BIA separations

Better purification. Faster.

Purification of single-stranded mRNA: The toolbox for the next generation

BIA Separations, Ajdovscina, Slovenia

June, 2020

Introduction

Messenger RNA is poised to become a major contributor in the fields of gene therapy and vaccines.

Making this a practical reality requires purification technology that accommodates its unique features and challenges.

Some of those challenges are inherent to mRNA.

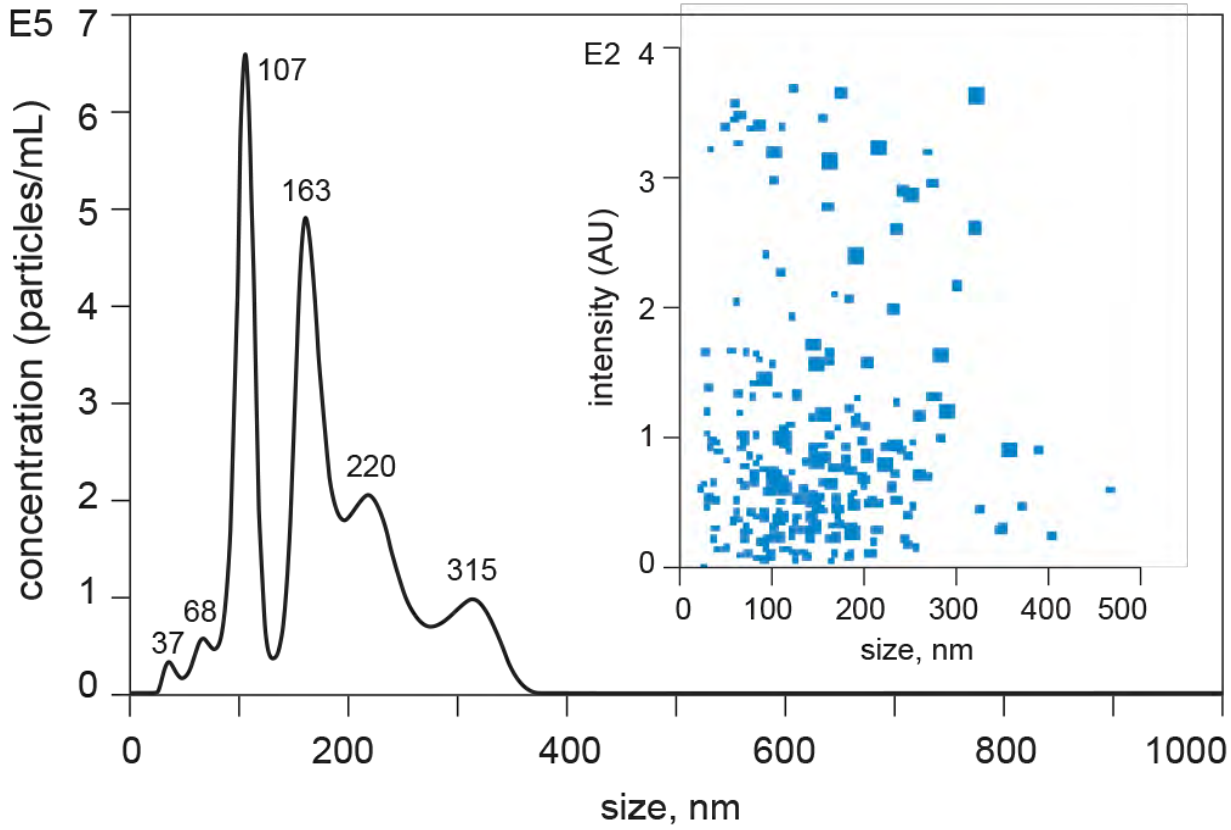
Others are byproducts of RNA synthesis.

This presentation addresses both and introduces a coordinated purification toolbox to advance the evolution of mRNA therapy.

Purification challenges: mRNA size

RNAs are large in proportion to their mass.

Nanotracking analysis (NTA). In vitro transcription mixture. ssRNA 1200b.



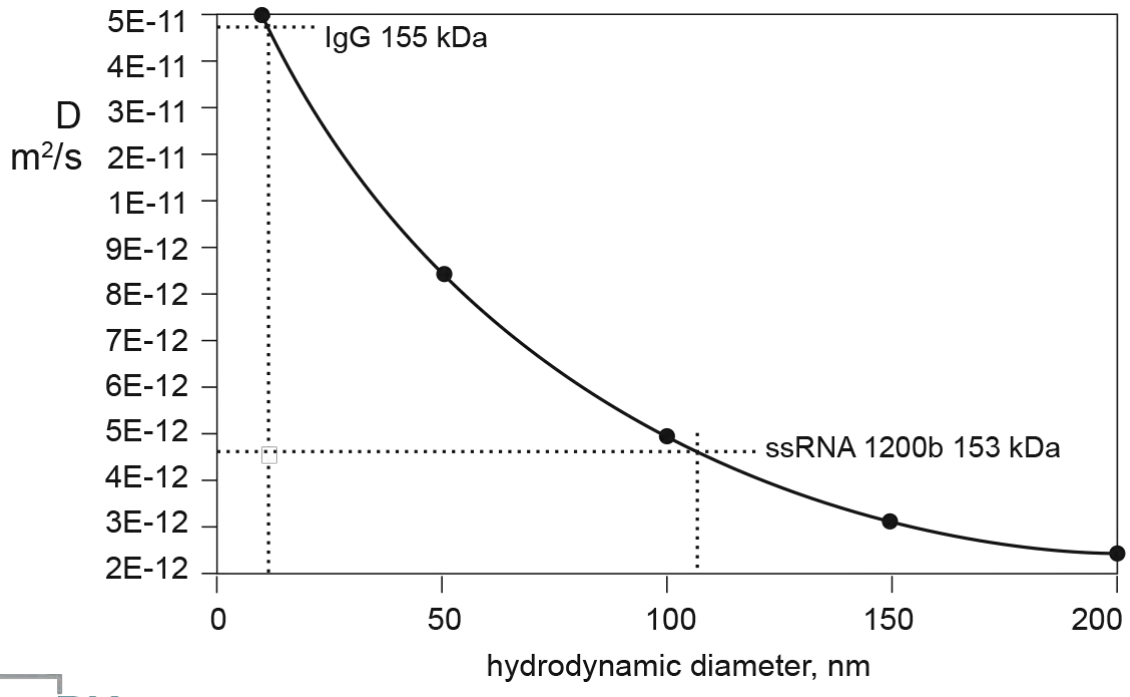
The free RNA population has an average size of about 107 nm.
The rest resides mostly in large heterogeneous associations.

Purification challenges: mRNA size

RNA size has important ramifications for chromatography.

Large size translates to slow diffusion constants. This is important for some kinds of chromatography media.

Size also determines the proportion of pores or channels RNA can enter into. The pore or channel size should be at least 10 times larger than the target molecule for unrestricted entry and exit.



1200b ssRNA has about the same mass as IgG but it diffuses 10 times more slowly because of its more linear structure.

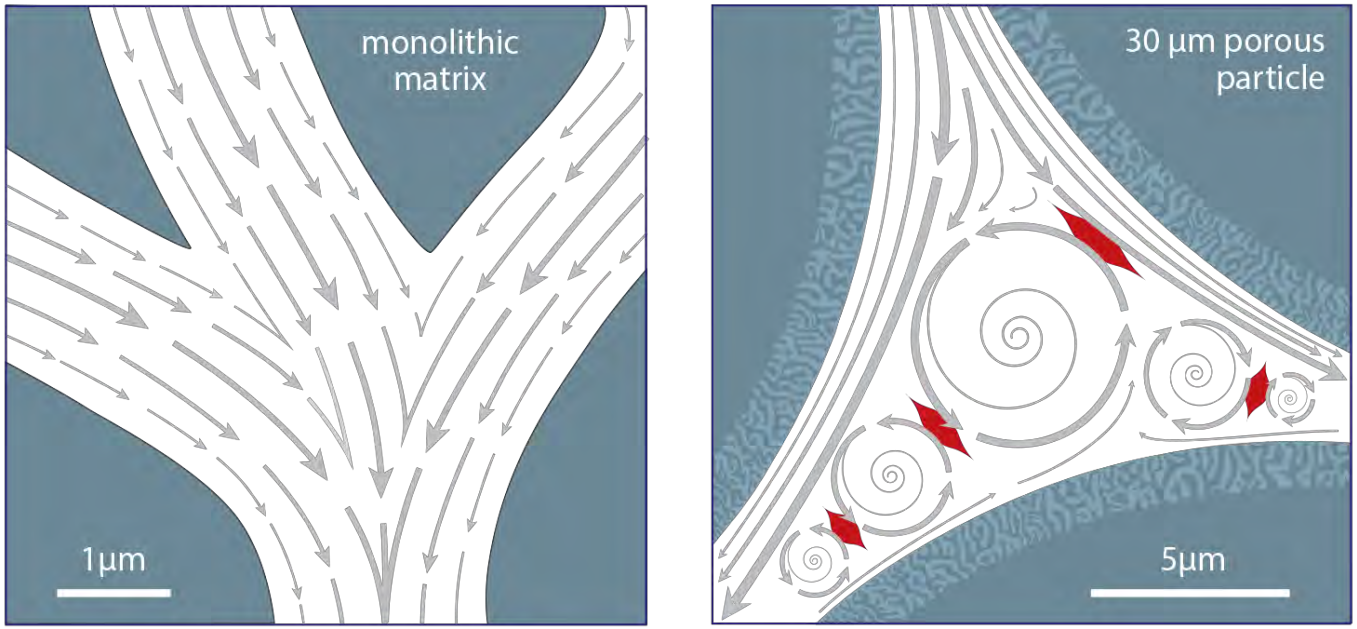
RNA size from NTA (previous slide).

Purification challenges: mRNA size

Size is also an indirect indicator of vulnerability to shear stress.

Friction causes flow rate to approch zero at surfaces but velocity increases with distance. This creates flow-velocity strata.

Larger molecules straddle a wider range of strata, each of which pushes the molecule at a different rate or different direction.



Laminar flow produces very low shear. Turbulent flow produces zones of countercurrent flow that create high shear stress (red).

Purification challenges: mRNA size

The architecture of monoliths was designed specifically to accommodate large biomolecules like nucleic acids.

They are characterized by 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 convective.

Convective mass transport also means that capacity and separation efficiency are independent of flow rate, even at flow rates of 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 chromatography media formats.

Purification challenges: mRNA chemistry.

The chemistry of mRNA has consequences for purification.

RNA is highly charged. Charges are long-range forces. They influence RNA conformation and interactions with other species.

Mutual repellency among negative charges on RNA‘s backbone pushes the molecule toward linearity. It also pushes away other RNA molecules and DNA.

High salt concentrations weaken those effects. With some salts, high concentrations promote precipitation of RNA.

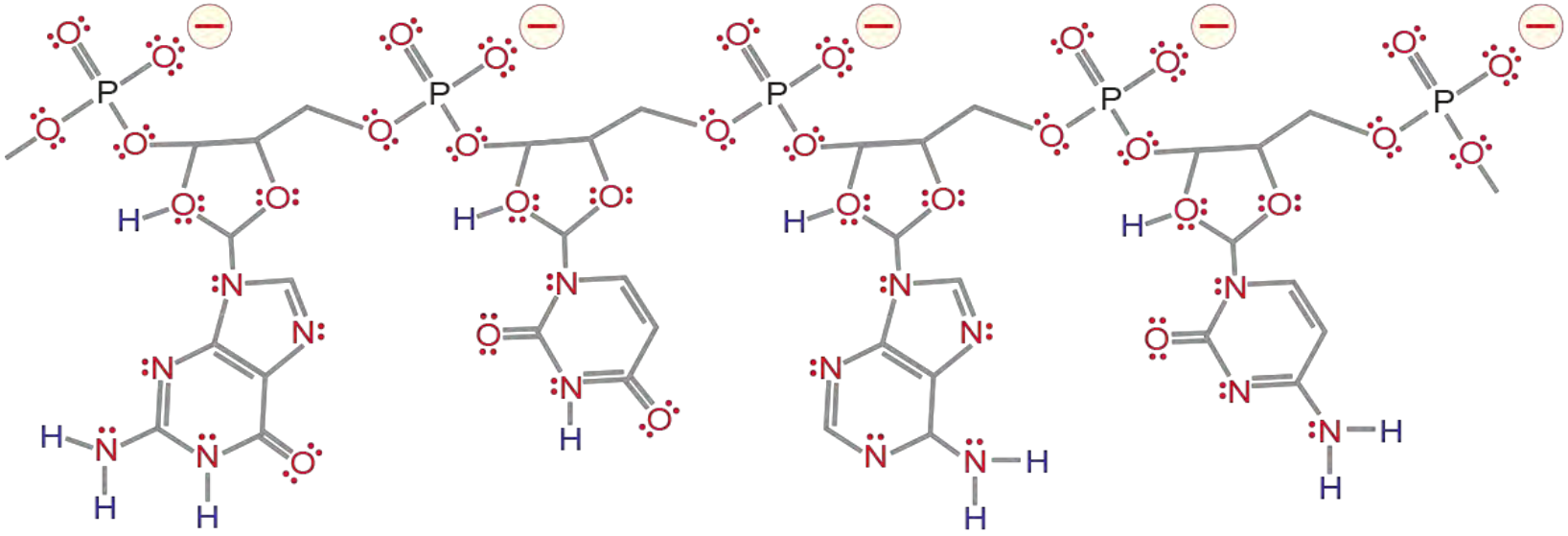
Low salt concentrations increase repulsion between negative charges but they enhance attraction to positively charged regions of contaminating biomolecules like proteins.

Purification challenges: mRNA chemistry.

Hydrogen bonds are short-range molecular forces. They are weaker individually than electrostatic interactions but RNA has many more hydrogen donor-acceptors than negative charges.

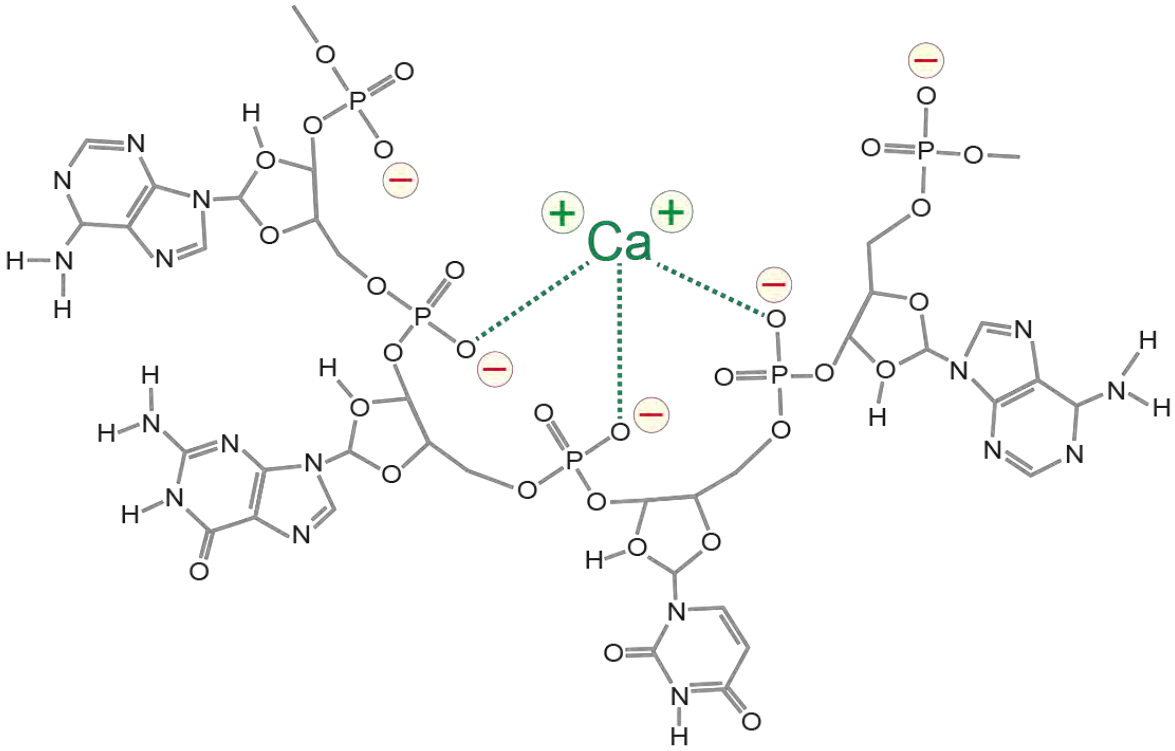
They influence RNA conformation, solubility, interactions with contaminants and with the surfaces of chromatography media.

Hydrogen donors are labeled in blue, acceptors in red. Paired red dots represent free lone-pairs of electrons. Yellow circles: negative charges.



Purification challenges: mRNA chemistry.

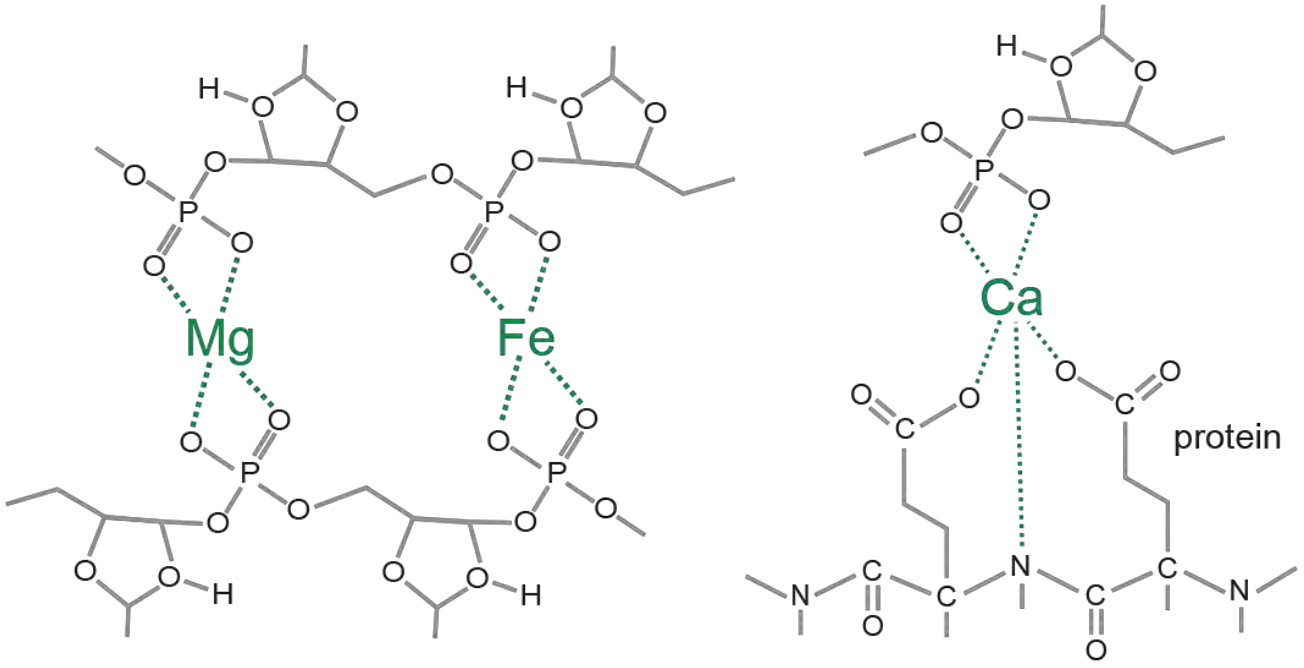
Multivalent metal cations participate in non-specific interactions that create charge and conformational heterogeneity in RNA populations.



Achieving the best purification performance requires that the product be homogeneous, so it responds uniformly to column chemistry and elution conditions. Heterogeneity contributes to peak broadening and losses.

Purification challenges: mRNA chemistry.

Metal coordination can also promote formation of intermolecular complexes that alter product solubility or enable contaminants to hitchhike along with the product into the elution fraction.



This and the previous slide highlight why metal ions represent such important contaminants. Chelating agents are critical for good process control, not only to maintain product homogeneity but also to suppress activity of nuclease enzymes.

Purification challenges: mRNA chemistry.

Charge interactions, hydrogen bonding, and metal coordination work cooperatively in IVT mixtures. Each enhances the others.

RNA, as the dominant component of the mix, serves as a nucleation center for development of heteroassociations between RNA and IVT contaminants.

These nonspecific complexes depress capacity, depress recovery, and depress the purification ability of every known purification method: filtration, precipitation, and chromatography. *Every* method.

The ability to achieve high recoveries of ssRNA, free of dsRNA, DNA, and proteins, requires strategic selection of chromatography methods and buffers to dissociate contaminants from ssRNA.

Purification challenges: contaminants.

IVT mixtures contain multiple classifications of contaminants:

I. RNA variants: dsRNA, truncations, fragments, aggregates.

2. Synthesis raw materials: The DNA plasmid and enzymes.

3. *The contaminants in the raw materials:*

   In the plasmid: E.coli proteins, DNA, and RNA.

   In the enzymes: fragments, host DNA, RNA, proteins.

4. Endotoxins, if introduced during processing.

If the plasmid is digested post-synthesis, there are two more:

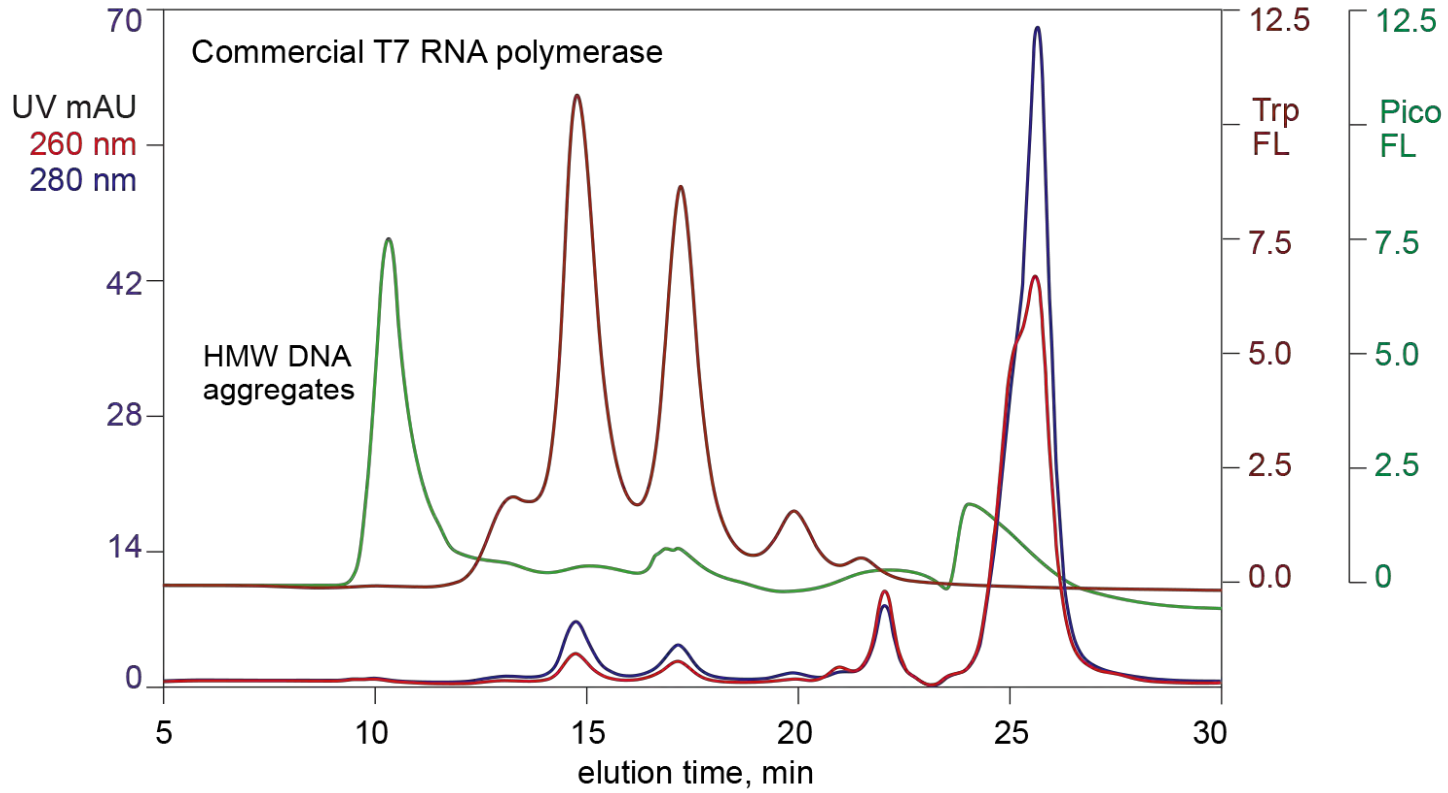
5. The DNAse enzyme and/or protease enzyme,

6. And the contaminants they carry, including metal ions.

Purification challenges: contaminants

Impurities and secondary contaminants in processing materials.

Analytical SEC. TSKgel™ G3000SWxl. 0.5 mL/min. Prestained with Picogreen.

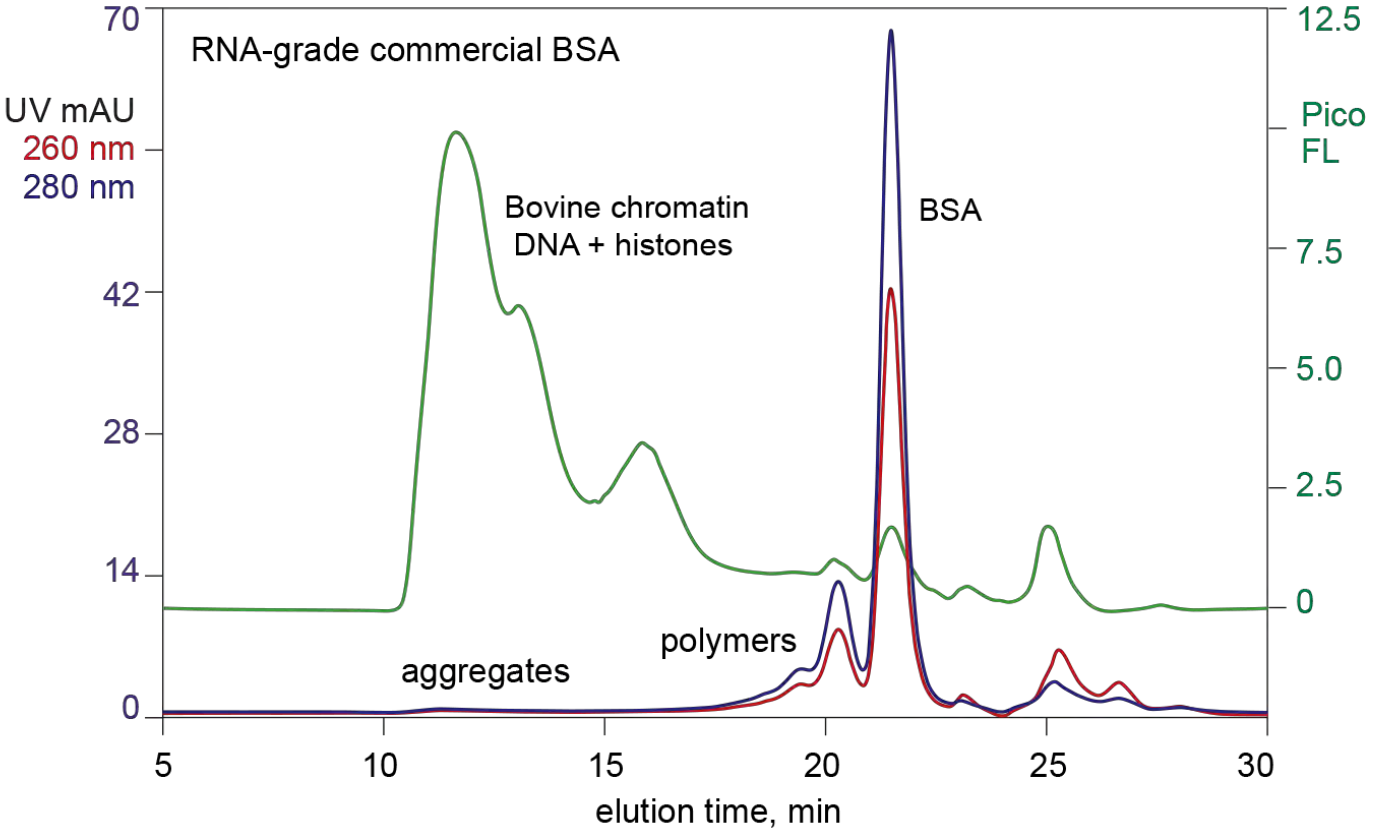


Tryptophan fluorescence increases sensitivity of protein detection 15–20 times over UV but does not detect nucleic acids. The DNA detected by Picogreen is from the host organism used to produce the enzyme.

Purification challenges: contaminants

Impurities and secondary contaminants in processing materials.

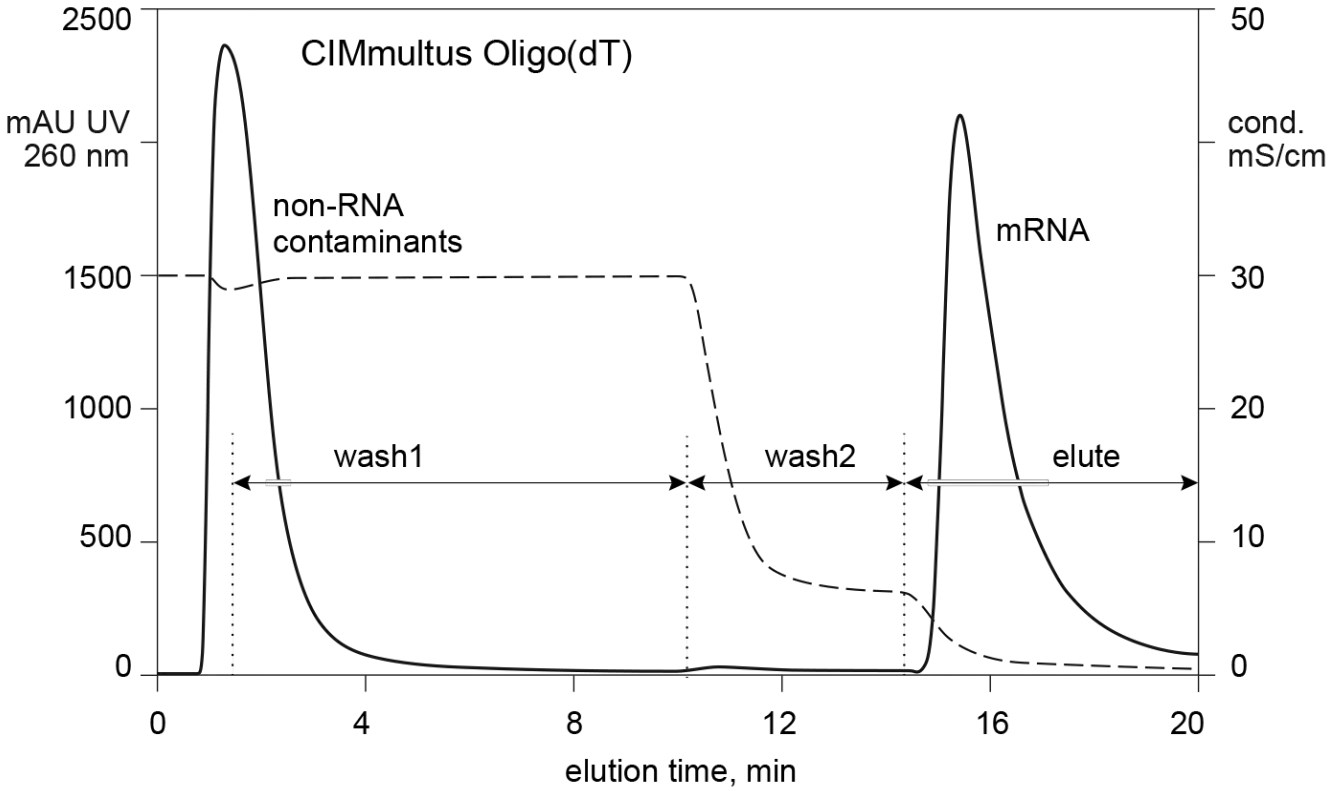
Analytical SEC. TSKgel™ G4000SWxl. 0.5 mL/min. Prestained with Picogreen.



This and the previous slide highlight the importance of robust raw material qualification. Caveat emptor.

The Tools: hybridization-affinity chromatography

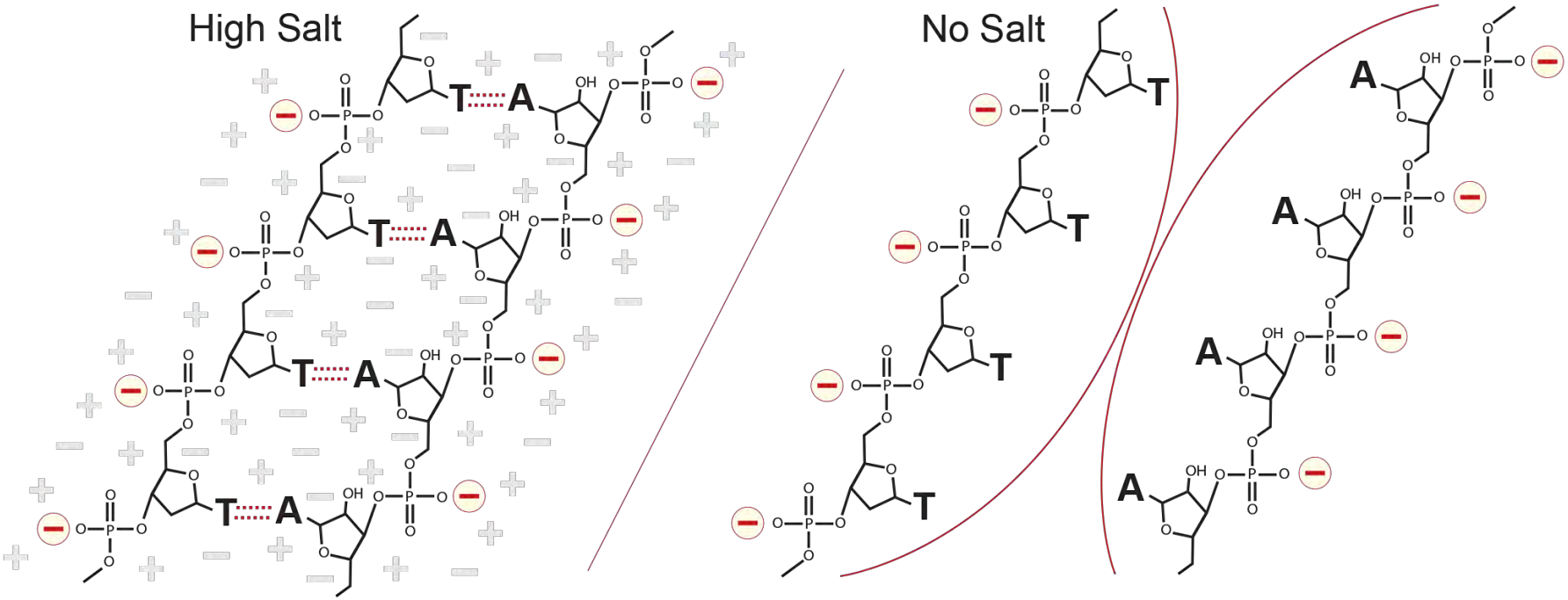
CIMmultus™ Oligo dT selectively binds the poly-A tail of mRNA. Species lacking a poly-A tail flow through. RNA is recovered in a single step.



Equilibrate/wash1: 50 mM sodium phosphate, 500 mM NaCl, 5 mM EDTA, pH 7.0. Wash2: 50 mM sodium phosphate, 5 mM EDTA, pH 7.0. Elute: 10 mM Tris, pH 7.5. Flow rate: 5 column volumes/minute.

The Tools: hybridization-affinity chromatography

CIMmultus Oligo dT works by balancing the relative contributions of electrostatic interactions and hydrogen bonding.



The presence of 250 mM NaCl suppresses repulsion among negative charges on the backbone of the ligand and RNA. This enables hydrogen bond base pairing that captures poly-A RNA.

Removing the salt restores charge repulson and overwhelms H-bonding.

The Tools: hybridization-affinity chromatography

Answers to FAQS about CIMmultus Oligo dT.

Oligo dT does not discriminate according to RNA size or conformation. Anything that contains poly-A is captured.

Oligo dT can be used to purify mRNA from IVT mixtures but heavy contamination may limit performance and column life.

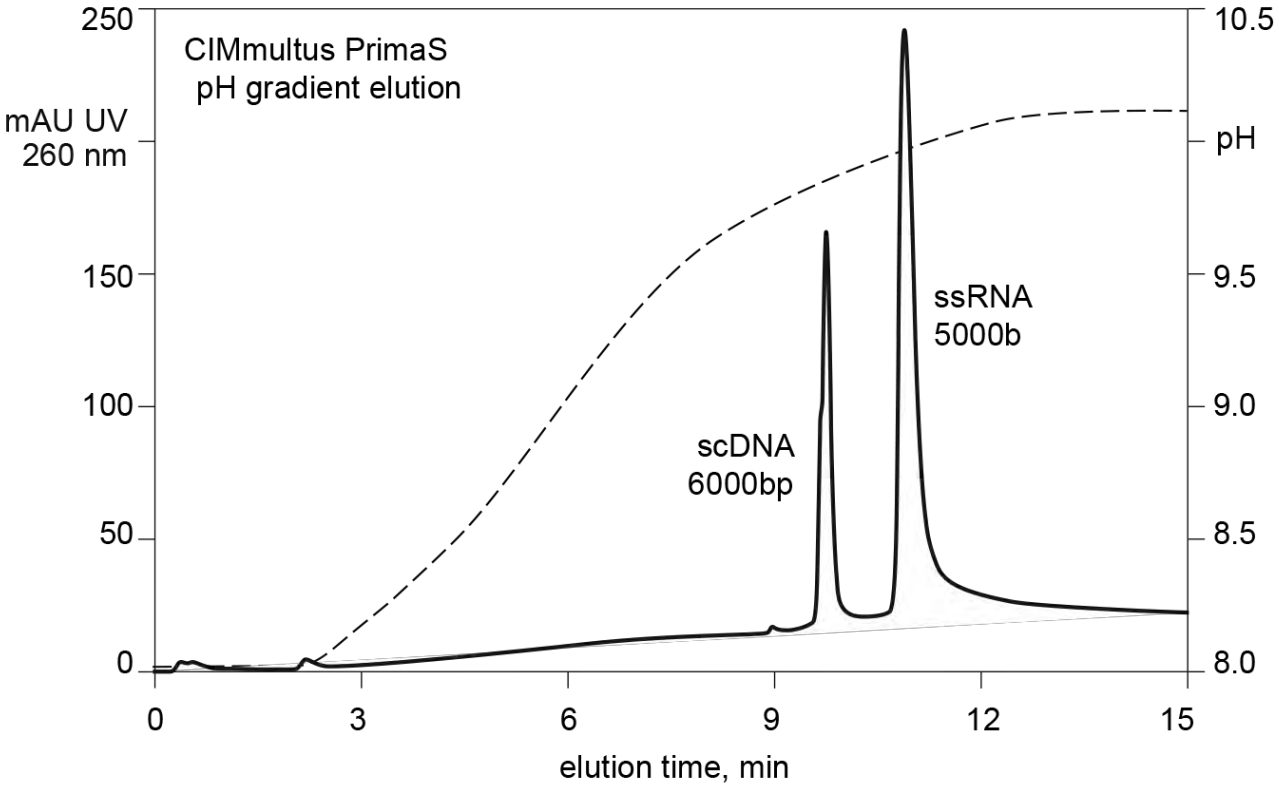
Oligo dT can be cleaned with 6 M guanidine or up to 0.1 M NaOH. This is adequate when it is used for polishing but it may not fully restore columns exposed to IVT samples.

Purity will be improved and fouling can be reduced by including chelating agents in the sample and equilibration/wash buffers.

Elution can be enhanced (sharper peaks, higher recovery) by the presence of nonionic hydrogen donor-acceptors like urea or sugars.

The Tools: anion exchange chromatography

CIMmultus PrimaS elutes ssRNA at ambient temperature in a pH gradient. DNA and dsRNA elute in advance of ssRNA.

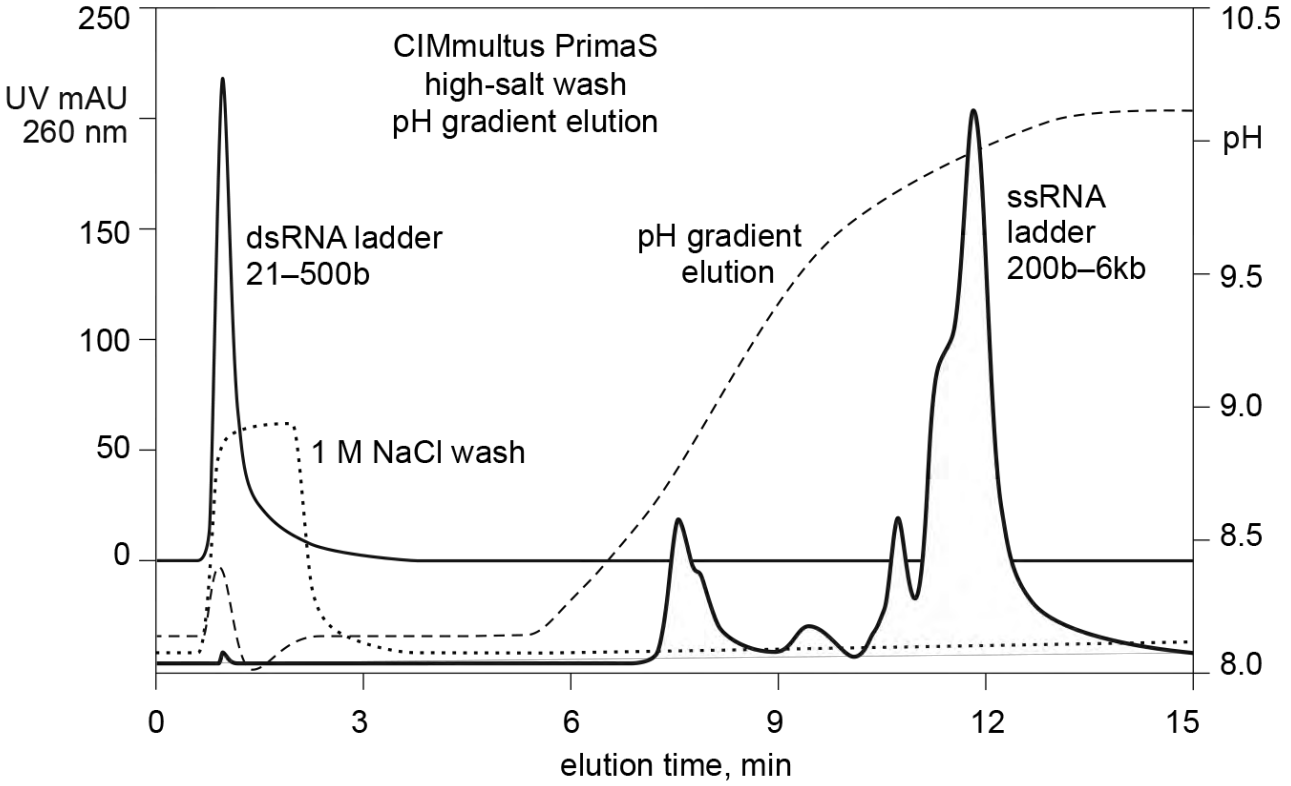


Including EDTA or other salts in the pH gradient buffers increases ssRNA recovery and lowers elution pH up to 0.5 pH units. Fractions neutralized immediately after elution show no adverse effects.

BIA separations

The Tools: anion exchange chromatography

Washing CIMmultus PrimaS with a combination of high salt and EDTA after sample application removes protein, DNA, and dsRNA. Elution with a pH gradient then separates ssRNA according to size.



Chaotropic salts like guanidine are typically more effective than NaCl. Single-stranded RNA remains bound even in saturated guanidine.

The Tools: anion exchange chromatography

Answers to FAQS about washing CIMmultus PrimaS.

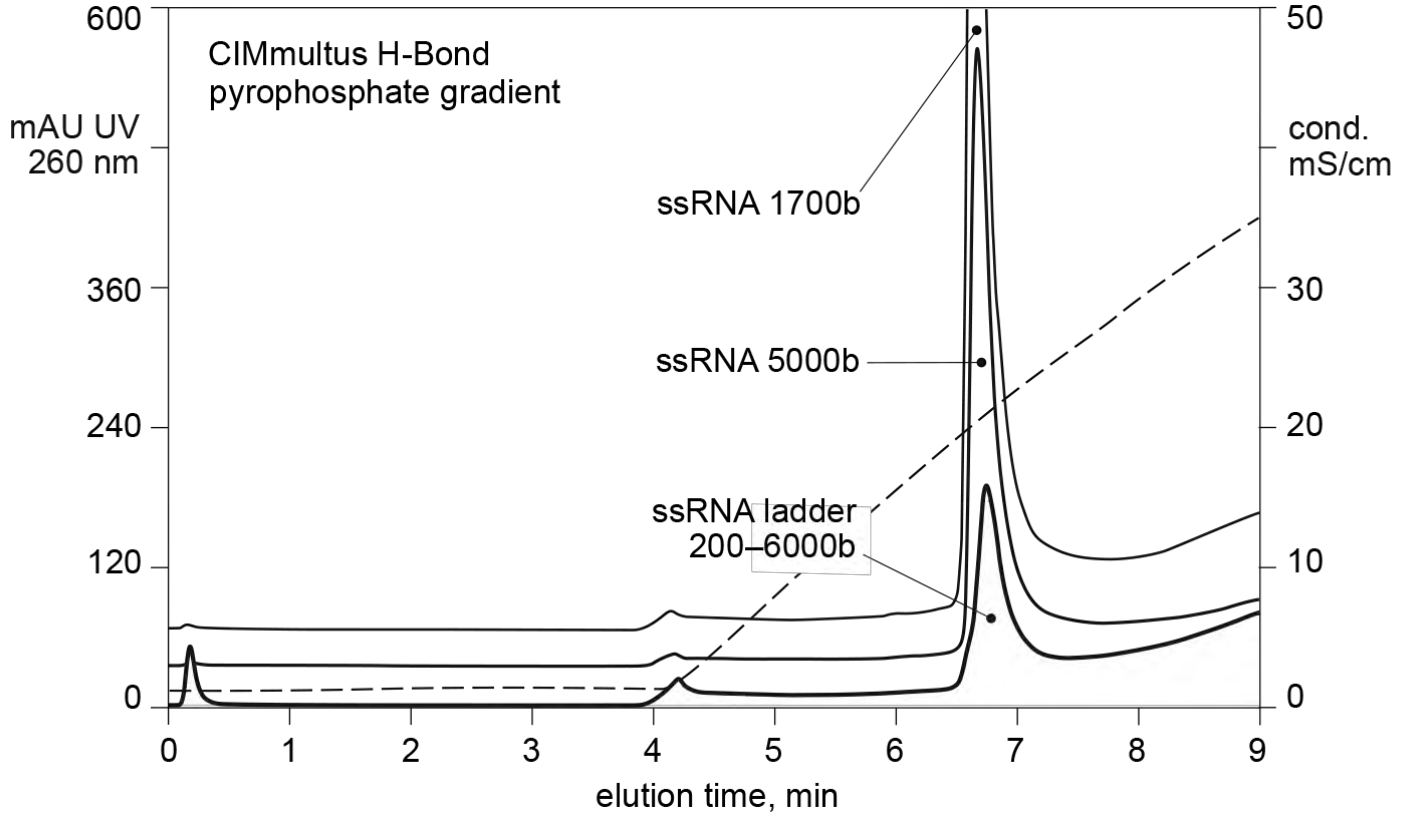
Single-stranded RNA remains bound at all concentrations of all known salts, including saturated guanidine-HCl.

Proteins, dsRNA, and DNA are eliminated easily with concentrated salt, especially in the presence of chelating agents. This permits washing with chaotropes up to full saturation, combined with chelating agents at high concentrations (20–50 mM).

Chaotrope-chelator washes are especially effective for dissociating RNA-contaminant heteroaggregates because they simultaneously suppress all their internal association mechanisms: charge interactions, hydrophobic interactions, hydrogen bonding, and metal-bridging. RNA remains soluble.

The Tools: Hydrogen bond chromatography

CIMmultus H-Bond also supports 1-step purfication of ssRNA at ambient temperature. It elutes ssRNA by a newly discovered affinity mechanism. All sizes of ssRNA elute at the same point.

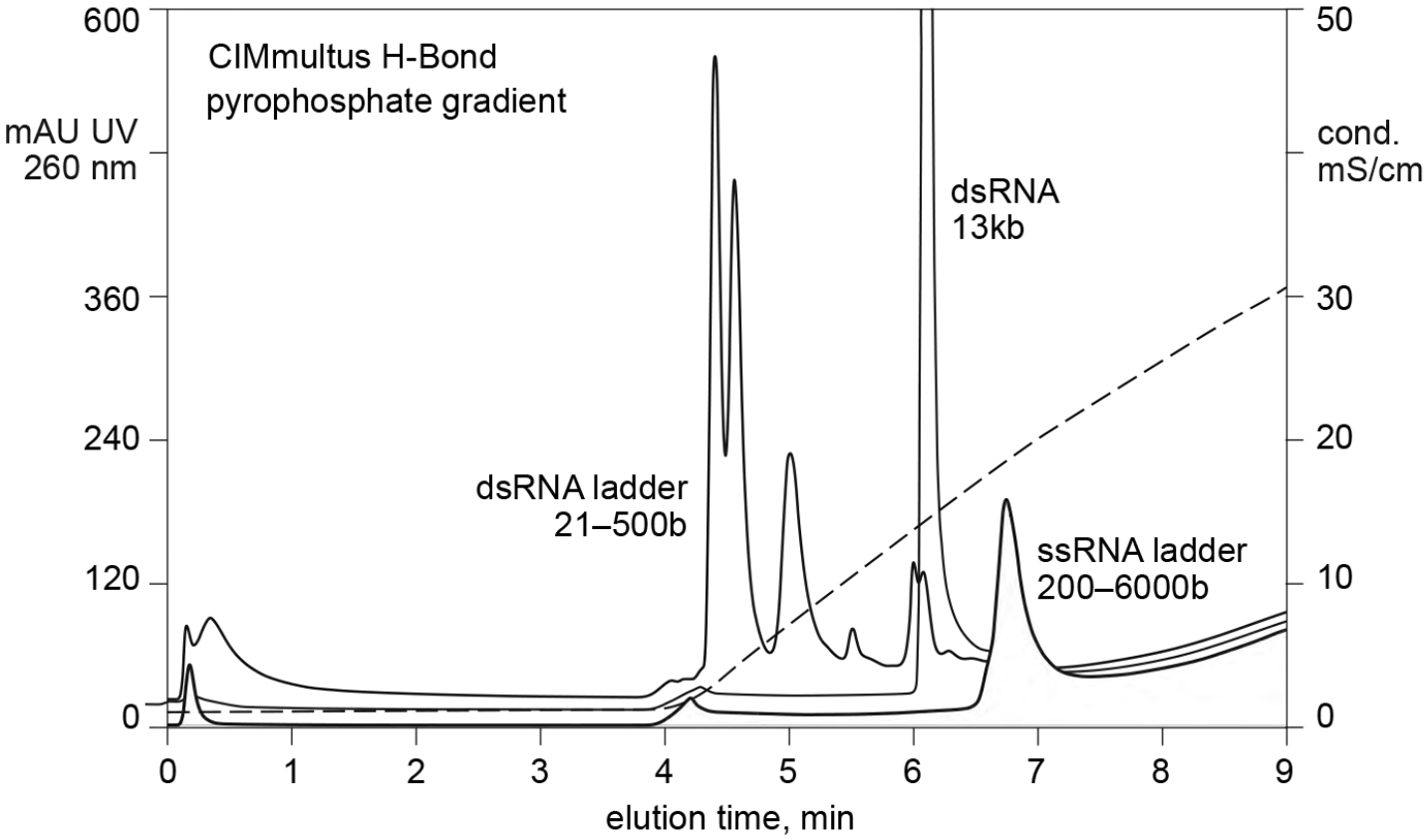


Elution with a linear gradient to 100 mM potassium pyrophosphate.

BIA separations

The Tools: Hydrogen bond chromatography

Double-stranded species elute in order of increasing to size but the largest dsRNA elutes before the smallest ssRNA.

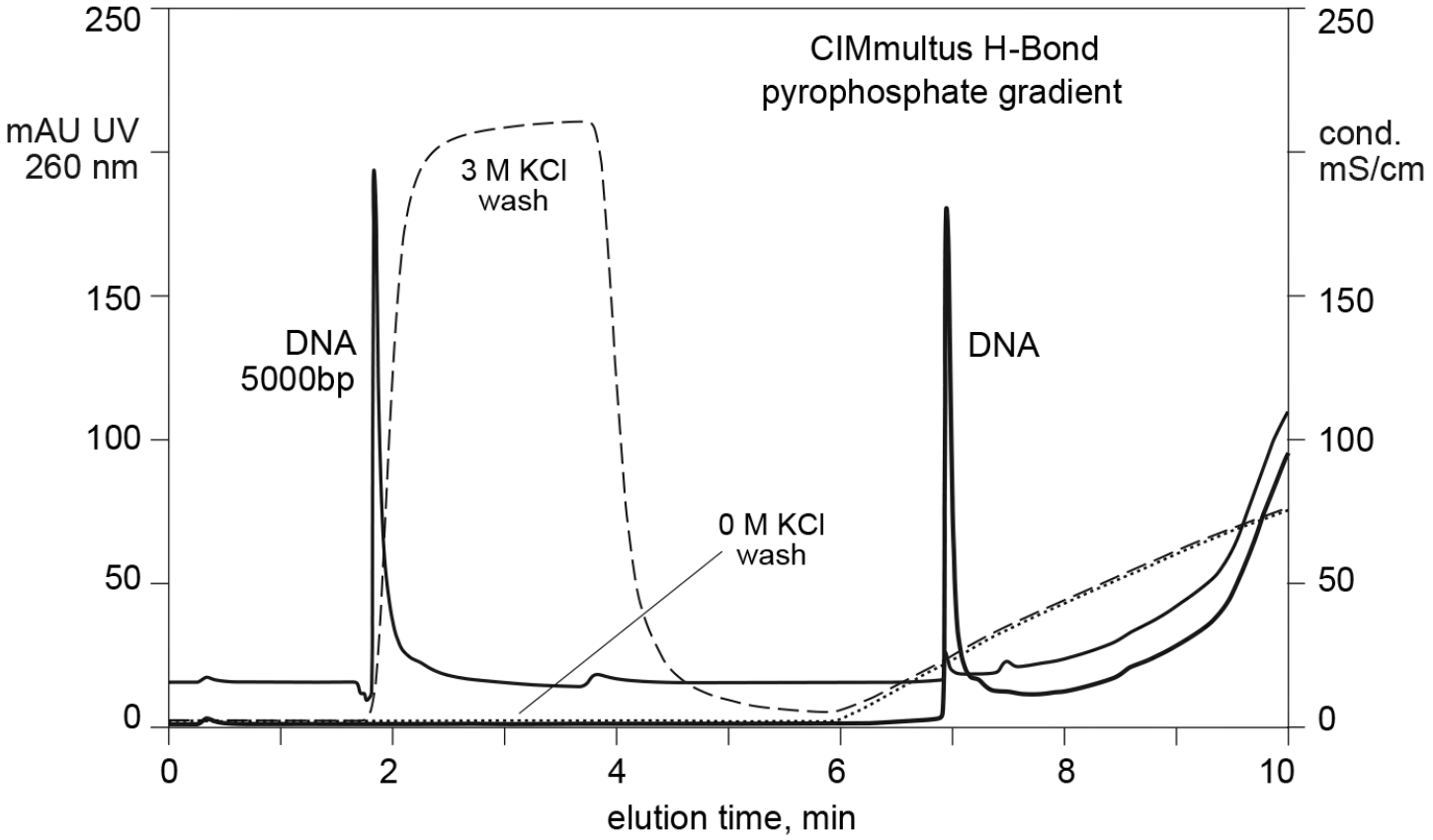


The presence of non-pyrophosphate salts shifts elution of all species to lower pyrophosphate concentration, as does increasing pH.

BIA separations

The Tools: Hydrogen bond chromatography

DNA, dsRNA, and proteins can be removed before elution with a high salt-chelator wash. Same conditions as for PrimaS.



Lacking a salt wash, DNA elutes before ssRNA in the pyrophosphate gradient. A salt wash eliminates it in advance. dsRNA too. ssRNA remains bound.

The Tools: Hydrogen bond chromatography

Answers to FAQS about eluting CIMmultus H-Bond.

Pyrophosphate needs to be removed before in vivo use. It forms crystals when it comes into contact with soluble calcium.

The most effective removal method is to follow CIMmultus H-Bond with CIMmultus PrimaS.

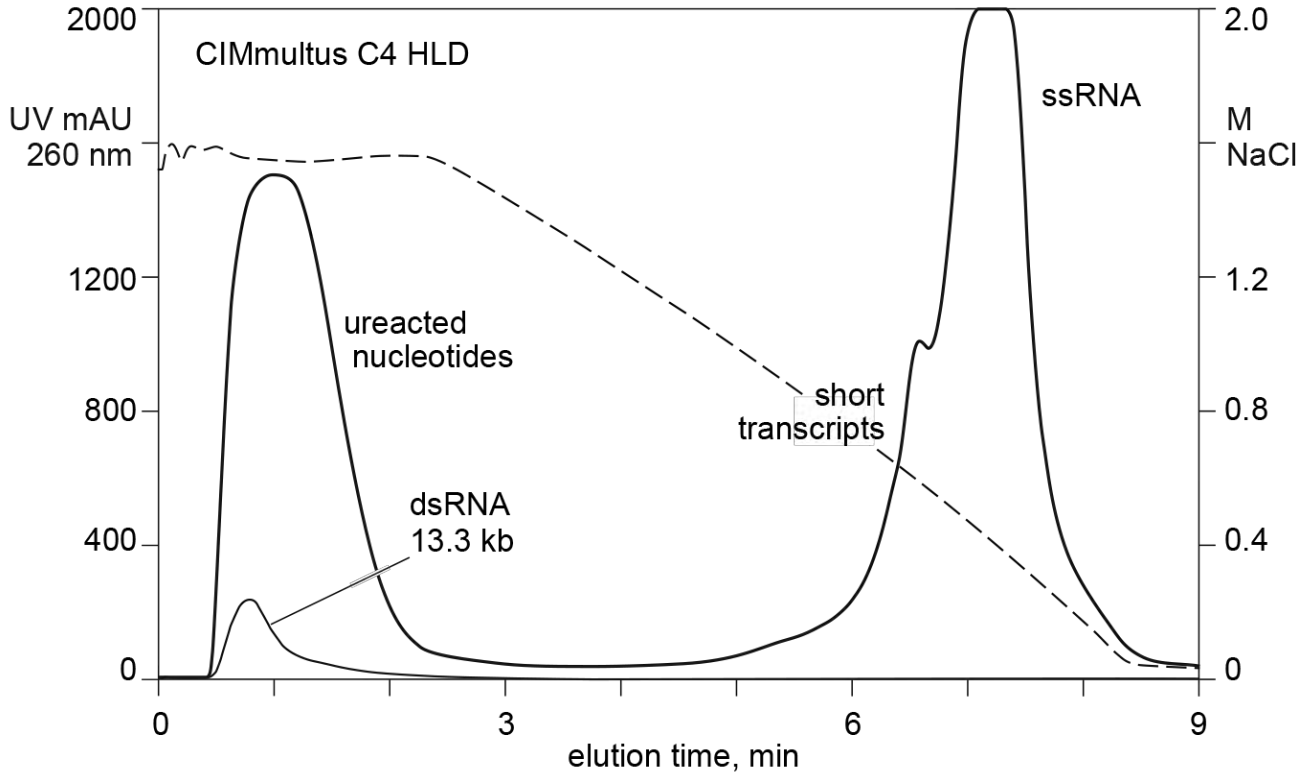
Load the ssRNA fractionation from H-Bond then wash with guanidine and EDTA. The guanidine suspends pyrophosphate binding to positively charged PrimaS. The EDTA suspends metal coordination between pyrophosphate and ssRNA.

Another approach uses hydroxyapatite particles (20–80 µm); flow-through or batch format. Pyrophosphates become part of the apatite crystal structure.

Sensitive assays to document pyrophosphate clearance are available commercially from on-line suppliers.

The Tools: hydrophobic interaction chromatography

CIMmultus C4 HLD separates ssRNA from truncated forms, dsRNA, DNA and proteins. Binding is in high salt, elution to low salt.



Binding can be performed with any salt that precipitates ssRNA. Each gives different selectivity but the general pattern is the same for all. DNA mostly flows through with dsRNA. Most proteins require NaOH for removal.

The Tools: Hydrophobic interaction chromatography

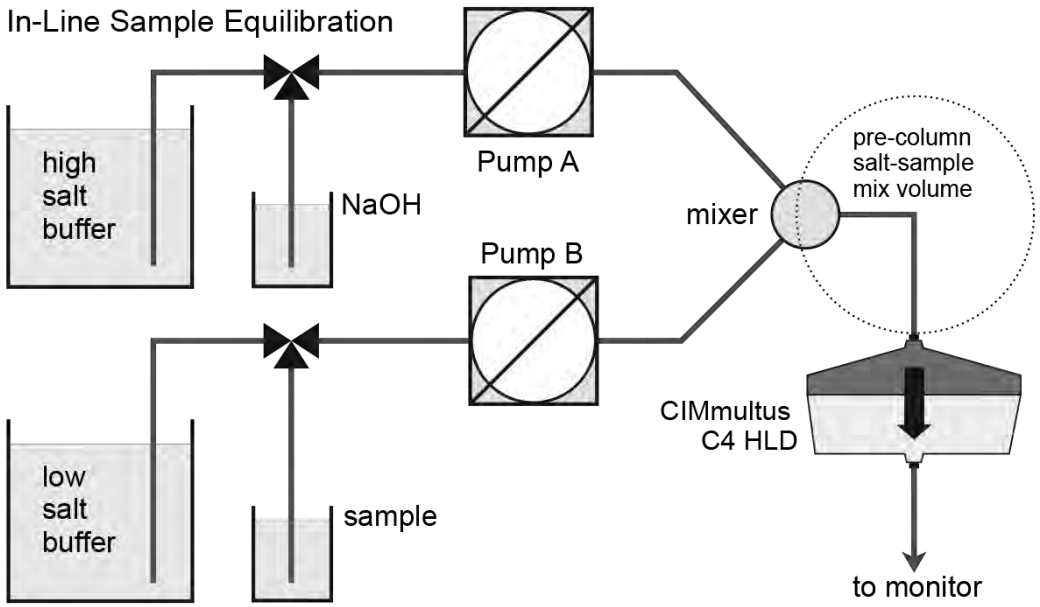
Answers to FAQS about loading sample onto C4 HLD.

The salt concentrations required to achieve high capacity binding of RNA tend to promote precipitation in the equilibrated sample.

Sample loading at industrial scale can be performed by in-line dilution.

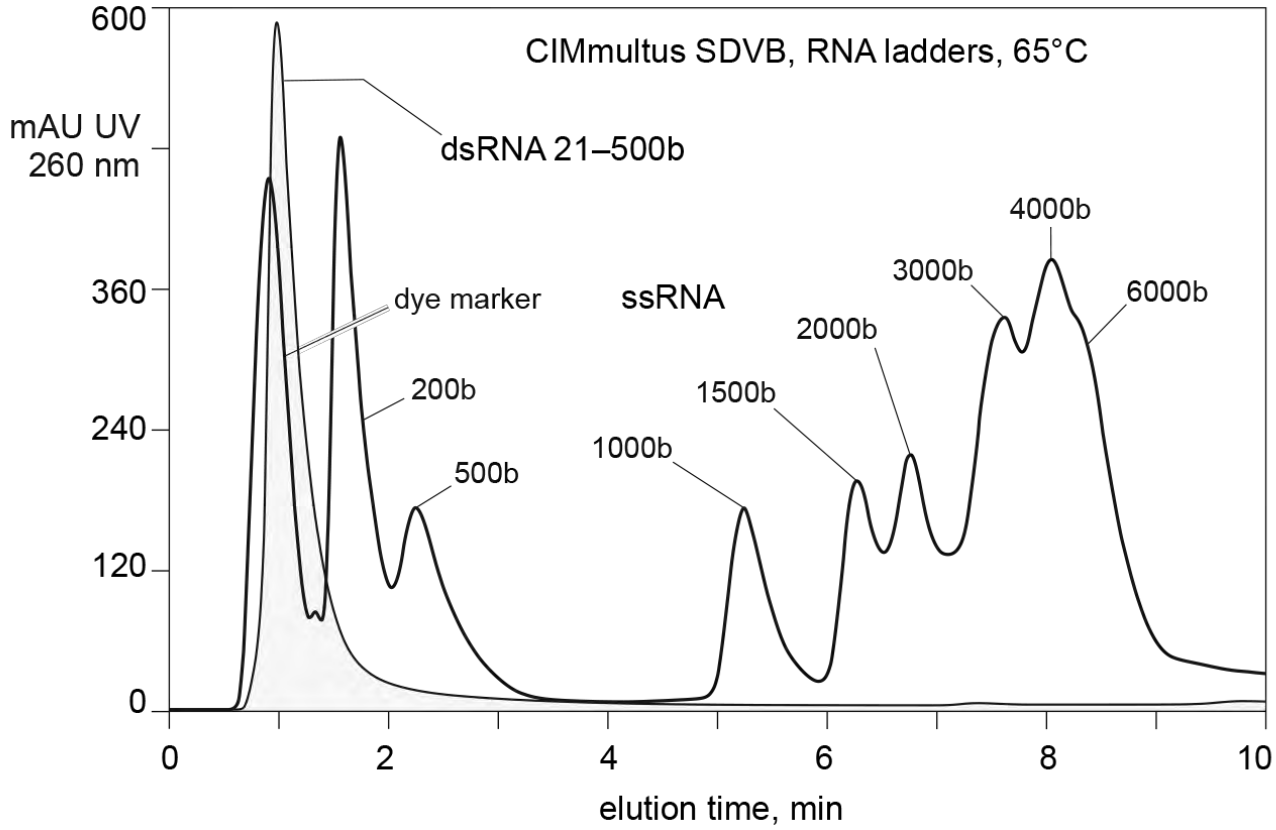
Ex: Load at 20% sample, 80% high-salt buffer.

The short duration of pre-column salt-contact prevents precipitation.



The Tools: Reverse phase chromatography

Reverse phase chromatography with SDVB is well documented for separation of ssRNA from truncated forms, dsRNA, and DNA.



Elution in an acetonitrile gradient. More information: Nwokeoji et al, J. Chrom. A 1484 (2017) 14–25 and J. Chrom. B 1104 (2019) 212–219.

The Tools: Reverse phase chromatography

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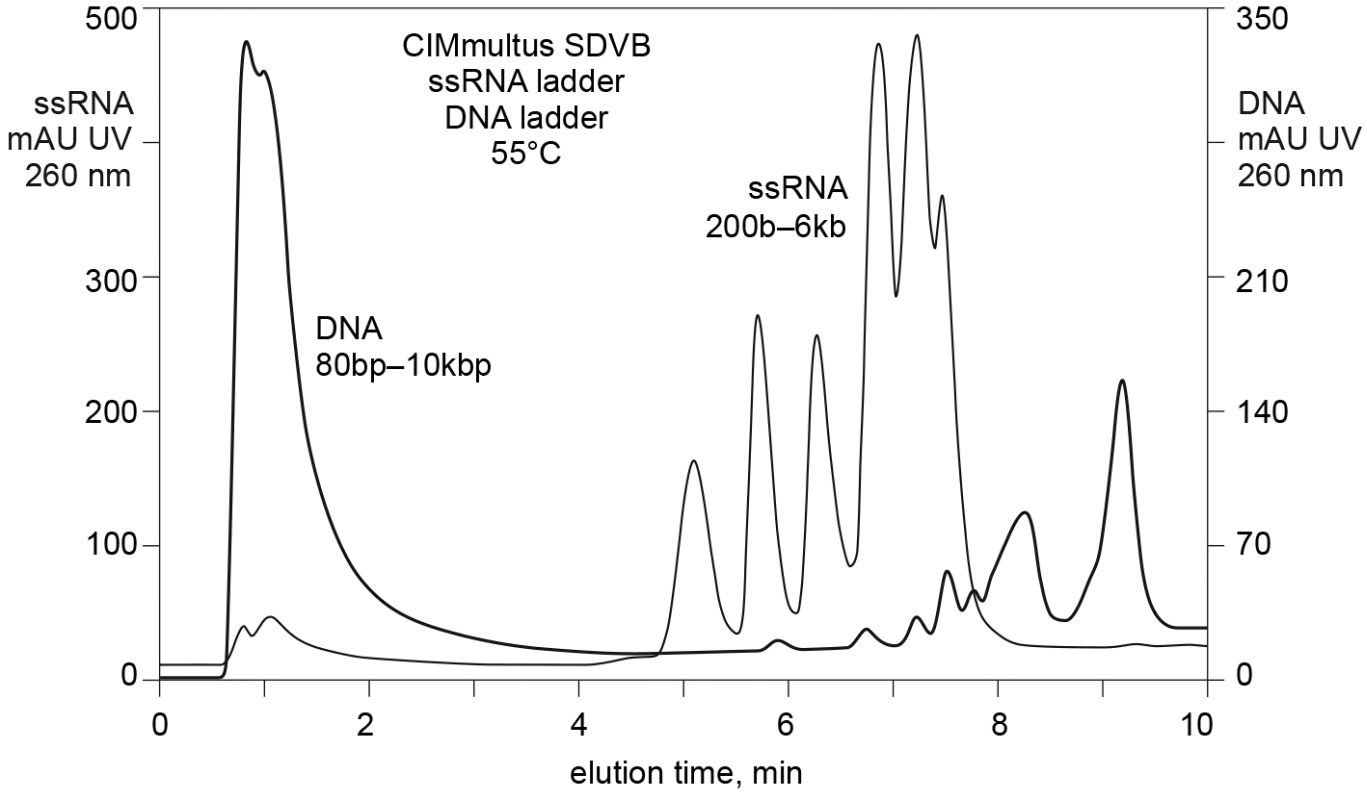

Temperature is an important process variable in RPC. Compare this profile with the previous slide at 65°C.

The Tools: Reverse phase chromatography

Answers to FAQS about eluting CIMmultus SDVB.

Reverse phase is generally best suited as a polishing method. Its intense hydrophobicity causes fouling by proteins and aggregates.

Fouling can depress capacity and separation performance.

However, SDVB is *extremely* tolerant of NaOH. Fouled columns can be cleaned with 1 M NaOH for a day or more if necessary.

Transitions between aqueous and organic solvents should always be performed as linear gradients, beginning at half the flow rate normally used for purification of ssRNA.

The gradient helps to dissiptate heat that can accumulate at aqueous-organic interfaces. The reduced flow rate is a precaution against swelling of the polymer. Sudden swelling or excess heat can damage the bed.

The New Toolbox

BIA‘s new RNA toolbox supports a variety of orthogonal two-step processes to accommodate any purification needs.

CIMmultus SDVB is best confined to polishing because of its vulnerabilty to fouling.

CIMmultus PrimaS and H-Bond are both attractive for capture because they deliver essentially 1-step purification of ssRNA. Their tolerance of chaotrope-chelator washes also helps to minimize fouling when loaded with crude feed streams.

CIMmultus C4 HLD can also be used for capture. It is less prone to fouling than SDVB but it can be a factor. The need for sample loading by in-line dilution also favors it use as a polishing step.

CIMmultus Oligo dT is also suitable for capture and does an excellent job clearing proteins and DNA. It must befollowed by a method that can remove dsRNA and short transcripts. Candidates include SDVB, PrimaS, and H-Bond.

The New Toolbox: analytics

Most of the chemistries discussed for preparative purification are also available in analytical HPLC formats to support raw material characterization, process development, validation, in-process monitoring, and charaterization of final drug product.

SDVB is well established for analytical characterization of dsRNA and discrimination of intact ssRNA from short transcripts. It can also be used to evaluate quality of raw material plasmids and enzymes.

PrimaS provides parallel ability but without the burden of requiring flammable organic solvents or elevated temperatures.

Oligo dT is ideal for fast affinity-determination of mRNA content when evaluating the impact of different materials and reaction conditions on efficiency of in vitro transcription.

All of these methods are enhanced by multi-detector monitoring, such as fluoresence and DLS or MALS in addition to UV.

Availability

CIMmultus	Beta-testing	Size	Delivery time	DMF/RSF
Oligo dT		all sizes available, all ligands available	up to 6 months depends on ligand availability)	Nov 2020
PrimaS	Available from stock	all sizes available by July 2020		Jan 2021
H-Bond	Available in July 2020	all sizes available by November 2020		June 2021
C4-HLD		all sizes available	up to 4 months	available
SDVB	Available from stock	all sizes available by November 2020		June 2021

For more information

BIA provides usage recommendations describing how to screen, optimize, clean, and store each of the tools discussed in this presentation for purification of mRNA.

These recommendations also include more extensive and detailed discussion of most of the issues raised in this presentation.

For product information, technical support, to inquire about beta testing, or to inquire about process development services, please contact:

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