SARTORIUS

Method Guide

CIM® C4 HLD for mRNA Purification

Convective Interaction Media (CIM®) chromatographic devices are available in different formats, from high throughput 96-well and 24-well plates, analytical chromatographic columns, and scalable purification columns. Chromatographic conditions such as buffer properties (type, pH, conductivity), gradients, or column cleaning may differ between applications. The equipment used for different formats (high throughput, analytical, and preparative devices) offers varying capabilities, such as ability to run gradient elutions. The following instructions can be directly applied to CIMmultusŏ preparative columns. Follow format-specific requirements when transferring the conditions outlined here to CIMacŏ or CIM® Plate formats.

The ligand in CIM® C4 HLD monoliths is a hydrophobic butyl group. RNA binds on the column under hydrophobic conditions. The majority of DNA and short transcripts elute earlier than intact ssRNA (Figure 1), while most of the proteins and aggregates bind very strongly and are eliminated by a cleaning step with NaOH. 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.

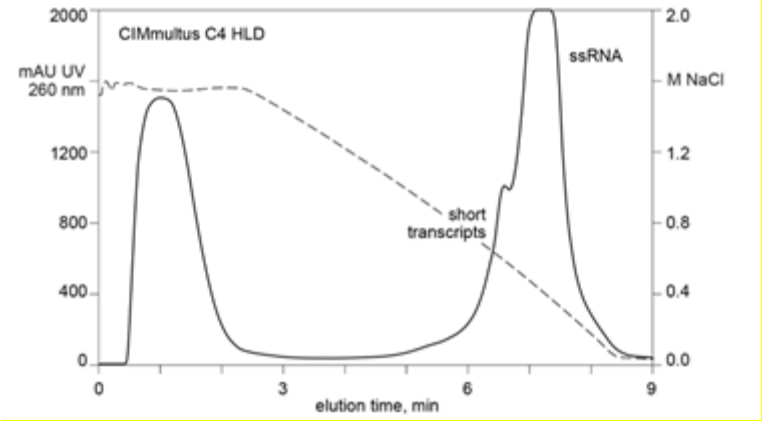

Figure 1: Separation of in vitro transcription mixture on CIMmultus C4 HLD.

For more information, contact
help.BIA@sartorius.com

The sample is applied at elevated concentrations of salts that precipitate RNA. The high salt concentration enables binding on the column. A decreasing salt gradient, or a step elution can be used to elute RNA from the column. Fractionation of the elution peak is recommended to analyse for purity (fragments).

Getting Started

Your column instruction manual can be downloaded by scanning the QR code on the right or by following this hyperlink. CIMmultus columns use a radial flow distribution inside the housing, requiring the column to be connected to the chromatograph with the correct flow direction. 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.

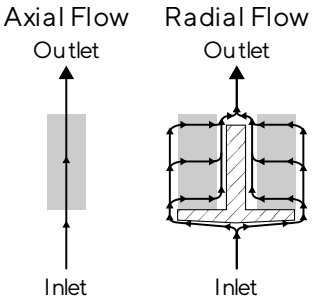

Figure 2: Comparison of axial flow and radial flow distribution within stationary phase of the chromatographic column.

Before applying any sample, prepare the column by removing the storage solution as instructed in the product sheet. It is also recommended to perform a run 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 recommended to evaluate the need for chelating agents in the sample and buffers. Multivalent metal cations may stabilize unnatural RNA conformations, and complexes between RNA and contaminants. If necessary, particulates can be removed by centrifugation or filtration (0.45 µm) before equilibrating the sample. Since the samples are loaded in high concentrations of salt, in-line dilution can be used to reduce the exposure time to salty conditions and thus avoid precipitation. This instruction document describes application of an Oligo dT eluate on a CIMmultus C4 HLD in 2 M NaCl using in-line dilution through system pumps A and B. Especially large volume samples benefit from in-line dilution through the pumps. More can be read below in “Scaling up sample application”. It is possible but not universal practice to pre-incubate samples to 50–70°C and cool them on ice prior to loading.

Purification of ssRNA with CIMmultus C4 HLD

The following guide anticipates the use of inline dilution and uses a binding salt concentration of 2 M NaCl. See the optimisation and trouble-shooting section for further guidelines.

- **Buffer A**. High salt buffer. 50 mM Tris, 2.75 M sodium chloride, pH 7.2.
- **Buffer B**. Stripping buffer. 50 mM Tris, pH 7.2.
- **Buffer C**. Elution buffer. 50 mM Tris, 0.4 M sodium chloride, pH 7.2.
- **Buffer D**. Loading buffer. 50 mM Tris, 2 M sodium chloride, pH 7.2.
- **Buffer E.** Cleaning buffer. 1 M NaOH.
- **Sample dilution buffer.** 50 mM Tris, 0.8 M sodium chloride, pH 7.2.
- **Flow rate.** Start with a flow rate of 5 CV/min on CIMmultus 1 mL. It is recommended to maintain the same flow rate in CV/min during scale-up.

Column equilibration. Wash the column with 20 CV of Buffer C, followed by 20 CV of buffer D. pH and conductivity detectors at the column outlet should match buffer specification.

Prepare the sample. To improve mixing of liquids during inline dilution, it is useful to start with a moderate salt concentration in the sample. Dilute the RNA sample in sample dilution buffer 1:1. This sample will be combined with Buffer A to achieve a salt concentration of 2 M NaCl in the next step.

Load the sample. With buffer A connected to pump A, and sample connected to pump B, apply 32% pump B. The final sodium chloride concentration will be 2 M. Observe operating pressure during application of large volume samples. Reduce flow rate if necessary to maintain operating pressure within acceptable limits.

Wash. Wash with buffer D to return UV signal to baseline. Wait at least 5-10 CV after the UV signal is stable.

Elution. For elution it is recommended to use lower flow rate (e.g. 2 CV/min on CIMmultus 1 mL). Perform a linear gradient (for example in 15 CV) from buffer D to 100% buffer C and collect the elution peak in several fractions until the UV260 signal returns close to the baseline.

Clean with buffer E. Was the column with 10 CV of buffer E. 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 neutralised 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.

Storage. To store the column, refer to the instructions provided in the Product Sheet.

Optimization and Troubleshooting

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.

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 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.

Cleaning. 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.

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

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.

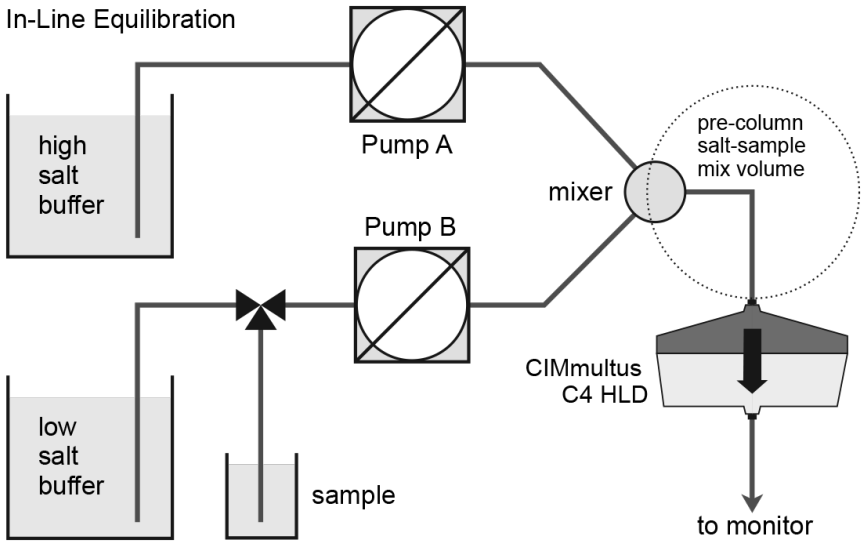

Figure 2: Sample equilibration by in-line dilution. Column equilibration is performed with the sample off-line. Sample application is performed with the sample in-line. The brief pre-column residence time of the sample in high salt minimizes precipitation prior to sample entering the column. Sample is 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 and 40 L column, please visit www.biaseparations.com or contact sales@biaseparations.com.

FAQ

What is the typical dynamic binding capacity of CIMmultus C4 HLD column?

Typical dynamic binding capacity of CIMmultus C4 HLD column is in the range of 2-3 mg/mL. Capacity may vary depending on chromatographic conditions, sample properties and degree of method optimisation. The binding capacity scales linearly with the volume of the column.

Can the CIMmultus C4 HLD column efficiently remove dsRNA impurities?

Hydrophobic chromatography may achieve a degree of separation between dsRNA and ssRNA if suitable conditions are identified. The standard conditions described in this Method Guide will not guarantee a separation. If dsRNA impurities removal is the primary objective, it is recommended to use ion pair reversed phase with CIMmultus SDVB.

Is the column reusable? How many times?

Yes, the column is reusable if appropriate cleaning after each run is performed. Column lifetime will be affected by sample properties, sample preparation, and column maintenance. The general Cleaning in Place (CIP) procedure is described in the Product Sheet, downloadable from: https://www.biaseparations.com/library/product-documentation/.

Germany
Sartorius Stedim Biotech GmbH
August-Spindler-Strasse 11
37079 Goettingen
Phone +49 551 308 0

USA
Sartorius Stedim North America Inc.
565 Johnson Avenue
Bohemia, NY 11716
Toll-Free +1 800 368 7178

For more information, visit
www.sartorius.com

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