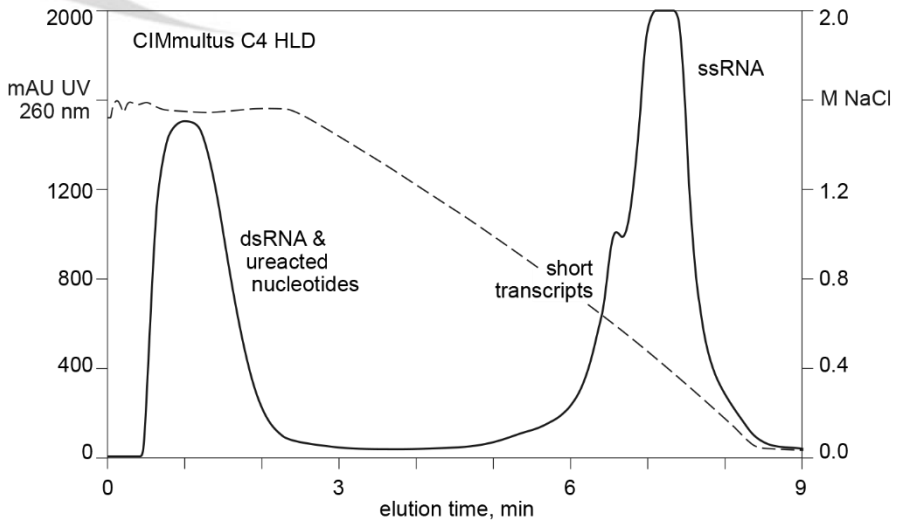
TN0010 Purification of mRNA with CIMmultus™ C4 HLD

Introduction

CIMmultus C4 HLD is a hydrophobic interaction chromatography monolith for purification of nucleic acids. Sample is applied at high ionic strength in salts that precipitate RNA. The column is eluted with a decreasing salt gradient. The majority of DNA, dsRNA, and short transcripts elute earlier than intact ssRNA (Fig. 1). The majority of proteins and aggregates bind very strongly and are eliminated by a cleaning step with NaOH. CIMmultus C4 HLD can be used to produce research grade ssRNA from in vitro transcription (IVT) mixtures but gives its best results as a polishing method.

Figure 1. Separation of in vitro transcription mixture on CIMmultus C4 HLD.



The monolithic architecture CIMmultus C4 HLD is ideally suited to purification of large mRNA. Monoliths are characterized by a highly interconnected networks of large channels, 2–6 µm, more than 10 times the size of large mRNA. Capacity and resolution are not compromised by the slow diffusion constants of large mRNA because mass transport is exclusively convective. Capacity and resolution are also independent of flow rate, even at many column volumes per minute. Flow through monolithic channels is laminar. Laminar flow does not generate the turbulent counter-current shear stress produced by other media formats.

Purification with CIMmultus C4 HLD is most effective following a capture method that eliminates the majority of hydrophobic contaminants, especially including proteins and nucleic acid-protein hetero-aggregates. Precipitation with salts or organic solvents partially achieve this result but chromatography methods do so more

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effectively and more reproducibly with better process control and scalability. Candidates include hybridization-affinity chromatography with CIMmultus Oligo dT, anion exchange chromatography with CIMmultus dsX, and hydrogen bond chromatography with CIMmultus H-Bond ADC. Contact BIA Separations for more information about any of these columns.

How to purify ssRNA with CIMmultus C4 HLD

CIMmultus C4 HLD monolith is a radial flow monolith with a cylindrical bed. It is designed to distribute flow from the outside of the cylinder to the inside. This has the effect of stabilizing its physical structure 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 a default reverse-flow subroutine programmed into their software that can reverse flow direction without warning. Make sure this function is disabled before conducting any experiments.

CIMmultus C4 HLD is delivered in 10 mM NaOH. 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 C4 HLD can be used to increase purity of any sample that contains ssRNA. However, a substantial degree of purification is recommended in advance. Strongly hydrophobic contaminants in IVT mixtures bind very strongly and potentially interfere with overall fractionation performance, especially if large sample volumes are loaded. Intact plasmid DNA may compete with the desired ssRNA for binding capacity and co-elute to some extent with ssRNA. It is also recommended that the sample be treated with a chelating agent. Even low levels of multivalent metal cations may stabilize unnatural RNA conformations and complexes between RNA and contaminants. Remove particulates by centrifugation or filtration (0.45 µm) before equilibrating the sample. The easiest way to prepare small volume samples is to dilute them 1:1 with a 2X concentration of the binding buffer immediately before loading. For example, to produce a final NaCl concentration of 1.6 M, dilute the sample 1:1 with 3.2 M NaCl immediately before sample application. Large volume samples benefit from in-line equilibration through the pumps (discussed below). It is common but not universal practice to pre-incubate samples to 50–70°C.

Buffer A: 50 mM sodium phosphate, 2.0 M sodium chloride, 5 mM EDTA, pH 7.0.

Buffer B. 50 mM sodium phosphate, 5 mM EDTA, pH 7.0.

Buffer C. 1.0 M NaOH. See below for more discussion.

Equilibrate column: 10 CV of 80% buffer A, 20% buffer B. Flow rate: 5 column volumes per minute (CV/min).

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

Wash with buffer A: 20 CV of equilibration buffer.

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Elute with a linear gradient to buffer B: 50 CV, starting with 80% buffer A, 20% buffer B. Ending at 100% buffer B. Then hold for 10 CV at 100% B.

Clean with buffer C. 10 CV. It is recommended to include a cleaning step 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 neutralized or buffer exchanged for further analysis. The presence of a low-to-moderate amount of RNA in the cleaning fraction is not necessarily cause for concern since it will tend to represent RNA-contaminant hetero-aggregates. A large amount of RNA in the cleaning fraction may indicate a need to optimize the gradient conditions.

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, earlier elution of the RNA, a change in the shape of the elution peak, increased contamination of the eluted RNA, and/or reduced recovery.

Columns loaded with IVT mixtures may require more aggressive cleaning. Begin by extending NaOH exposure to 1 hour. Incubation time may be further extended to 16–24 hours for badly fouled columns. Maintenance of minimal flow rate during cleaning tends to produce better results since it washes foulants out of the column instead of merely hydrolyzing them in place. Combining 2–3 M NaCl with NaOH may be helpful in some cases but may also create an antagonistic effect by continuing to promote binding of RNA-contaminant aggregates.

Columns may be cleaned with chaotropic agents like guanidine-HCl up to 6 M or guanidine thiocyanate up to 12 M but they are generally less effective than 1 M NaOH.

Surfactants should be avoided at all process steps since they may bind to the solid phase and alter capacity and separation performance.

Optimization

Use the initial scouting chromatogram as a guide for optimizing the composition and duration of the individual steps described above. A higher concentration of salt may be required for smaller ssRNA, and a lower concentration for larger ssRNA.

Gradient configuration. Resolution may be enhanced by decreasing the slope of the gradient. Eluting product concentration may be increased by making the gradient steeper. Once the elution conditions of the desired ssRNA are defined, the gradient may optionally be converted to a step format.

Effects of different salts. CIMmultus C4 HLD will bind RNA in any salt that precipitates it. Qualified salts include sodium chloride, potassium chloride, and lithium chloride, among others. Different salts will give the same general selectivity with respect to ssRNA and major contaminant classes but at different salt concentrations. Some may produce worthy results.

Effects of pH. RNA behavior on CIMmultus C4 HLD is not affected dramatically by variations in pH. Operating pH values close to neutrality are recommended.

Effects of temperature. Hydrophobic interaction chromatography is most commonly performed at ambient temperature but it needs to be controlled. Uncontrolled operating temperature may compromise reproducibility. Higher temperatures increase the intensity of hydrophobic interactions. They may increase

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capacity but they will also affect selectivity and resolution. Lower temperatures may reduce capacity and cause RNA to elute at a higher salt concentration than expected.

Scaling up sample application

Bulk equilibration of sample immediately before sample application is convenient at lab scale but it can compromise reproducibility across increasing process scales. This is because RNA precipitation with salts is time-dependent. A sample that contains little turbidity immediately after dilution with a high-salt buffer may show obvious turbidity after a period of time. If it takes 60 minutes to load the bulk-diluted sample, that means higher turbidity sample will be loaded at the end of sample application than was being loaded at the beginning. Higher turbidity corresponds to a higher content of aggregates and precipitates. Precipitates can clog monolith channels and increase operating pressure.

Reproducible control of sample equilibration and application can be achieved by a method known as in-line dilution. Undiluted sample is loaded through one pump. High-salt buffer is loaded simultaneously through another pump. The two streams meet at a mixer immediately before the column (Fig.2). Pre-column residence time of the sample in the high salt environment is a function of the chromatography system volume from the point where the sample and high-salt diluent meet, to the entrance of the column. Pre-column residence time of the sample in high salt remains constant even if it takes hours to load the entire sample. Binding capacity and eluted product purity are typically higher compared to experiments in which samples are equilibrated by bulk dilution.

It is important to be wary of the temptation to use as high a salt concentration as possible in the diluent buffer with the idea of achieving the lowest volumetric dilution factor. Salt solutions of different concentrations have different viscosities that tend to reduce mixing efficiency. Good mixing efficiency is critical for in-line dilution. A good starting point is to have the concentration in the high-salt buffer about 20% higher than the target binding concentration. Subsequent experimentation will reveal if and how much the salt concentration can be increased. Keep in mind that temperature control is also important when optimizing sample application conditions since it affects both solubility and binding to the solid phase.

Note that bubble-traps must be absent or at least off-line during sample equilibration by in-line dilution. They act as large dead-volume mixing devices that vastly prolong the period of time required to load the sample onto the column and ensure formation of precipitates. They also interfere with the ability of the chromatography system to accurately deliver programmed gradients. Differences in the ratio of bubble-trap volume to column volume are also a major cause failure during scale up. Bubble-traps are not necessary with monoliths because monoliths do not trap air and the passage of air has no effect on packing quality or separation performance. Entrapment of air or its passage through a UV monitor may create artefacts on the chromatogram but the monolith sustains no damage. Simply displace the air with buffer and continue.

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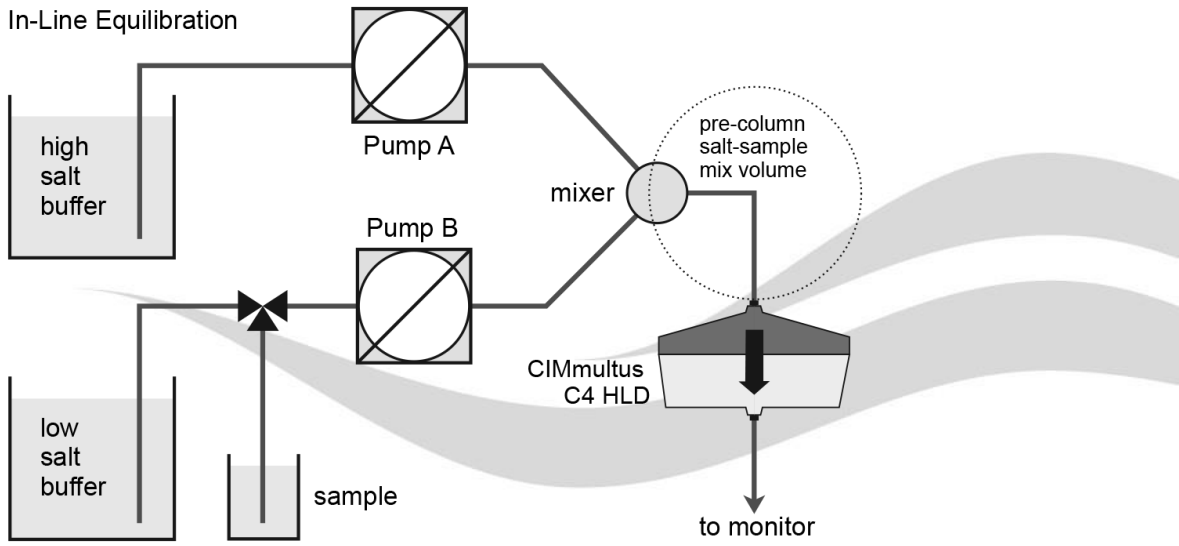
Figure 2. Sample equilibration by in-line dilution. Column equilibration is performed with the sample off-line. Sample application is performed with the sample in-line. The brief pre-column residence time of the sample in high salt minimizes precipitation prior to sample entering the column. Sample is taken off-line during the wash and throughout the elution gradient.



Ordering information

Cat no.	Product name
311.8136-2	CIMmultus™ C4 HLD 1 mL Monolithic Column (HLD Butyl) (2 µm channels)
414.8136-2	CIMmultus™ C4 HLD 4 mL Monolithic Column (HLD Butyl) (2 µm channels)
411.8136-2	CIMmultus™ C4 HLD 8 mL Monolithic Column (HLD Butyl) (2 µm channels)
614.8136-2	CIMmultus™ C4 HLD 40 mL Monolithic Column (HLD Butyl) (2 µm channels)
611.8136-2	CIMmultus™ C4 HLD 80 mL Monolithic Column (HLD Butyl) (2 µm channels)
814.8136-2	CIMmultus™ C4 HLD 400 mL Monolithic Column (HLD Butyl) (2 µm channels)
811.8136-2	CIMmultus™ C4 HLD 800 mL Monolithic Column (HLD Butyl) (2 µm channels)
1014.8136-2	CIMmultus™ C4 HLD 4000 mL Monolithic Column (HLD Butyl) (2 µm channels)
1011.8136-2	CIMmultus™ C4 HLD 8000 mL Monolithic Column (HLD Butyl) (2 µm channels)

For cGMP compliant columns, please visit www.biaseparations.com or contact sales@biaseparations.com.

For any additional information or further enquiries please contact us:

Tel.: +386 59 699 500

help@biaseparations.com
sales@biaseparations.com

www.biaseparations.com