SARTORIUS

Purification of mRNA With CIMmultus® Oligo dT

TN0009

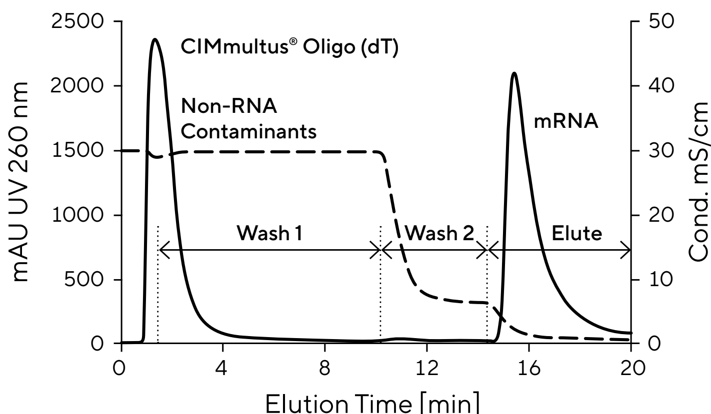
Technical Note

Introduction

CIMmultus® Oligo dT is a hybridization-affinity chromatography monolith for purification of mRNA that terminates in a poly-A tail. Non-RNA contaminants flow through the column during sample loading. RNA with a poly-A terminus binds. The column is washed then the mRNA is recovered in a single elution step at neutral pH (Fig.1).

CIMmultus® Oligo dT can be used for one-step purification of research grade ssRNA, as a high-resolution capture method in a multi-step purification process, as a polishing method, or as an analytical method for estimating quantity and purity of mRNA in a sample. It can be used with mRNA up to 10 kb or more.

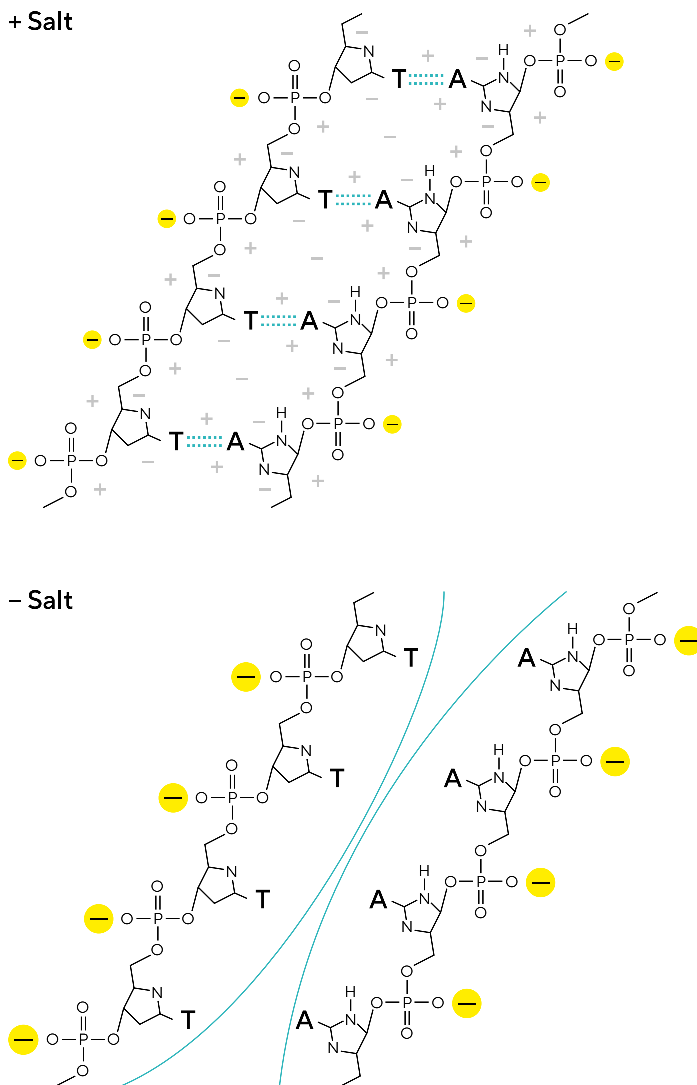
Figure 1: *Chromatogram of IVT Mixture Applied to CIMmultus® Oligo dT. Buffers and Conditions as Described Below.*



Sample is applied at elevated ionic strength to suppress charge repulsion between the phosphatidic acid residues on the ligand and the target RNA. Binding occurs through hydrogen bonding, with the oligo-deoxythymidine ligand forming a stable hybrid association with the terminal poly-adenine sequence of the target. Unreacted nucleotides, partial transcripts, plasmid DNA, and enzymes are not retained. RNA is recovered by reducing ionic strength, which restores charge repulsion sufficiently to dissociate the hydrogen bonds (Fig. 2).

After capture by CIMmultus® Oligo dT, the ssRNA fraction can be further purified by anion exchange chromatography using CIMmultus® dsX, which eliminates dsRNA along with the majority of other contaminants. It can also be followed with hydrogen bond chromatography using CIMmultus® H-Bond ADC, with hydrophobic interaction chromatography using CIMmultus® C4 HLD, or with reverse phase chromatography using CIMmultus® SDVB. Contact BIA Separations for more information about any of these columns. CIMmultus® Oligo dT also supports smooth workflow as a polishing method since high-salt samples may be loaded without further sample preparation or salt can be easily added to low-salt preparations. These features also make CIMmultus® Oligo dT an easy follow-on partner to salt or organic solvent precipitation methods.

Figure 2: *Binding and Elution of mRNA's Poly-A RNA Terminus to Oligo dT*



Note. Elevated conductivity created by excess salt ion disperses the influence of RNA's charged residues over a large area and weakens local charge interactions. This permits same-charge molecules like nucleic acids to approach each other closely enough for hydrogen bonds to form (indicated in teal). At low conductivity, mutual repulsion between same-charge molecules is strong enough to prevent formation of hydrogen bonds, or to dissociate existing hydrogen bonds.

How to Purify ssRNA With CIMmultus® Oligo dT

Your CIMmultus® Oligo dT monolith is a radial flow chromatography device. It is designed to distribute flow from the outside of the bed cylinder to the inside. This has the effect of stabilizing the physical structure of the bed cylinder and also has a concentration effect during elution that improves separation performance. Before conducting any experiments, be sure to connect the unit to the chromatograph so that the direction of flow follows the markings on the device. Note that some chromatographs have default reverse-flow functions built into their software that can cause the flow direction to be reversed without warning. Make sure this function is disabled before conducting any experiments.

CIMmultus® Oligo dT is delivered in 20% ethanol. Before applying sample, It is recommended that a run be performed without sample to provide a baseline against which to compare experimental results. Some buffer components absorb UV, such as EDTA, and some transitions between buffers may create refractive index artefacts that can confuse interpretation of experimental results.

Sample and Preparation

CIMmultus® Oligo dT can be used to process in vitro transcription (IVT) mixtures, including after digestion with DNAse and | or proteinase K, as well as ssRNA resuspended from salt or organic solvent precipitates, or partially purified ssRNA from other purification methods. Some degree of pre-purification will often be desirable to reduce fouling since the ligand will not tolerate cleaning with 1 M sodium hydroxide. Sample pH should be roughly neutral. If the sample lacks salt, or contains a low concentration of salt, add sodium chloride to a final concentration of about 250 mM. Also add EDTA to at least 5 mM. Particulates must be removed by centrifugation or filtration (0.45 µm) in advance of injection.

Buffer A

Equilibration | wash buffer. 50 mM sodium phosphate, 250 mM sodium chloride, 5 mM EDTA, pH 7.0.

Buffer B

Second wash buffer. 50 mM sodium phosphate, 5 mM EDTA, pH 7.0.

Buffer C

Elution buffer. 10 mM Tris, pH 8.0.

Buffer D

Cleaning buffer. 3 M Guanidine-HCl, 5 mM EDTA, pH 7.0. See also discussion below.

Equilibrate Column With Buffer A

Pump equilibration buffer (A) through the column until output pH and conductivity are the same as the input buffer.

Flow Rate

5 column volumes per minute (CV/min). It may be possible to increase flow rate to 10 CV/min when injecting small volumes of substantially purified samples.

Inject Sample

Observe operating pressure during application of large volume samples, especially with crude samples like in vitro transcription mixtures. Reduce flow rate if necessary to maintain operating pressure within acceptable limits.

Wash 1 With Buffer A

10 CV of equilibration buffer.

Wash 2 With Buffer B

10 CV second wash buffer.

Elute With Buffer C

Until UV absorbance returns to baseline.

Clean With Buffer D

Treatment with at least 10 CV of cleaning buffer is recommended after every run since it will reveal if a significant amount of material remains bound to the column at the end of the elution step. The contents of the cleaning step may be collected and buffer exchanged for further analysis. The presence of moderate amounts of RNA in the cleaning fraction is not necessarily cause for concern since it will tend to represent RNA-contaminant aggregates. Large amounts of RNA in the cleaning fraction may indicate a need to optimize the composition of the elution buffer.

Inadequate cleaning may be indicated by a gradual increase of operating pressure over a series of runs, increased tailing during the post-load washing step, a change in the shape of the elution peak, increased contamination of the eluted RNA, and reduced recovery. These effects may be especially evident if the column is loaded with IVT mixtures.

Columns loaded with IVT mixtures may require more aggressive and | or more frequent cleaning. The concentration of guanidine-HCl can be increased to 6 M without damaging the ligand. Guanidine thiocyanate is more effective mole per mole and it can be applied at concentrations up to 12 M. Cleaning with 10 CV 50 – 100 mM NaOH often removes foulants that guanidine does not. Keep NaOH exposure brief and follow immediately with a neutralizing buffer, such as buffer A. Repeated or prolonged exposure to excess NaOH will reduce column performance over a series of cycles, but so will fouling. Seek a balance that serves your needs.

Storage

After flushing the cleaning agent out of the column, store it in 20% ethanol. Take special precautions to avoid following NaOH directly with ethanol as this will form ethoxide radicals that may significantly degrade the ligand in minutes. The column must be at near-neutral pH before introducing ethanol.

Optimization

Use the initial scouting chromatogram as a guide for optimizing the composition and duration of the individual steps described above.

Effects of pH

Hydrogen bonding becomes stronger with decreasing pH due to increasing protonation of biomolecules. This means that RNA will bind more strongly with increasingly acidic pH. It also creates a potential risk of promoting non-specific binding by contaminants and depressing RNA recovery during elution. Increasing pH should have the opposite effect, which may be useful to enhance recovery during elution.

Effects of Salts

Experimentation with different species of salts and concentrations can be beneficial but it is important to keep in mind that they have different effects on the chemical mechanisms that control binding and elution: electrostatic repulsion and hydrogen bonding. It is also important to keep a third variable in perspective: many salts precipitate RNA.

Neutral salts like sodium chloride and potassium chloride have a strong effect on electrostatic repulsion but they have a relatively weak effect on hydrogen bonding. This implies that sodium chloride should be applicable at very high concentrations, with a hypothetical benefit of helping to suppress non-specific binding by contaminants. This is impractical however because the RNA precipitates they form would likely interfere with flow through the column.

Chaotropic salts like guanidinium chloride have an equivalent effect on electrostatic repulsion but a much stronger effect on hydrogen bonding and they maintain solubility of RNA. Their presence in the sample may eliminate more contaminants by suppressing non-specific binding to a greater degree, but there is a risk that they will reduce binding capacity for the desired RNA. They can be added to the elution buffer with the likely effect of enhancing recovery and causing the RNA to elute in a narrower peak but consider that their presence may subsequently affect the efficiency of sample binding in a follow-on chromatography method.

Salts comprising multivalent anions like phosphate, sulfate, and citrate have intermediate characteristics. Mole per mole, they more effectively suppress electrostatic interactions than monovalent salts. They are also more effective hydrogen donor-acceptors than neutral salts because of the oxygen residues they carry. On the negative side, they have a tendency to promote associations among biomolecules, leading to formation of complexes and precipitates, so their concentrations should be kept low.

A more subtle issue is that they are often highly contaminated with multivalent metal cations that can alter the conformation of ssRNA and promote formation of metal-bridge associations with dsRNA, DNA, and proteins. Phosphate buffers in particular may also be contaminated with pyrophosphates (diphosphates). In living systems, pyrophosphates form precipitates with calcium that can lead to adverse health consequences. Phosphate buffers are typically not labeled to indicate pyrophosphate content. Anhydrous phosphate buffers carry the highest proportion of pyrophosphates because of the high temperature used to drive off hydration water. If phosphates are used at all, it is recommended to use hydrated phosphates.

Chelating salts have special capabilities for enhancing the performance of CIMmultus® Oligo dT. They dissociate metal-bridge complexes among mulitivalent metal cations, nucleic acids, and proteins. In other words, they tend to disaggregate large non-specific complexes between ssRNA and contaminants. They are most effective when present in sample and in the equilibration | wash buffer. Consider concentrations in the range of 5 – 10 mM.

Effects of other additives: Nonionic chaotropes like urea weaken hydrogen bonding. Urea will likely reduce binding capacity if present in samples but it may improve RNA recovery during elution and | or cause it to elute in a narrower peak. It may be used at concentrations up to 10 M, though more commonly in the range of 4 – 8 M. Sugars, including sorbitol and xylitol, are also strong hydrogen donor-acceptors and may be more effective at lower concentrations, such as up to 200 mM. Sugars will increase viscosity but this is not a problem for monoliths since efficiency of convective mass transfer is independent of viscosity.

Effects of temperature: Increasing temperature weakens hydrogen bonding. RNA samples are often exposed to elevated temperature in advance of being applied to chromatography columns and sometimes during chromatography. Higher temperature during sample application risks reducing binding capacity. Higher temperature during elution may improve RNA recovery and cause it to elute in a sharper peak. This is also a warning that uncontrolled operating temperature may compromise reproducibility.

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