CELL & GENE THERAPY INSIGHTS

LIVE30 TRANSCRIPT

Optimizing mRNA purification conditions by using a high-throughput screening approach

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To support the development of mRNA-based therapies, Thermo Fisher Scientific has developed a platform chromatography solution for the purification of mRNA. The POROS™ Oligo (dT)25 Affinity Resin helps to address the selectivity and capacity requirements for the large-scale manufacturing of mRNA used in vaccine and gene therapy applications. Typically, mRNA binds to the Oligo (dT)25 affinity resin using high ionic strength conditions and neutral pH and is eluted from the column using low ionic strength solutions such as water. Although water works well for most mRNA constructs, a need to identify alternative elution buffers to optimize mRNA recovery exists. This article describes experiments conducted to optimize mRNA purification using high-throughput screening.

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RNA THERAPEUTICS & PURIFICATION

Synthetic mRNA has diverse applications, including cancer immunotherapy, vaccines, allergy tolerization, protein replacement, gene editing, and genetic reprogramming. Methods for mRNA delivery include direct injection, *ex vivo* injection of the transfected cells, and transfection of genome editing enzymes. These diverse applications and delivery

methods require a robust and easy-to-use purification platform.

The approval of an mRNA COVID vaccine in 2021 – the first approved vaccine against the virus – accelerated research into mRNA therapeutics for all applications. Despite the ever-growing list of applications for mRNA therapeutics, many challenges remain in mRNA purification. Current chromatography methods for RNA purification are detailed in Table 1.

mRNA capture is the critical first step in the downstream process before moving on to the polish, formulation, and fill and finish steps. The POROS Oligo (dT)25 affinity resin can be used as the capture step in the purification of mRNA in the downstream workflow to remove process-related impurities and some product-related species.

POROS OLIGO (dT)25 AFFINITY RESIN

The POROS Oligo (dT)25 resin has been specifically designed for the purification and isolation of mRNA from the in vitro transcription (IVT) reaction. The mRNA is captured through AT base pairing and contains a dT-25 (poly-deoxythymidine) ligand with a propriety linker attached to a 50 µm POROS™ bead. The resin can achieve a dynamic binding capacity of up to 5 mg/ml for a 4000 nucleotide (nt) mRNA with greater than 90% recovery. The resin also has excellent scalability and does not contain animal-derived components.

The POROS Oligo (dT)25 resin has three key attributes that differentiate it from other chromatography resins. First, the bead is made of poly(styrene-divinylbenzene), a rigid material that provides a linear relationship between pressure and flow on packed columns. This permeability is independent of column diameter, which facilitates scalability and enables the use of high flow rates with moderate pressure drops. Additionally, due to the polymeric nature of the backbone and the robust covalent chemistries of the beads, the resin shows physical and chemical stability from pH 1 to 14. This enables the use of standard cleaning solutions like 0.5 M NaOH, to meet resin lifetime targets.

The second attribute is the large pore structure that results in reduced mass transfer resistance. This is particularly important for large biomolecules like mRNA which diffuse into the bead at longer residence times.

The third attribute is the 50 µm average particle size. The relatively small beads allow for band broadening in packed beds which translates into smaller elution pool volumes.

TABLE 1

Chromatography methods of RNA purification.

Method	Advantages	Disadvantages
Reversed phase	▶ Native purification possible ▶ High resolution ▶ Some selectivity for product impurities	▶ Limited column capacity ▶ Use of expensive/ flammable/toxic chemicals
Ion exchange chromatography (IEX)	▶ Native purification possible ▶ Scalable ▶ Some selectivity for product impurities	▶ High pH may be needed for elution ▶ May need toxic chemicals for denaturation
Hydrophobic interaction chromatography (HIC)	▶ Native purification possible ▶ Scalable ▶ Potential replacement for reversed phase	▶ High salt concentration needed for binding may compromise stability ▶ Unproven approach for mRNA purification
Affinity chromatography	▶ Native purification possible ▶ Scalable ▶ Platform solution for a wide range of mRNA molecule sizes – selective to poly(A)	▶ Requires additional polishing step to remove product-related impurities

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TABLE 2

Experimental summary.

Resin volume	20 µL
Column load density	1 mg/mL of resin
Equilibrium buffer	10 mM Tris, pH 7.4, 1.0 mM EDTA, 0.8 M NaCl
Wash buffer	10 mM Tris, pH 7.4, 1.0 mM EDTA, 0.1 M NaCl
Elution buffer	Variable

FINDING AN ALTERNATIVE ELUTION BUFFER

Experimental summary

The objective of this study was to determine an alternative elution buffer for the POROS Oligo (dT)25 resin that would have similar or better recovery than water. Traditional methods have shown that mRNA binds to the affinity resin using high ionic strength conditions and is eluted from the column using low ionic strength solutions like water. Although water works well for most mRNA constructs, alternative elution buffers are necessary when water does not result in sufficient recovery or stability of the RNA.

A high-throughput screening (HTS) approach was implemented to test various elution buffer conditions on a 96-well plate format. An automated liquid handler instrument was used to execute the experiments. The purified mRNA sample was diluted with the equilibration buffer before loading onto the resin. The purified, 1000 nt mRNA sample was incubated on the resin for 1 hour at room temperature while shaking at 1000 rpm. After incubation, the resin was washed with equilibration buffer and a low-salt buffer. The elution buffer was varied to study the effect on recovery. The absorbance at 260 nm was used to quantify the eluted sample. A summary of the experimental conditions used in this study is shown in Table 2.

The buffers used in the study were chosen to evaluate various pH levels and ionic strengths to determine an alternative elution buffer to RNase-free water and included:

- 1 and 5 mM citrate with and without EDTA at pH 5 and 6
- 5, 10, and 25 mM Tris with 1 mM EDTA at pH 7 and 8
- 1 and 5 mM citrate in combination with 5, 10, and 25 mM Tris

Results

Comparing citrate-only elution buffers to RNase-free water, lower recoveries were observed for citrate buffer at pH 5, independent of the ionic strength or presence of EDTA. Comparable recoveries to water were observed for citrate at pH 6, with similar recoveries for 1 mM and 5 mM citrate. Interestingly, the addition of EDTA to 5 mM citrate pH 6.0 buffer resulted in a 25% decrease in recovery. The superior elution recovery of citrate at pH 6 could be explained by the charge of the citrate molecule, which has a pKa of ~6.

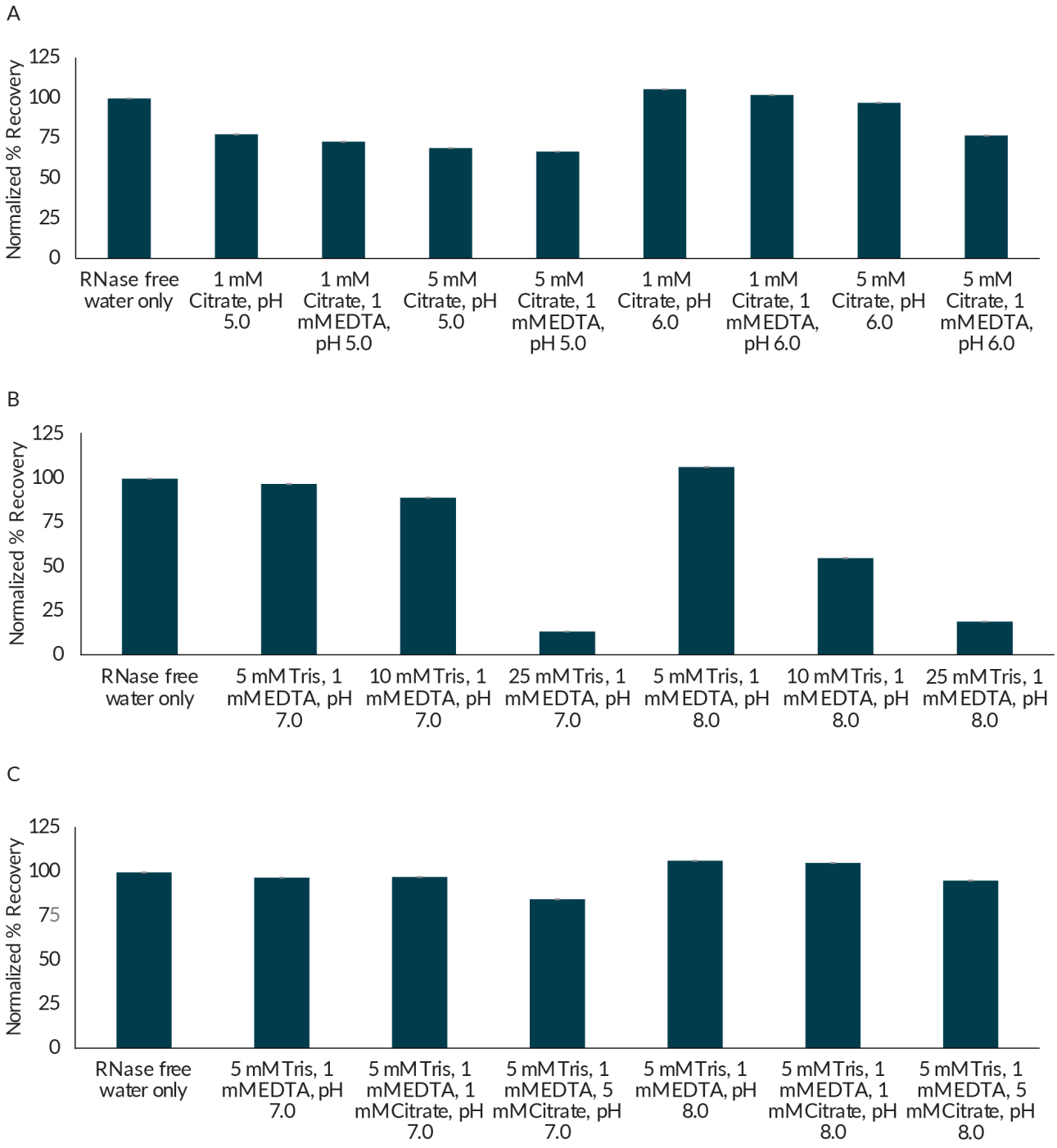
In observing the recoveries from Tris elution buffers only, Tris buffer at pH 7 showed similar recovery for 5 and 10 mM Tris, though a large decrease in recovery was observed for 25 mM Tris. At pH 8, 5 mM Tris resulted in the greatest recovery. An increase in ionic strength from 5 to 10 mM Tris resulted in a 50% loss in recovery. Even poorer recovery was observed for 25 mM Tris.

When citrate buffer was added to 5 mM Tris (Figure 1A), it was found that the combination of 5 mM Tris with 1 mM citrate does not have a significant impact on recovery, while the combination of 5 mM Tris with 5 mM citrate at pH 7.0 has the lowest recovery in comparison to RNase-free water.

In contrast, the addition of citrate to 10 mM Tris buffer had a notable impact on the elution recovery (Figure 1B). The addition of citrate to 10 mM Tris at pH 7.0 resulted in

FIGURE 1

Recovery results for Tris and citrate elution buffers in combination.



(A) Comparison of 5 mM Tris with citrate elution buffers. (B) Comparison of 10 mM Tris with citrate elution buffers. (C) Comparison of 25 mM Tris with citrate elution buffers.

a decrease in recovery, while the addition of citrate to 10 mM at pH 8.0 resulted in increased recovery, with 1 mM citrate having the best recovery relative to RNase-free water.

Tris buffers with a concentration of 25 mM showed the poorest recovery relative to RNase-free water (Figure 1C). In this case, the addition of citrate to the Tris buffer

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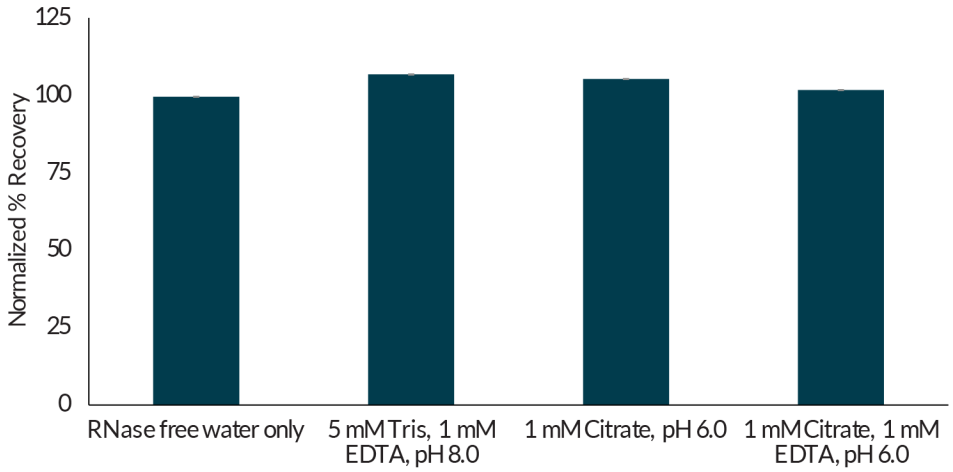
FIGURE 2

Best performing alternative elution buffer candidates from HTS.



did not show significant improvements in recovery.

The best performing alternative elution buffers from the HTS experiments are shown in Figure 2. These data show that the presence of EDTA does not have an impact on the elution recovery for citrate buffers. However, some improvement in recovery could be made with the addition of 1 mM citrate to a low-recovery elution buffer.

Column verification

The results from the HTS experiments were verified by testing the alternative elution buffers with column runs. Pre-purified, 1000 nt mRNA was diluted in equilibration buffer before loading onto a 1 mL pre-packed PO-ROS™ Oligo (dT)25 column. The column load density was 2 mg of RNA per mL of resin, and the sample load concentration was 0.25 mg/ml. The conditions of the experiment are shown in Table 3.

In the mL column verification runs, water showed the best recovery at 100%. This was followed by 1 mM citrate at pH 6, and 1 mM citrate plus 1 mM EDTA at pH 6 with an 89 and 88% recovery, respectively. The results confirm that no difference in recovery was observed with the addition of EDTA to the 1 mM citrate buffer. The lowest recovery of 84% was observed for 5 mM Tris plus 1 mM EDTA at pH 8.0.

To test if the recovery could be improved for the alternative elution buffers,

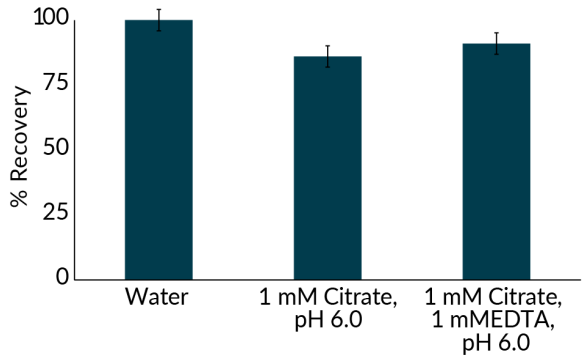
TABLE 3

Column experimental summary.

Step	Buffer	Column volumes	Residence time (min)
Equilibration	10 mM Tris pH 7.4, 1mM EDTA, 0.8 M NaCl	10	1.0
Load	1000 nt RNA	–	5.0
Wash 1	10 mM Tris pH 7.4, 1 mM EDTA, 0.8 M NaCl	15	1.0
Wash 2	10 mM Tris pH 7.4, 1 mM EDTA, 0.1 M NaCl	15	1.0
Elution	Variable	15	1.0
CIP	0.1 M NaOH	5	1.0
Re-equilibrate	10 mM Tris, 1mM EDTA, 0.8 M NaCl	5	1.0

FIGURE 3

Impact of elution buffer on recovery of a 2500 nt mRNA.



the residence time was increased from 1 to 2 min for the low-salt wash and elution steps. The same conditions as the previous runs were used. Comparable recoveries were observed for alternative buffers independent of residence time. Therefore, all three alternative elution buffers can be used to purify a 1,000 nt molecule with a 90% or greater recovery.

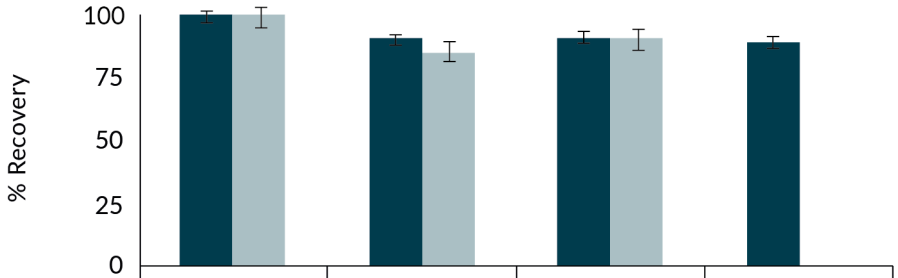
To further assess the alternative elution buffers, the recoveries of a 2,500 nt mRNA construct using these eluents were tested. The same sample preparation was carried out for these experiments, in which a purified 2,500 nt mRNA molecule was diluted to 0.25 mg/ml in equilibration buffer before loading onto a 1 mL prepacked column at a 2 mg/ml column density. After the sample load, no peak was observed in the equilibration wash step, but a small peak was observed in the low-salt wash. After washing with low salt, a narrow elution peak was observed with high absorbance, followed by a small peak in the CIP fraction.

When comparing the recovery of a 2,500 nt mRNA molecule for the POROS Oligo (dT)25 resin with various elution buffers, the broadest elution peak was observed in the presence of EDTA for the citrate buffer. It is hypothesized that EDTA causes a conformational change in the mRNA molecule that may result in entrapment in the pores of the resin and would require a greater elution volume to remove it from the resin. However, more studies would need to be done to investigate this phenomenon further.

A summary of the recoveries of a 2,500 nt mRNA molecule as a function of elution buffer from the POROS Oligo (dT)25 resin is shown in Figure 3. Citrate buffers can serve as alternative elution buffers with comparable recoveries in the absence and presence of EDTA for a 2,500 nt mRNA construct.

FIGURE 4

Most successful alternative elution buffers to RNase-free water for various mRNA sizes.



	Water	1 mM Citrate, pH 6.0	1 mM Citrate, 1 mM EDTA pH 6.0	5 mM Tris, 1 mM EDTA pH 8.0
1000nt	100	91	92	90
2500nt	100	86	91	Not tested

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CONCLUSIONS

In conclusion, low concentration citrate can serve as an alternative elution buffer to RNase-free water for various mRNA constructs. **Figure 4** shows there are comparable recoveries with 1 mM citrate pH 6 elution buffer for 1,000- and 2,500 nt mRNA molecules. Similar recoveries are observed for both size constructs for 1 mM citrate, 1 mM EDTA, pH 6. The elution buffer consisting of 5 mM Tris, 1 mM EDTA, pH 8 also showed good recovery for the 1,000 nt mRNA molecule and can be another alternative.

Based on this work 1 mM Citrate, pH 6.0 would be the recommended buffer as an alternative elution buffer to RNase-free water for various mRNA sizes. The addition of EDTA to the citrate buffer resulted in a larger elution pool with similar recoveries.

Q&A with Jenny England

Jenny England (Thermo Fisher Scientific) answers your questions on mRNA purification with POROS Oligo (dT)25

JENNY ENGLAND is a Staff Scientist in the Applications and Innovation group in Purification and Pharma Analytics at Thermo Fisher Scientific. Jenny is a biophysicist by training and earned her PhD from Georgetown University. After graduate school, she did a post doc at the National Cancer Institute that focused on structure-based drug design for protein kinase complexes. Jenny currently leads the application group for process development of the POROS resin products for antibody, mRNA, plasmid, and viral vector purification. Additionally, Jenny evaluates new and emerging technologies that can be applied to solve unmet customer needs in the bioproduction workflow.

Q What impurities remain after the Oligo (dT) purification, and what would you recommend as a polishing step for further removal of these impurities?

JE: Although the POROS Oligo (dT)25 resin works well in removing process-related impurities from the IVT reaction, it does not separate single-strand (ss) RNA from double-stranded (ds) RNA, if present in the load sample. dsRNA is the major impurity after affinity capture, and we would suggest including an additional polishing step such as ion exchange or hydrophobic interaction chromatography for further purification.

Q Why wasn't the 5mM Tris, 1mM EDTA, pH 8 elution buffer tested for the larger mRNA construct? Would you recommend this as an alternative elution buffer?

JE: We tested it for the 1,000 nt mRNA, but we did not move it into the 2,500 nt due to the peak broadening effect that we observed in the elution step, and therefore we recommended 1 mM citrate as an alternative elution buffer to water. However, this could be specific to your mRNA molecule, and it could be tested if citrate does not work well for your recovery needs.

Q Would you say that pH or ionic strength has a stronger impact on mRNA elution and recovery?

JE: This is dependent on the elution buffer used. For citrate, we saw that there was a greater effect of citrate between pH 5 and 6. For Tris, we saw a greater effect on ionic strength, where the 25 mM Tris had the lowest recovery.

It is important to keep in mind that these results may be dependent on the specific mRNA construct to be tested and are intended only as a guide.

Q Is there a minimum or maximum length of mRNA that the POROS Oligo (dT)25 resin can work with?

JE: The POROS Oligo (dT)25 resin works across a variety of mRNA lengths. We are always looking to work with customers to generate data with large mRNA constructs and have worked with customers on the purification of large self-amplifying mRNA molecules.

However, keep in mind that you will optimize the POROS™ Oligo (dT)25 purification to your specific mRNA molecule, and you may observe differences in binding capacity as a function of size.

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AUTHORSHIP & CONFLICT OF INTEREST

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