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# 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.

**TABLE 1**

**Methods of RNA purification.**

| Method | Advantages | Disadvantages |
|---|---|---|
| Reversed phase | ▶ High resolution<br>▶ Some selectivity for product impurities | ▶ Limited column capacity<br>▶ Use of expensive/flammable/toxic chemicals<br>▶ Column fouling impacts resolution |
| Ion exchange chromatography | ▶ Native purification possible<br>▶ Scalable | ▶ Column capacity and recovery (HPLC)<br>▶ May need toxic chemicals for denaturation<br>▶ Purified product can contain traces of elution salts |
| Size exclusion chromatography | ▶ Native purification possible | ▶ Separation efficiency affected by alternative folding<br>▶ Flow limited |
| HIC | ▶ Native purification possible<br>▶ Scalable<br>▶ Replacement for reversed phase | ▶ Non-selective |
| Affinity chromatography | ▶ Native purification possible<br>▶ Scalable<br>▶ 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.


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## 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 market faster, and decreasing the overall cost of goods.

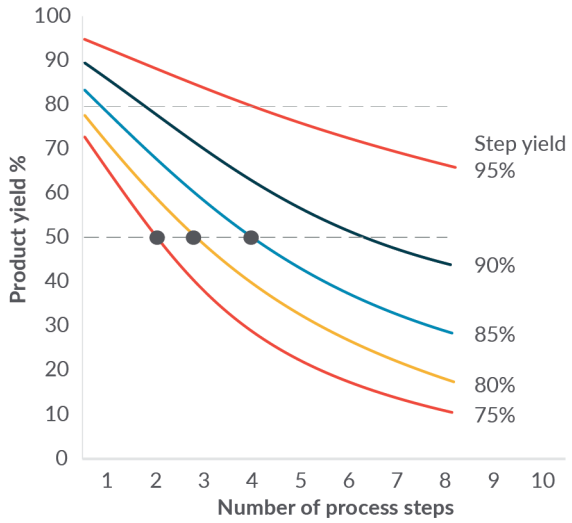
## THE THERMO SCIENTIFIC POROS™ OLIGO (dT)25 AFFINITY RESIN

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.

**FIGURE 1**

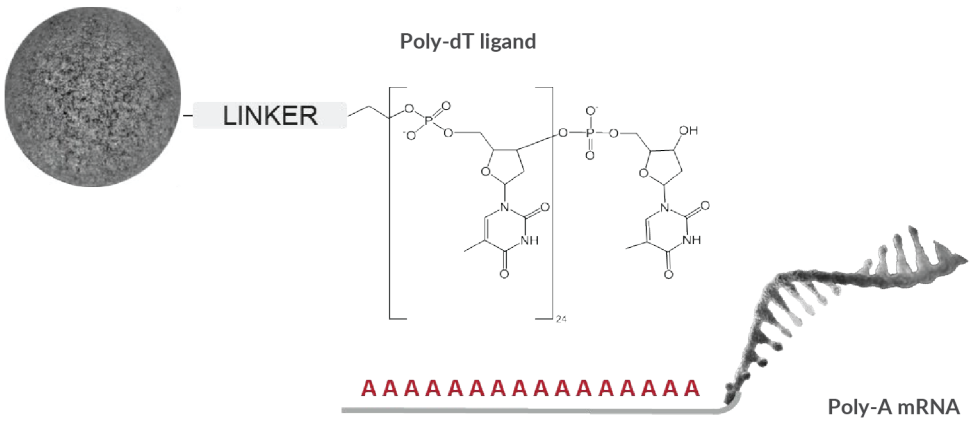
**Product yield declines with increasing number of process steps.**




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.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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.

## THE POROS™ BEAD

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

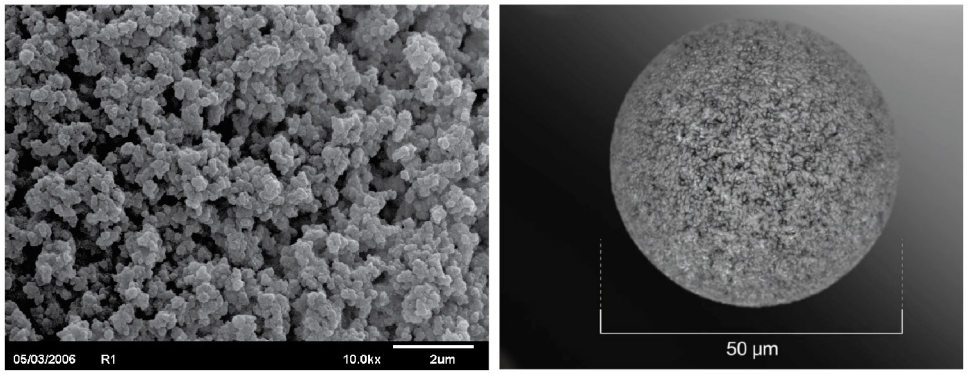
1. **Poly(styrene-co-divinylbenzene) backbone.** The beads are rigid and incompressible compared to agarose type resin. This results in stable column beds as well as linear pressure-flow profile over a wide range of column dimensions, allowing the user to maintain high operational flow rates with a modest 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.

**FIGURE 3**

**Scanning electron microscope images showing a POROS™ bead (left) and the large through-pores of the bead surface (right).**



## 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 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

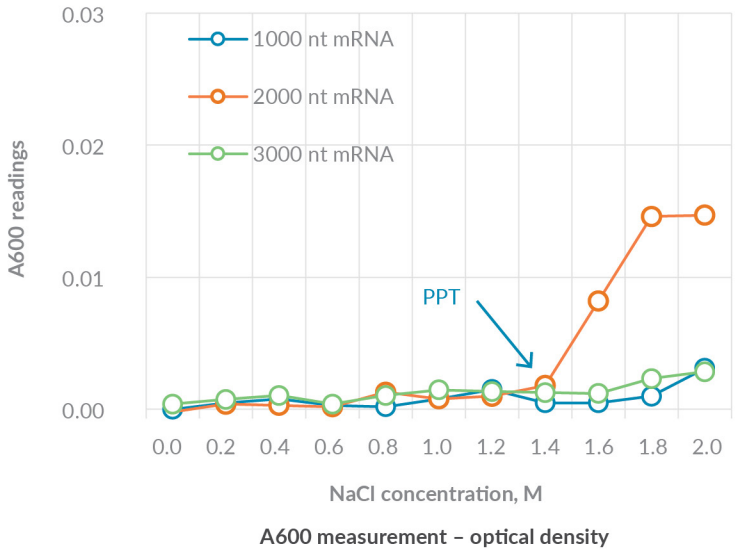
The goal of process development was to first understand how a range of mRNA molecules behaved, in order to more effectively optimize binding capacity without impacting the mRNA. Utilization of a high throughput screening approach allowed rapid optimization over a range of conditions. Once favorable conditions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

**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.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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.

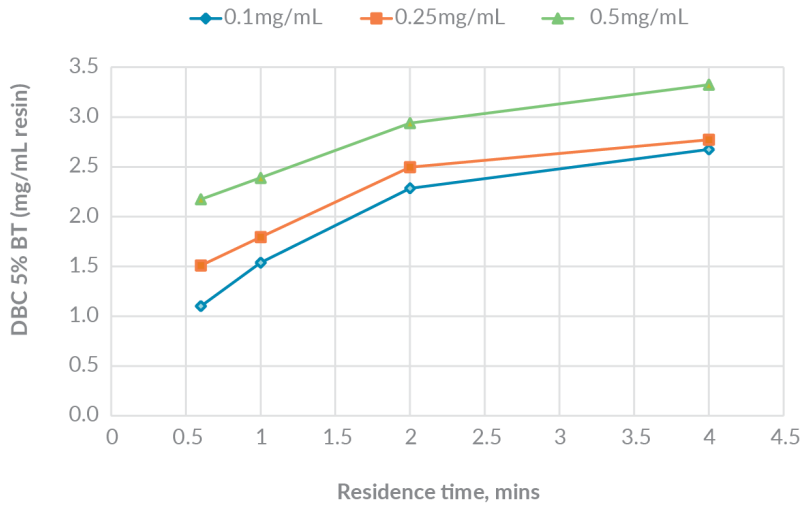
When considering buffer choice, the impact of binding across a range of pH in Tris buffer was studied. Again, optimal binding conditions were not consistent across the range of mRNA sizes used. These differences can be used to further optimize later column experiments, which will in turn assist in optimizing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

**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.

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.

## 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.

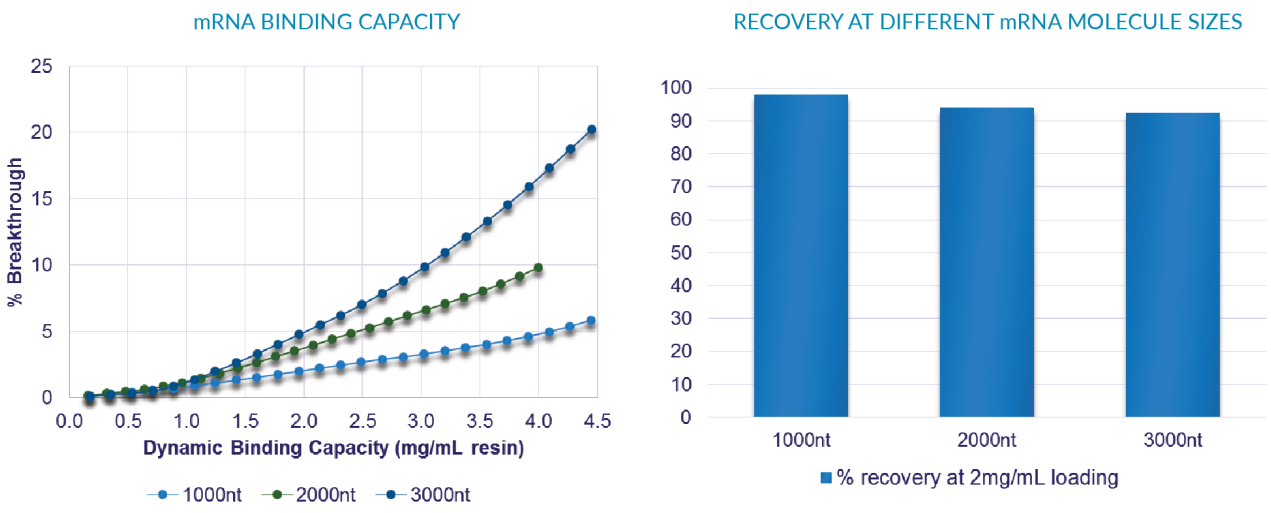
## REUSE, CLEANING & STABILITY OF THE OLIGO (dT)25 AFFINITY RESIN

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.

## FIGURE 6

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




mRNA molecule size impacts binding capacity but not final recovery

Smaller mRNA has a higher binding capacity (left) but size does not impact final recovery (right).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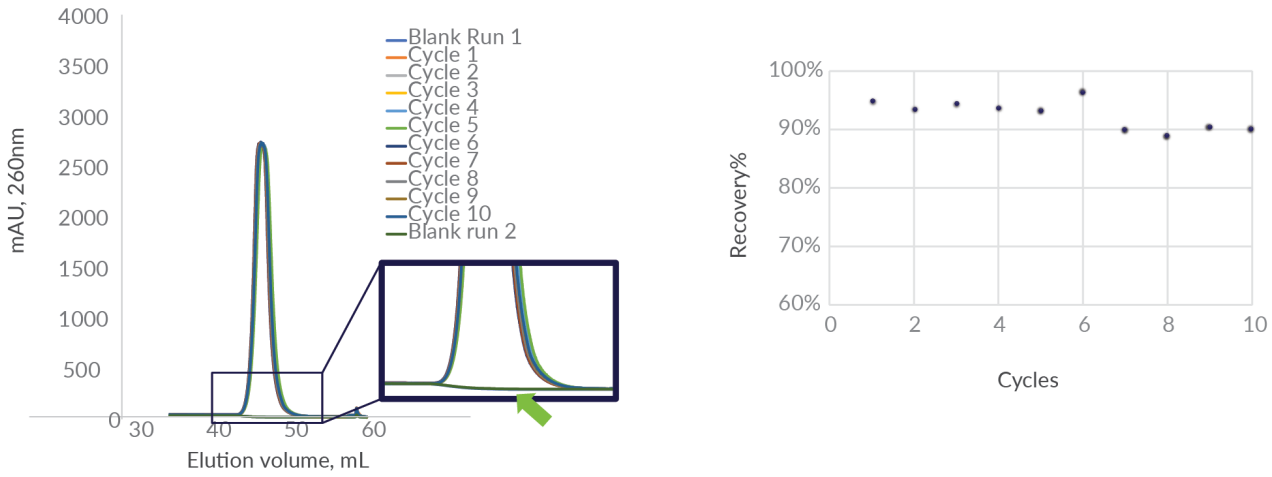
## FIGURE 7

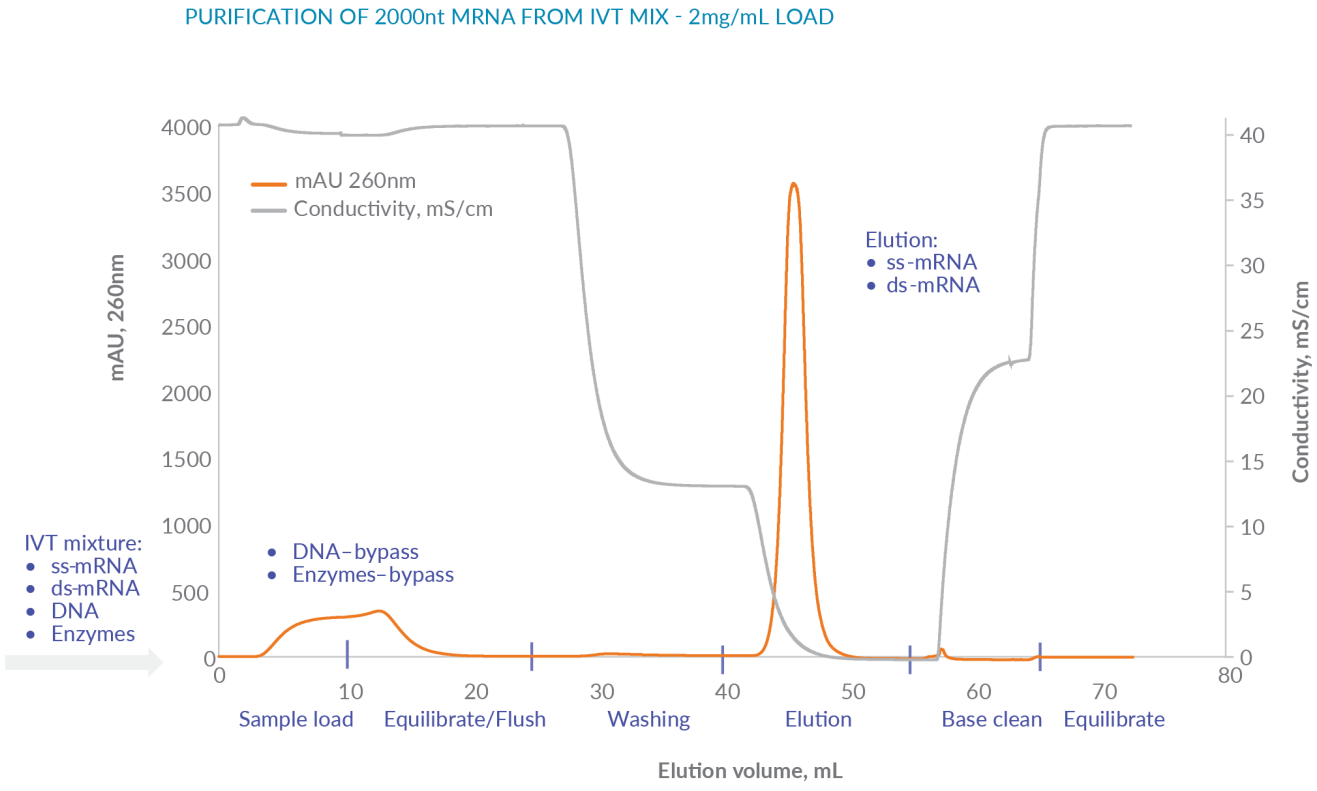
**Effect of resin reuse and cleaning on mRNA purification.**




**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

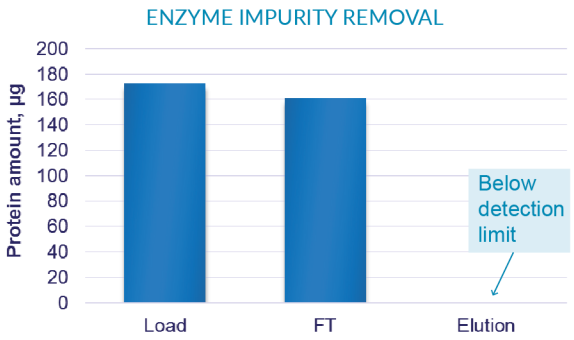
**Output of a chromatographic purification run.**




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.

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).

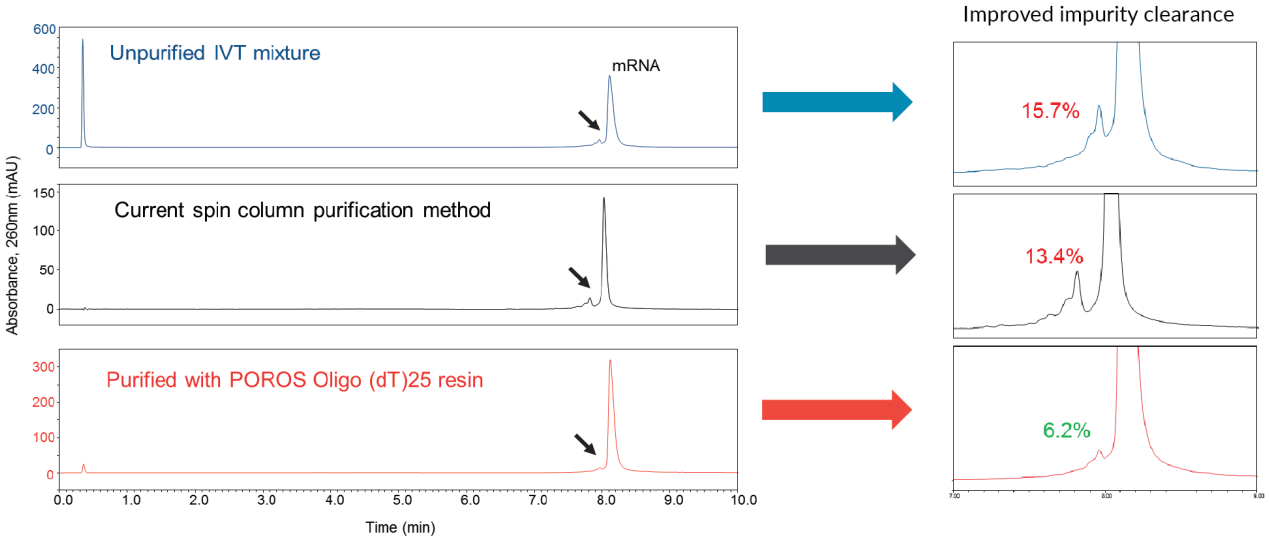
## PURIFICATION VERIFICATION

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

## 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.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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.

## 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.

# Q & A

**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.


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**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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