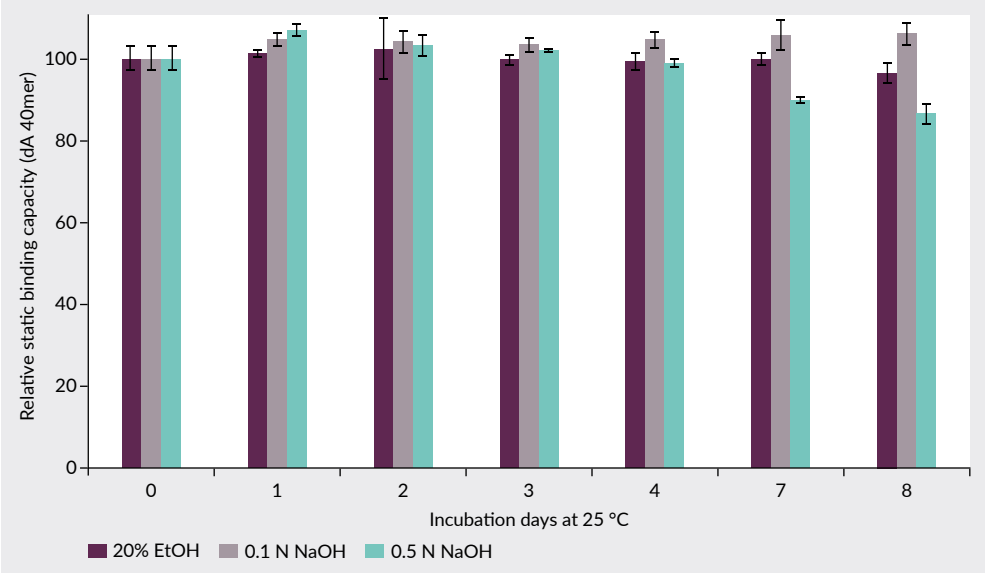
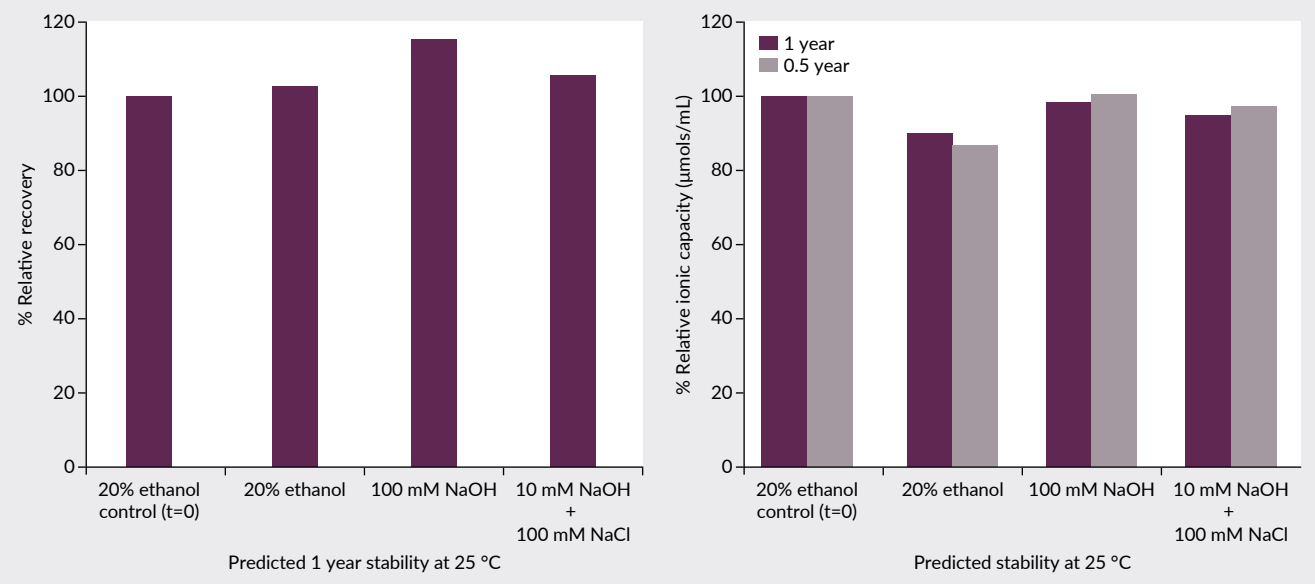


FIGURE 16

Long-term predicted stability in various storage solutions.



and can be extrapolated to the equivalent of approximately 96 cleaning cycles.

The study was then expanded to test the stability and performance of the resin after cleaning and storage in NaOH compared to ethanol over a longer period of time (up to 8 days). This particular study was important for demonstrating simplicity of use where employing ethanol cleaning and storage are challenges. The data in **Figure 15** shows that the resin has good caustic stability and can be cleaned and sanitized using alkaline conditions as an alternative to ethanol. Although a decrease was observed with 0.5 M NaOH, the number of sanitization cycles where no drop in static binding capacity was observed exceeded 200 cycles (assuming 48 × 30 min sanitization cycles per day). A higher cycle count could be achieved by using either a lower concentration of hydroxide or shorter cleaning steps.

To further investigate the resin's stability and performance after storage for longer periods of time, an accelerated stability study was performed **(Figure 16)**. High-temperature accelerated studies for a duration equivalent to 1 year of storage were performed at 25 °C. On the left of **Figure 16**, a 4,000 nt construct was bound and eluted from resin that was incubated in one of each of the following storage solutions:

- 20% ethanol
- 100 mM NaOH
- 10 mM NaOH plus 100 mM NaCl

Compared to 20% ethanol storage at t=0, mRNA recovery was consistent across all three conditions. The graph on the right shows that similar performance based on ionic capacity was observed in the three different storage conditions for both the equivalent of 6 months and 1 year at 25 °C.

These data support the use of POROS Oligo (dT)25 affinity resin for alternative storage conditions.

Q&A

Chantelle Gaskin

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

CG: No, you do not need heat to use the Oligo (dT). In some cases, though, an end user will opt to use heat for disruption of higher order structures and things of that nature. All of the studies discussed above were carried out at ambient room temperature, but Oligo (dT) is stable up to 65 °C.

Q Is the Oligo (dT) resin only available as loose resin, or do you have columns as well?

CG: We do have columns. We are stocked with 0.2 mL, 1 mL, and 5 mL pre-pack columns, as well as RoboColumns. In addition, we also just launched the 96-well plate formats for screening. The one thing that we do not carry is the analytical size columns, so if you are interested in that, you should reach out to your local technical sales specialist.

Q What sizes of RNA can be purified? Is there a construct size limit?

CG: There is no limit. We have tested various sizes in-house, including the 1,000–4,000 nt constructs I discussed today. We also have customers who have tested this resin with much larger constructs such as self-amplifying (sa)RNA constructs, for example. Though you can obtain high recoveries in these cases, we do recommend opting for a higher residence time that will help with binding capacity. Furthermore, as with any process, optimization is usually required, so I also recommend reaching out to your local field application scientist. They should be able to help with determining process conditions for those larger constructs.

Q Does the length of the poly(A) tail impact the capacity of the resin and the final purity?

CG: No, it does not. By far, the largest constructs we have seen have around a 100–120 mer poly(A) tail, and with that, we have seen consistent results specifically in terms of capacity and purity. The resin only has a 25 mer Poly(dT) ligand, which is enough to bind the construct. We have also seen some shorter tail constructs that perform decently with this resin.

Q Will POROS Oligo (dT)25 remove truncated mRNA from IVT reactions for longer constructs?

CG: If the poly(A) tail is damaged or missing, it will not bind. This would ensure the removal of truncated mRNA.

Q Is proteinase K treatment necessary?

CG: Proteinase K is not needed when using the POROS Oligo (dT) resin.

Q Does POROS Oligo (dT) remove double-stranded mRNA?

CG: The POROS Oligo (dT) will not remove double-stranded mRNA. It will bind the poly(A) tail, so some forms of double-stranded mRNA will bind and co-elute from the resin. There are some preliminary data showing that either hydrophobic interaction chromatography or anion exchange chromatography could be used as a polish step for removing double-stranded mRNA, but most customers tend to optimize the IVT reaction to reduce the production of double-stranded RNA instead.

Q Is POROS Oligo (dT) compliant with CGMP, and is it scalable?

CG: This resin is fully scalable, up to commercial scale. As far as CGMP goes, we do have regulatory support files that you can request from either your local technical sales representative or field application scientist. They can provide you with the documentation needed to support your regulatory process.

Q What is a typical lifetime for mRNA purification with the column?

CG: We have data showing little significant decrease in resin performance for >70 cycles. It is likely that you can reach a higher number of cycles than that, however, in the event that a sufficient cleaning protocol is established.

BIOGRAPHY

CHANTELLE GASKIN is a Field Applications Staff Scientist, specializing in protein and viral vector purification and downstream process development. She held leadership positions at Applied Genetic Technology Corporation and Brammer Bio, prior to joining the Thermo Fisher Scientific Bioproduction Division, Alachua, FL, USA in 2020. With over 10 years of experience in gene therapy, Chantelle has accumulated comprehensive knowledge of standard industry practices and regulatory standards, applying this knowledge to advance development of therapies for a variety of indications including ocular, CNS and systemic disease. Chantelle holds an MSc in Chemistry from University of Florida, Gainesville, FL, USA and a BSc in Chemistry from Smith College, Northampton, MA, USA.

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AUTHORSHIP & CONFLICT OF INTEREST

Disclaimer: Pharmaceutical grade reagent. For manufacturing and laboratory use only.

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