Increasing Dynamic Binding Capacity of Oligo(dT) for mRNA Purification

Experimental Results Using CIM 96-Well Plates

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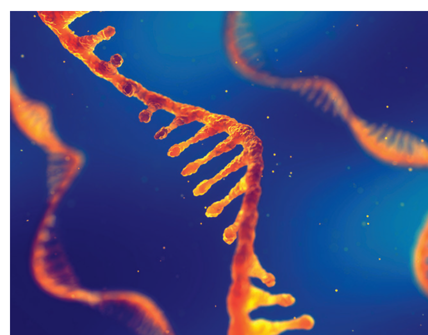
Messenger RNA (mRNA) has emerged as a powerful therapeutic tool for treatments in gene therapy, oncology, infectious diseases, and (as was recently demonstrated) vaccines such as those against SARS-CoV-2 during the COVID-19 pandemic. The synthetic process for mRNA production can be designed and scaled up rapidly in a platform that is highly adaptable to different targets. One major challenge in such manufacturing operations is the removal of process-related impurities stemming from the in vitro transcription (IVT) reaction. Those include residual nucleoside triphosphates, DNA templates, enzymes, abortive transcripts, and double-stranded RNA.

Affinity Purification of mRNA Using Oligo(dT)

Affinity-based chromatographic isolation of mRNA is a robust and straightforward technique that is useful as an industrial platform. An mRNA construct contains a 3' polyadenylic acid (polyA) tail to increase in vivo stability. That enables the use of affinity purification with oligo-deoxythymidinic acid (oligo(dT)) probes that are covalently coupled to solid supports (**1**). Polyadenylated mRNA forms a stable hybrid with oligo(dT) under high-salt conditions, and that can be destabilized when the salt is removed, allowing for mRNA to be released (Figure 1).



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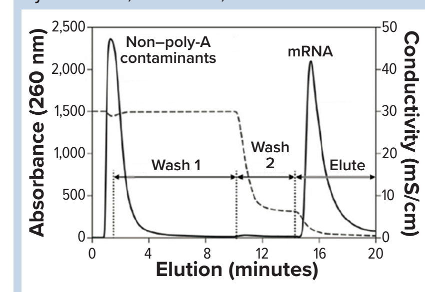
A number of oligo(dT) chromatographic resins are available commercially, differing not only in resin type and bead size, but also in ease of use, binding specificity, and binding capacity. Stationary phases that use convective mass transfer — such as CIM monoliths from BIA Separations (Sartorius) — are better suited for large biomolecules, such as mRNA. Spanning a molecular-weight range of 400 kDa – 2 MDa, mRNA molecules are not well suited to porous particle purification because of their slow diffusion and large size.

Macroporous polymethacrylate monoliths offer high binding capacity and resolution for mRNA through their convective and interconnected flow-through channels (>1.3 µm), which are modified with appropriate ligands as binding sites that are easily accessible for mRNA (**2**). Low shear forces coupled with chemically inert mobile phases used for affinity purification prevent degradation through chemical and/or enzymatic activity, which may take

Figure 1a: Representative chromatogram for purification of mRNA on a CIMmultus oligo(dT) column — reproduced with permission from Gagnon P. *Purification of Nucleic Acids.* BIA Separations: Ajdovščina, Slovenia, 2020

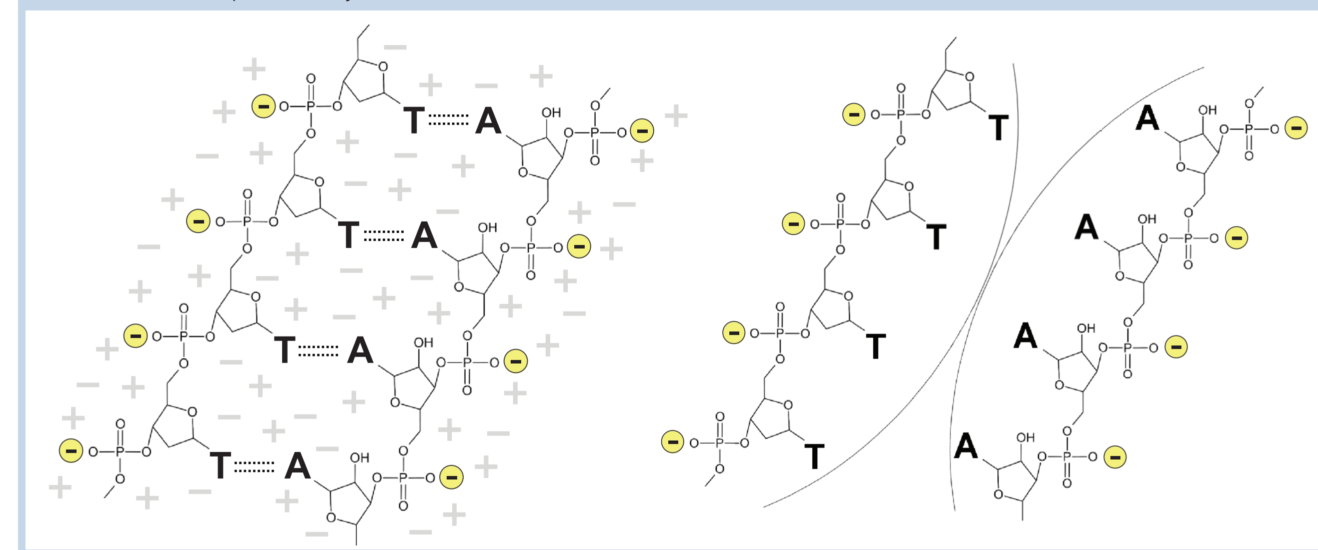


place when other purification means are used. Stability of oligo(dT)-purified mRNA drug substance has been shown at 37 °C for at least one month — by contrast with precipitation techniques that lead to degradation of mRNA (**3**).

The Question of Capacity

With increasing productivity of IVT reaction protocols, which routinely reach 5–10 mg/mL, elucidation of conditions that can increase binding capacity of oligo(dT) has been an intense focus of many developers (**4**). Depending on construct length, maximum binding capacities of 3–4 mg/mL have been reported (**5**). Caution is advised in interpreting literature data, however; some recovery studies have been interpreted erroneously as binding capacity data (**6**), leading some people to underestimate the binding

Figure 1b: Binding interactions between mRNA and oligo(dT) molecules — reproduced with permission from Gagnon P. *Purification of Nucleic Acids.* BIA Separations: Ajdovščina, Slovenia, 2020

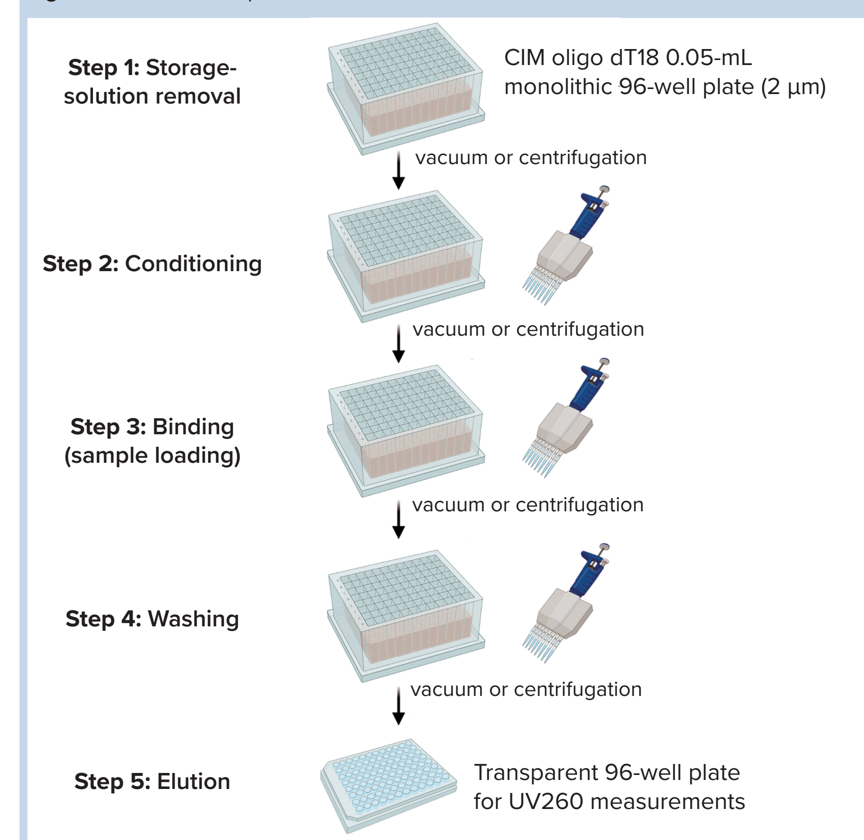


capacity of selected oligo(dT) products by several orders of magnitude.

Typical capacity for CIMmultus oligo(dT) for mRNA is 2–4 mg/mL, depending on construct length and loading concentration of NaCl. To further improve that capacity, users can apply multiparallel approaches to reduce development time by screening multiple conditions at once in a selected chromatography matrix. CIM 96-well plate oligo(dT) was developed to facilitate a smooth workflow for optimization of binding conditions to oligo(dT). CIM 96-well plates can be operated easily with a vaccum manifold for 96-well plates, a centrifuge compatible with 96-well plates, or a positive-pressure pump (Figure 2). The process can be scaled up to preparative scale using, for example, CIMmultus products operated by chromatographic skids.

96-Well Plate-Based Screening of Factors to Increase Dynamic Binding Capacity (DBC): We performed screening experiments in CIM 96-well oligo(dT) format to elucidate factors that affect CIMmultus oligo(dT) binding capacity for mRNA. For model systems, we used mRNA constructs of 995 nt (encoding for eGFP) and 4,000 nt (encoding a proprietary sequence). Table 1 lists the factors tested in a custom two-level screening on eGFP mRNA. Because we needed a design that enabled identification of main factors and some second-order interactions to perform 84

Figure 2: CIM 96-well plate workflow



experiments with a predefined combination of listed factors, the 96-well oligo(dT) plate was a suitable format for multiparallel testing of conditions.

We used a multiple-regression model to fit our experimental results and refined them with a backward-selection approach using a *p*-value threshold of <0.05. A simplified model identified NaCl, guanidine hydrochloride (Gu-HCl), and $MgCl_2$ concentration as the key factors contributing to DBC. Buffer chemistry and pH, salt type, and mRNA concentration had little or no effect on DBC, so we excluded those parameters from further evaluation (Figure 3). The effect of NaCl on binding capacity of oligo(dT) for mRNA already has been described (**5**). We found the effect on

Figure 3: Prediction graphs of factors with significant contribution to oligo(dT) dynamic binding capacity (DBC)

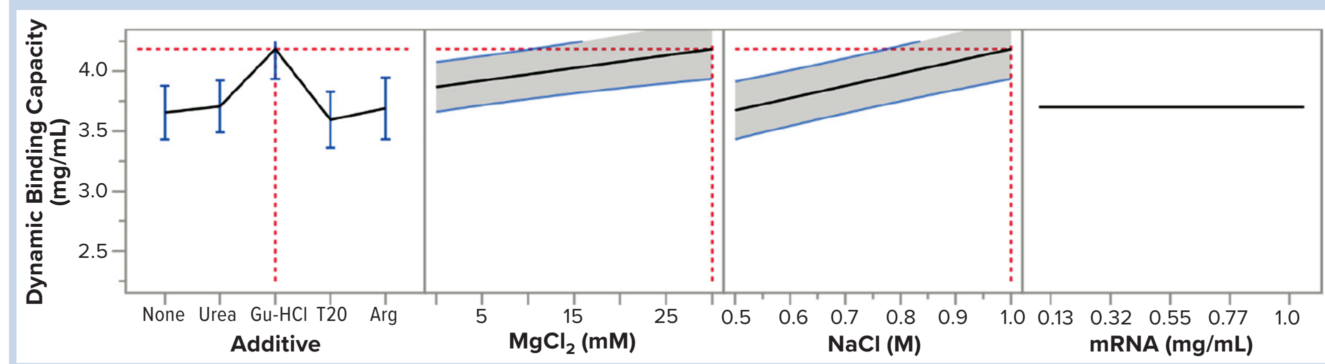
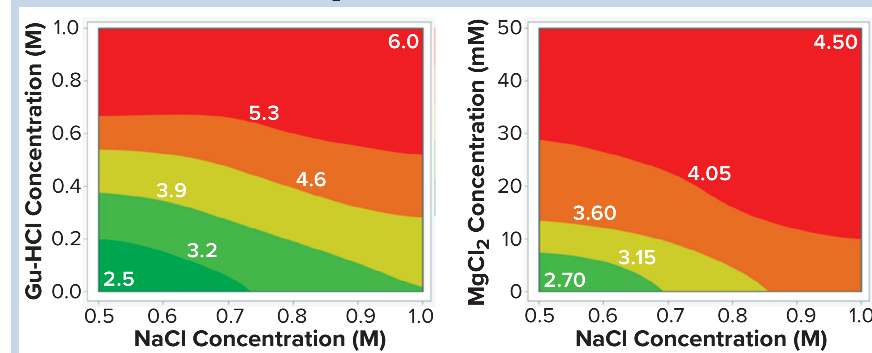


Table 1: List of factors evaluated for their contributions to the binding capacity of an oligo(dT) monolith chromatography support

Factor	Variable
Buffer type	Tris, phosphate
Buffer pH	7.2, 7.6, 8.0
Salt type	NaCl, KCl, CH_3COONa
Salt conc.	0.5 M, 0.75 M, 1 M
Additive	None, urea, Gu-HCl, arginine, and Tween-20 (T20)
$MgCl_2$ conc.	0 mM, 15 mM, 30 mM
mRNA conc.	0.25 mg/mL, 0.5 mg/mL

Figure 4: Contour plots of oligo(dT) dynamic binding capacity (DBC) with mRNA loaded in Gu-HCl–NaCl (LEFT) and $MgCl_2$–NaCl (RIGHT) combinations



DBC of both Gu-HCl and $MgCl_2$ to be greater than that of NaCl.

Guanidinium and Mg^{2+} Effects in Combination with NaCl on DBC: In a second set of experiments, we evaluated the combinatorial effects of Gu-HCl–NaCl and $MgCl_2$–NaCl in 96-well format. Consistent with initial screening results, loading buffer containing Gu-HCl combined with NaCl (in 50 mM Tris at pH 7.5) positively influenced DBC, with the effect of Gu-HCl being significantly more pronounced. A binding capacity of 6 mg/mL was reached with multiple combinations of Gu-HCl and NaCl (Figure 3, left panel). Precipitation of mRNA was observed with >1 M concentration for both. The effect of Mg^{2+} was also positive, though less pronounced, with a capacity of 4.5 mg/mL reached for $MgCl_2$ and NaCl concentrations of 30 mM and 1 M, respectively (Figure 4, right panel).

Contour plots of the Gu-HCl–NaCl combination indicate a significantly more pronounced effect on DBC of Gu-HCl than NaCl, so next we titrated Gu-HCl alone as a loading salt. A dose response was observed between 0.1 M and 1.0 M Gu-HCl, with 6.4 mg/mL binding capacity achieved with 1 M Gu-HCl used as a loading salt (Figure 5).

We also tested the effect of Gu-HCl on a larger mRNA construct (4,000 nt). A significant but smaller increase in DBC was observed in comparisons of 1 M NaCl (to 2.9 mg/mL) and 0.75 M Gu-HCl (to 3.8 mg/mL) as loading salts (Figure 5).

Note that with Gu-HCl concentrations over 1 M, mRNA precipitated when it was present at 1 mg/mL. Diluting the mRNA to 0.25 mg/mL prevented that occurrence.

Scaling Up 96-Well Plate Output: Next we transferred the effect of Gu-HCl to chromatographic separations using a CIMmic oligo(dT) column of 0.1-mL bed volume. We diluted 800 µg mRNA in binding buffer containing 0.75 M Gu-HCl to a final concentration of 0.16 µg/µL, then loaded that solution on the column at 5 CV/min. Recovery in the elution fraction was 550 µg, corresponding to a binding capacity of 5.5 mg/mL (Figure 6).

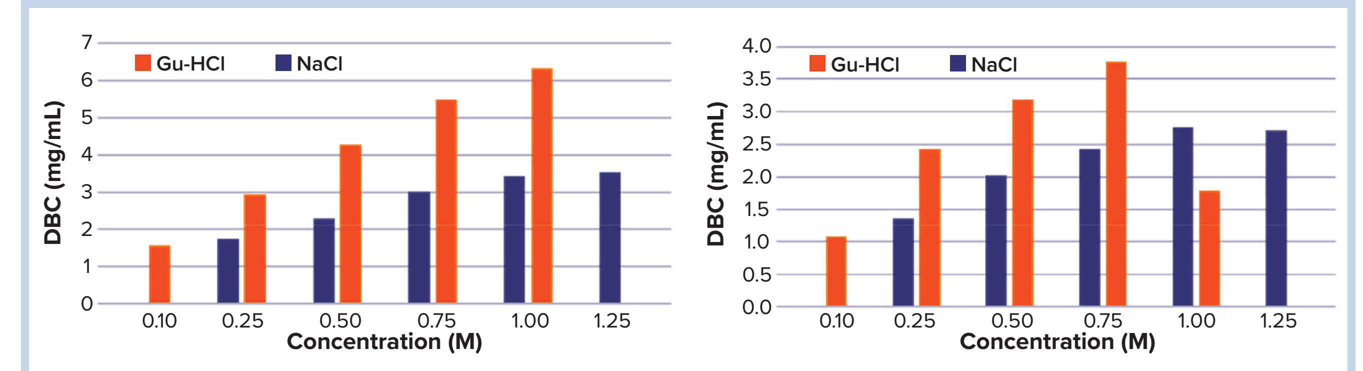
Finally, we performed a scale-up run by loading eGFP mRNA diluted in 0.75 M Gu-HCl onto a 1-mL CIMmultus oligo(dT) column. A binding capacity of 5.5 mg/mL was determined by UV content measurement of the elution fraction.

Additives Make the Difference

We used CIM oligo(dT) 0.05-mL monolithic 96-well plates for multiparallel screening of binding conditions to the oligo(dT) monolith. Three main contributing factors to DBC were identified: NaCl, Gu-HCl, and $MgCl_2$.

Although the positive effect of NaCl concentration on binding of mRNA to oligo(dT) is well known, the effects of Gu-HCl and Mg^{2+} have not been described before. Gu-HCl and Mg^{2+} can be used as additives to NaCl-containing binding buffer. Besides isolation of mRNA from an IVT mixture, the approach can be extended to isolation of mRNA from cell cultures and tissues. In such cases, Gdn historically has been used for cell lysis and inactivation of RNAses, but this new method would eliminate the need for buffer exchange to NaCl-containing buffers for loading onto oligo(dT) (**7, 8**).

Figure 5: Titration of Gu-HCl as a loading salt for binding mRNA to an oligo(dT) column in 96-well format; (LEFT) mRNA coding for eGFP (995 nt) (RIGHT) mRNA encoding a proprietary sequence (4000 nt)

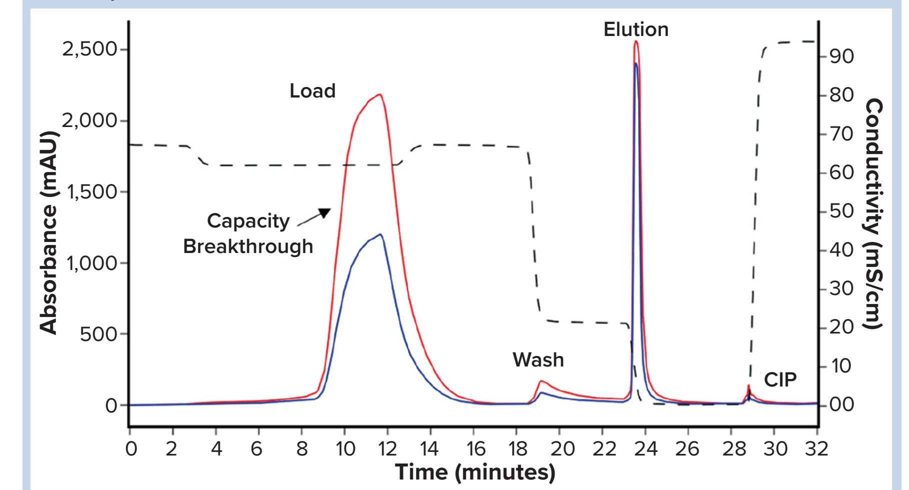


Gu-HCl also can be used in place of NaCl. We presume that the higher chaotropicity of Gu-HCl compared with NaCl enables stronger binding of mRNA to the oligo(dT) solid support. That effect probably is mediated by reducing the hydration shells around these molecules, thus minimizing repulsive interactions between mRNA and the solid support.

We observed no reduction of selectivity when replacing NaCl with Gu-HCl. Note that there is a threshold for Gu-HCl concentration in combination with mRNA concentration in load, beyond which precipitation can occur. That threshold needs to be evaluated for each mRNA construct.

DBC of CIMmultus oligo(dT) is 2–4 mg/mL when NaCl is used as loading salt, with capacity depending on mRNA sequence and length. Capacity can be increased significantly if NaCl is replaced with Gu-HCl, however, with DBC values of >6 mg/mL demonstrated herein. Future studies will evaluate the effects of stronger chaotropes (e.g., guanidinium thiocyanite and perchlorate). Although those are less applicable to industrial settings, such work could drive a better understanding of mRNA–oligo(dT) interactions, and thus development of improved technologies for working with these promising molecules.

Figure 6: Chromatogram of mRNA DBC determination on a 0.1-mL CIM oligo(dT) column in the presence of 0.75 M Gu-HCl; UV_{260} nm in red, UV_{280} nm in blue, and conductivity traced by the dashed line



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