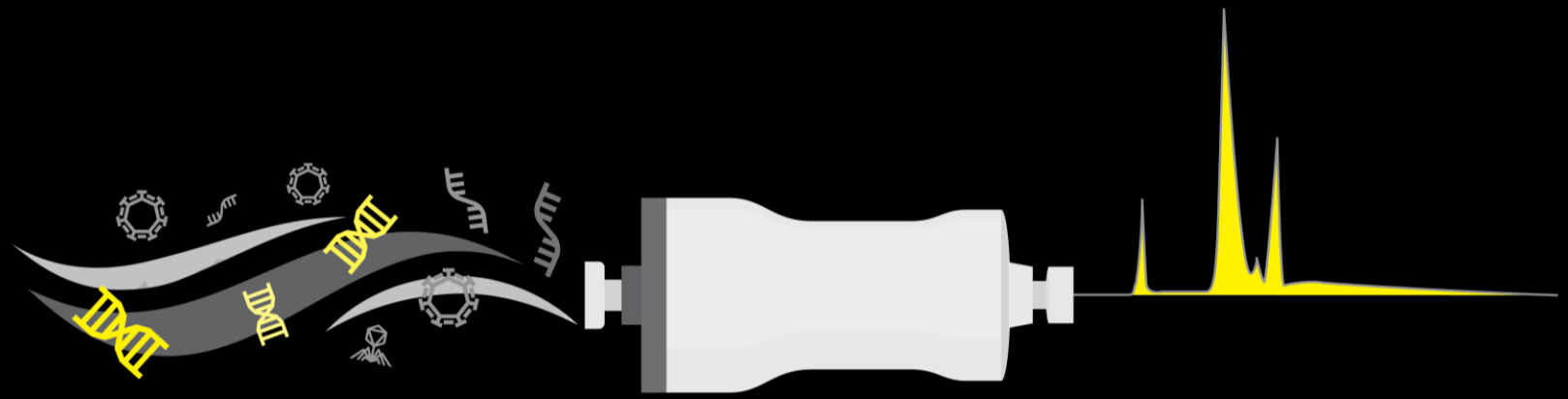
Simplifying Progress



From *E. coli* to mRNA Drug Substance – Development of Analytical and Purification tools for a scalable mRNA production process

mRNA Therapeutics Summit, 14 July 2021

Rok Sekirnik, Head mRNA/pDNA Process Development

BIA separations

SARTORIUS

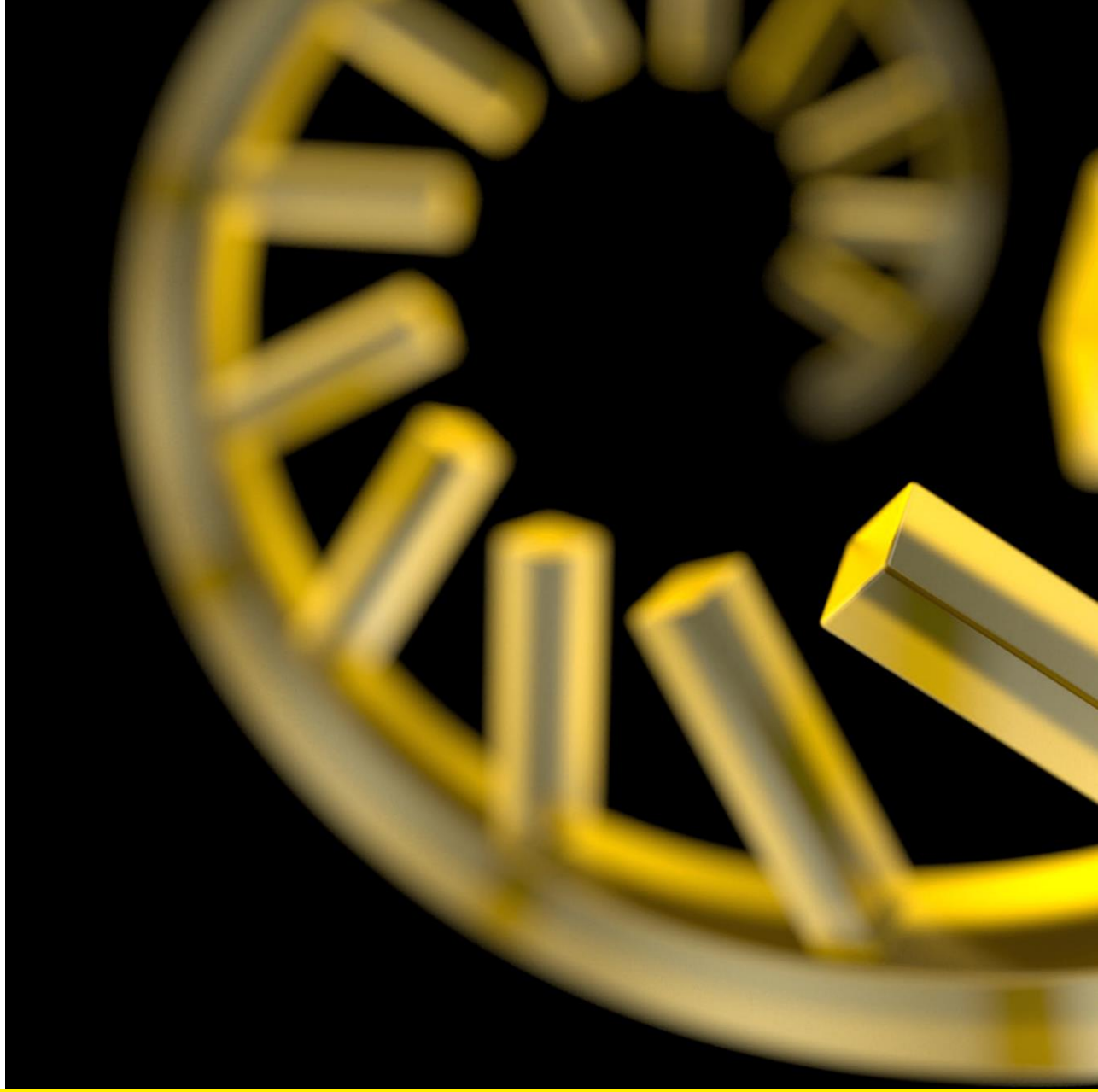
Agenda

1 Looking ahead

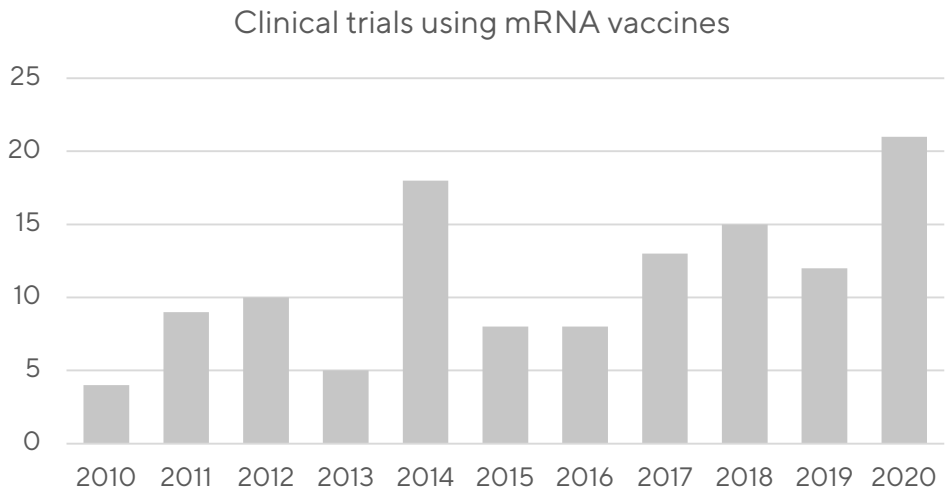
2 Structure of mRNA

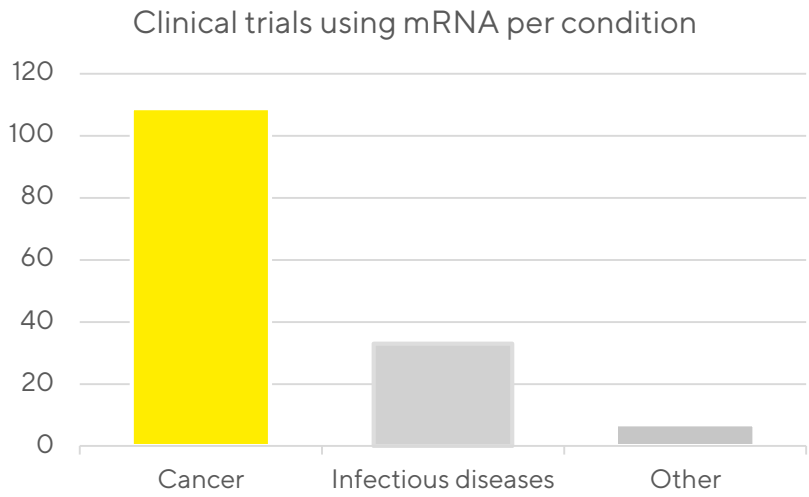
3 Analytical Bottlenecks in mRNA production

4 Adapting to Purification Challenges



Looking Ahead of the Covid-19 Pandemic



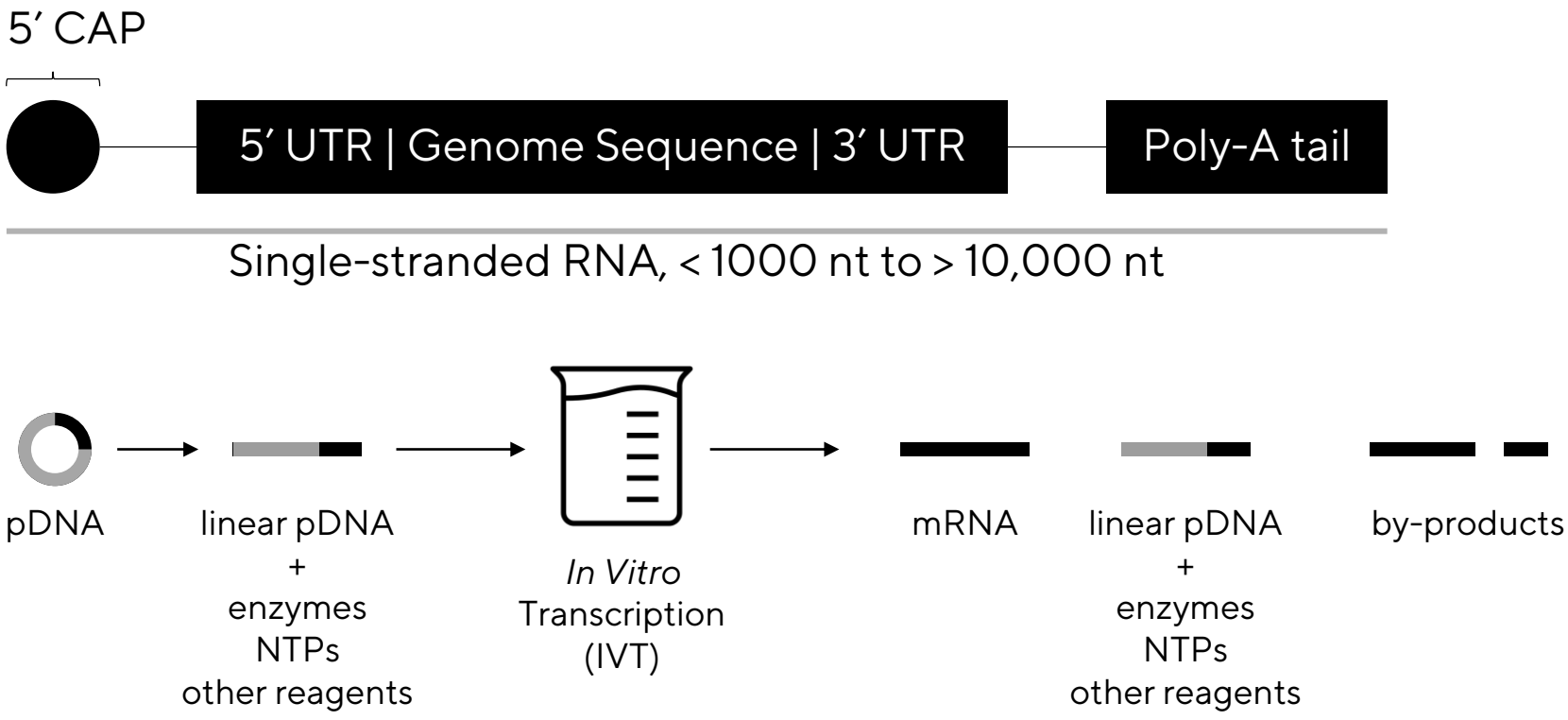


- Current demand for mRNA is generated primarily from COVID-19. Approval of Pfizer/BioNTech and Moderna vaccines brings closer to reality using mRNA in other therapeutic areas, such as cancer and infectious disease.
- Scalable purification methods using chromatography will replace laboratory scale methods such as precipitation. Early implementation can lead to faster development and shorter time to clinic.

Adapted from Rosa, Sara Sousa, et al. "MRNA Vaccines Manufacturing: Challenges and Bottlenecks." *Vaccine,* vol. 39, no. 16, 2021, pp. 2190-2200.

mRNA – What and Why?

Mature mRNA Structure



- 5′ Cap and poly-A tail are required for successful protein expression in cells.
- *In Vitro* transcription produces RNA from a DNA template, often a plasmid DNA
- 5′ Cap can be added co-transcriptionally (during IVT), or post-transcriptionally
- Poly-A tail can be encoded in the DNA template, or added enzymatically after IVT

Good analytical understanding of IVT reaction is fundamental to maximise productivity and improve robustness.

mRNA
Analytical Bottlenecks in Production

Building Up IVT Understanding With Analytics

Analytical method	Parameter
RiboGreen	RNA content
qPCR	Identity
AGE	RNA, DNA size and nucleic acid contamination
Capillary electrophoresis, Fragment analyzer	RNA size, purity
SDS PAGE, NanoOrange, BCA	Protein content
HPLC	Purity, content
LC-MS	Capping

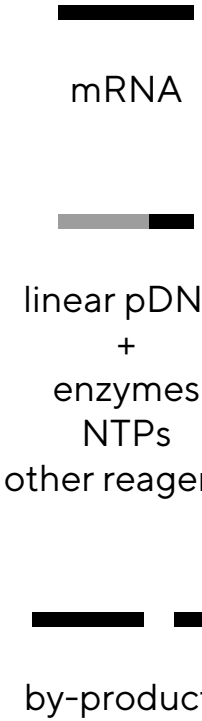
Common laboratory methods for characterisation of IVT products.

- Optimisation of reaction conditions for different RNA constructs can lead to improved process performance, notably for longer RNA molecules, such as saRNA
- IVT contains a large number of variables to measure and adjust: ratio of individual reagents (nucleotides, capping reagent, polymerase, pyrophosphatase, etc), buffer conditions (Mg^{2+} ion, DTT, etc), reaction time, temperature
- Modelling of reaction kinetics in relation to the interaction between reagents can further improve our process understanding.
- Most methods are time consuming, lack automation, and most of all, measure one parameter at a time.

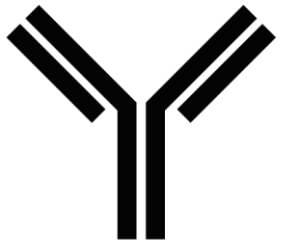
mRNA
Challenges with Purification

What Are We Separating mRNA From?

- IVT reaction components (DNA template, enzymes, NTPs, capping, other reagents)
- Contaminants in the raw materials:
  - Plasmid: *E.coli* proteins, DNA, and RNA.
  - Enzymes: fragments, residual DNA, residual RNA, residual proteins.
- Endotoxins, if introduced during processing
- Reagents used for any additional processing, such as plasmid digestion post-synthesis or capping enzymes
- RNA variants: dsRNA, truncations, fragments, aggregates.

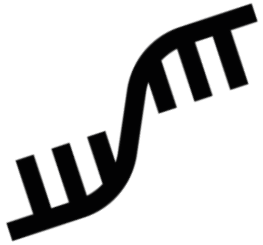


mRNA is large



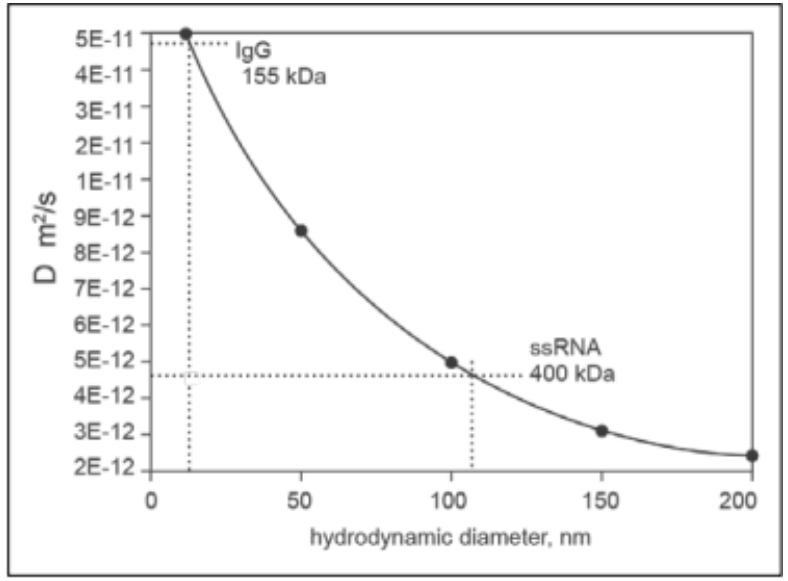
IgG – 150 kDa

~11.5 nm



1200 nt mRNA – 400 kDa

~107 nm hydrodynamic size



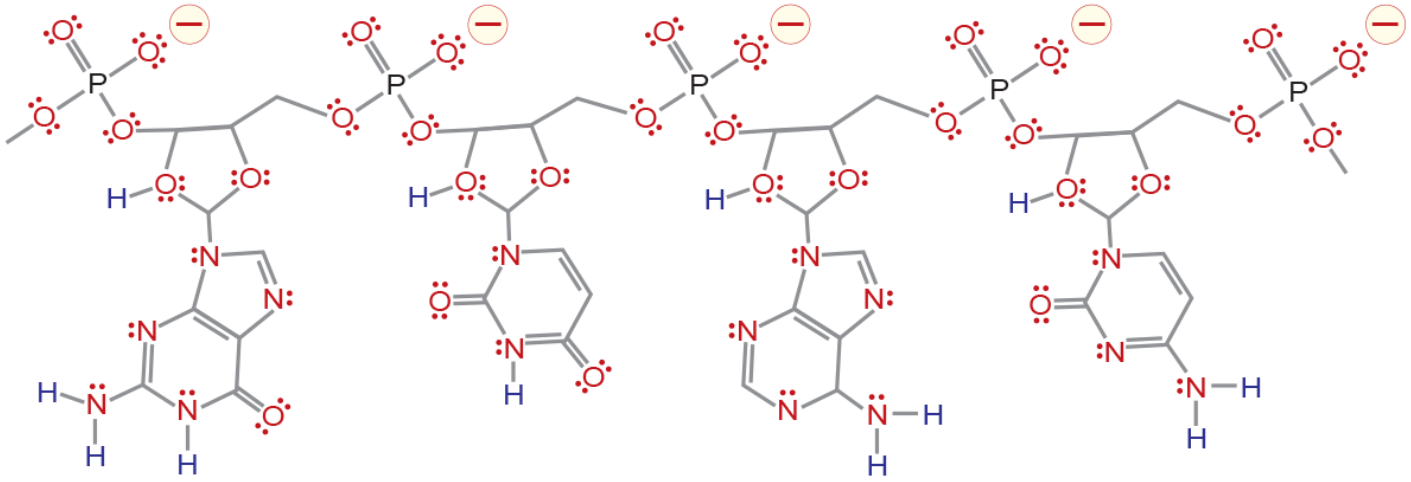
Relative diffusion constants of 11.5 nm IgG and 107 nm mRNA

Size is an indicator of RNA behaviour in solution, and also its vulnerability to *shear stress*.

Selection of filtration and purification media are critical to ensure molecular stability and purification performance.

Complex Chemistry of mRNA

- Evenly distributed negative charge along the backbone, with pKa of about 2.6 (i.e. charged across working pH) provides a good basis for ion exchange chromatography.



Base pairing

Adenine (A) – Uracil (U)/Thymine (T)

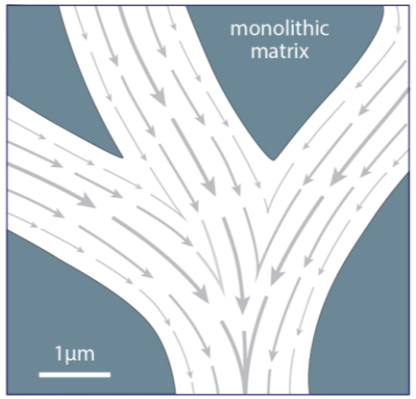
Guanine (G) – Cytosine (C)

- The nucleoside moiety contains both hydrogen donors and acceptors. High density of hydrogen-bonding nucleic acid residues determines the conformation, solubility, and interactions with contaminants and chromatographic surfaces. It also provides a basis for alternative purification, exploiting hydrogen bonding.

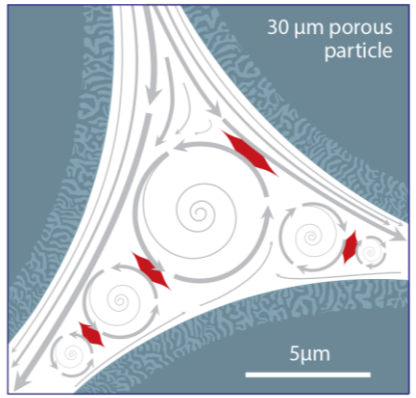
Chromatography Media Selection for Capture from IVT mixture

Accessible surface area for large mRNA molecules ⟩ Low shear environment ⟩ Convective mass transport ⟩ High capacity and easy scalability ⟩ Suitable chemistry selection

Monolith



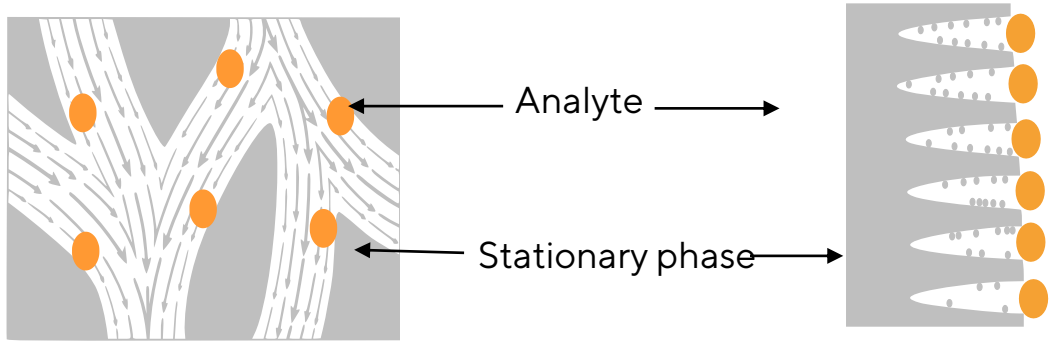
Porous particle



Laminar flow in **monoliths** produces very low shear. **Turbulent flow** in **porous particles** produces zones of countercurrent flow that create high shear stress

Monolith

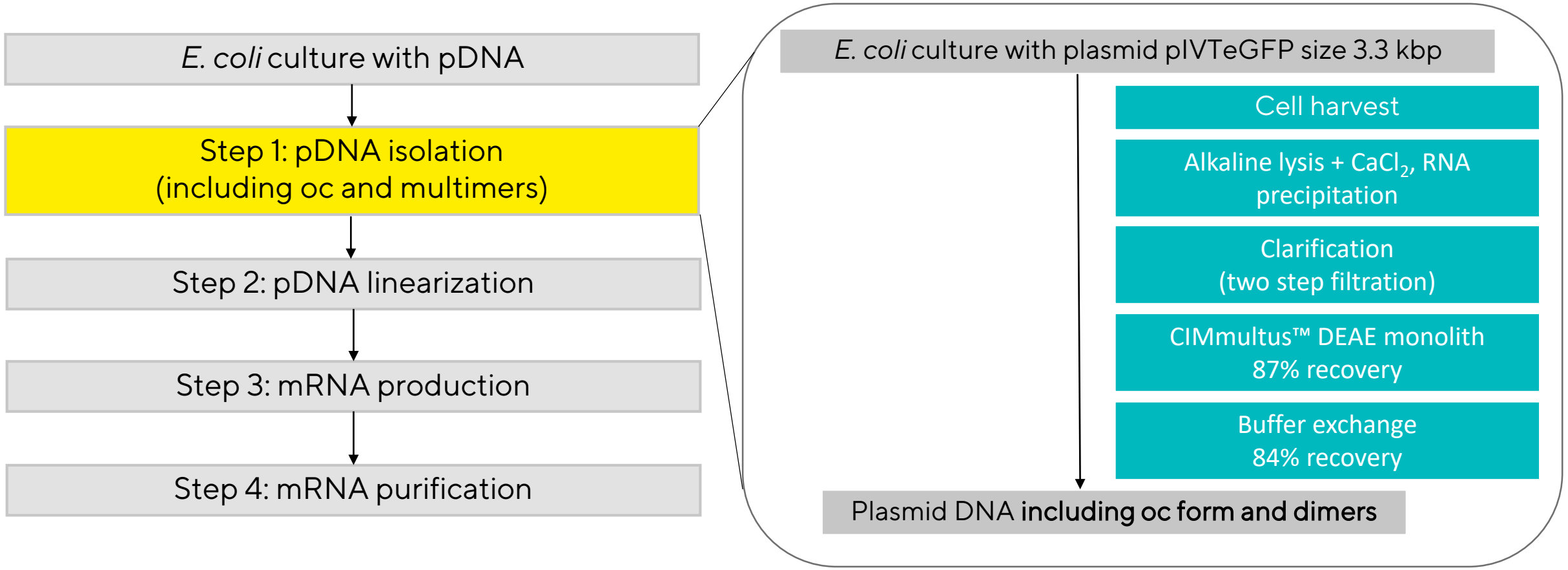
Porous particle



Large flow-through channels provide complete surface accessibility to large mRNA molecules. This enables high capacity. Lack of dead-end pores (no diffusion) provides flow-independent performance.

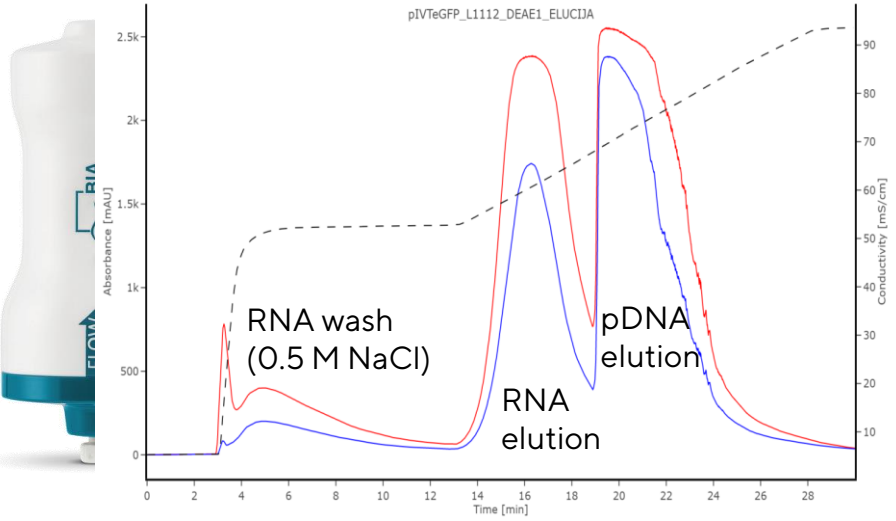
mRNA
From cell lysate to mRNA DS

From *E.coli* to pure mRNA



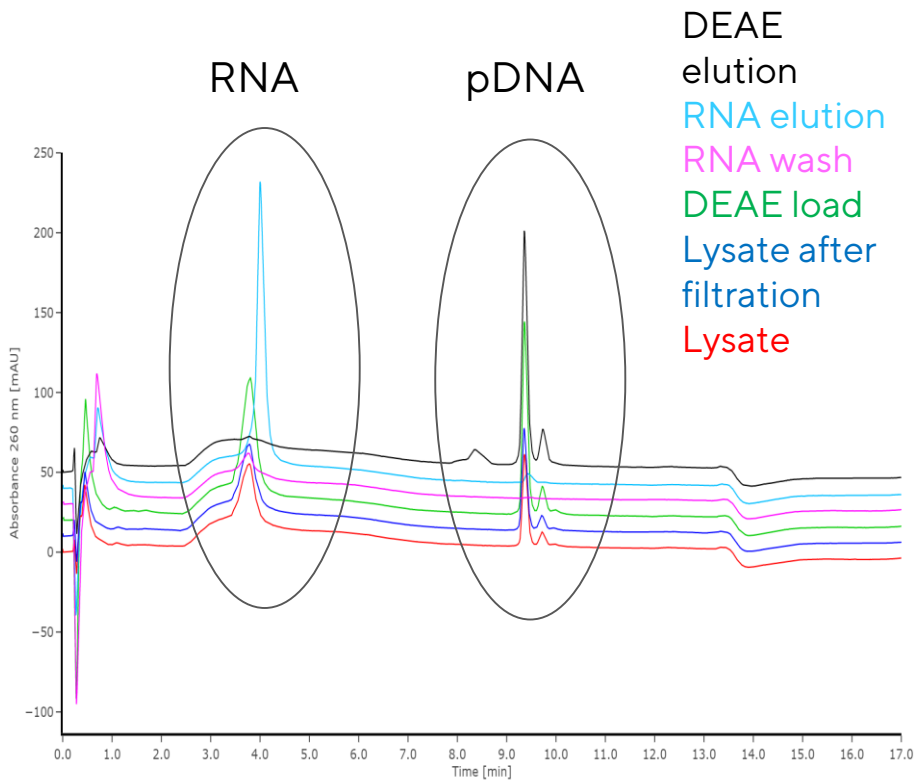
Purification of pDNA (3.3 kbp) Using CIMmultus DEAE Column

CIMmultus ™ DEAE - preparative

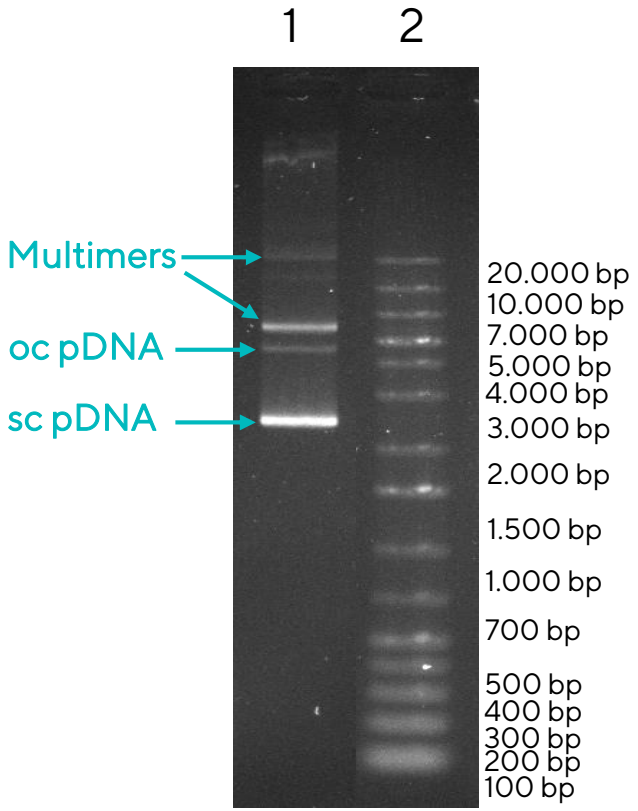


pDNA purification	
pDNA recovery in elution (%)	87
RNA removal (%)	> 99 %
oc pDNA (%)	3
Multimers (%)	25

HPLC CIMac™ pDNA - analytical column

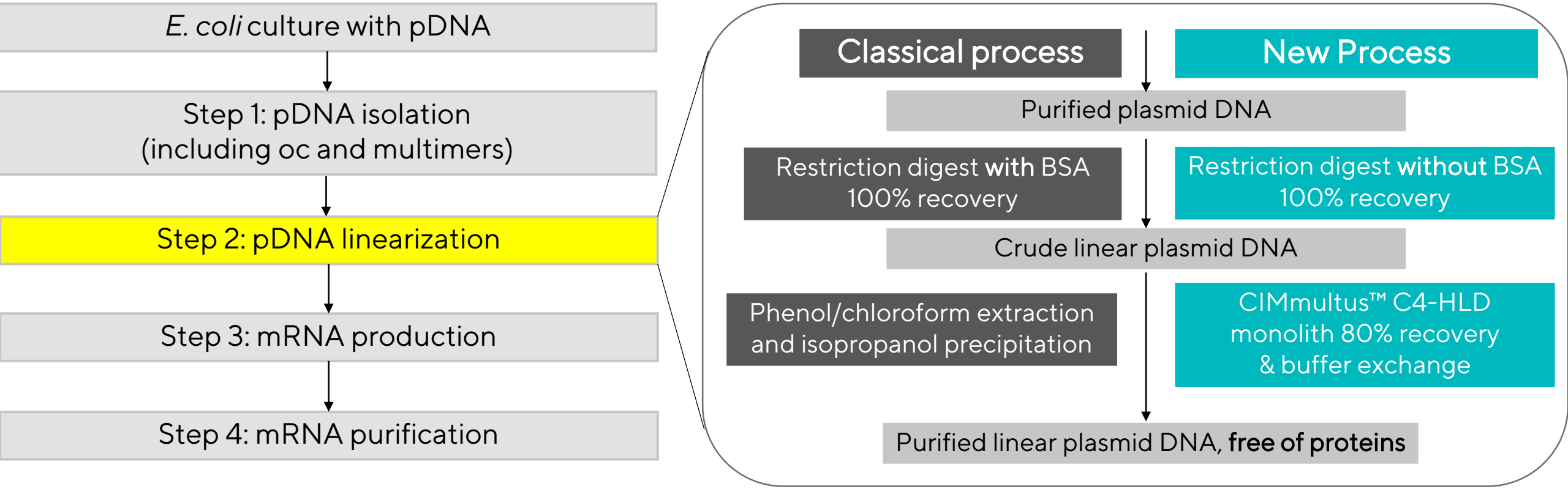


AGE



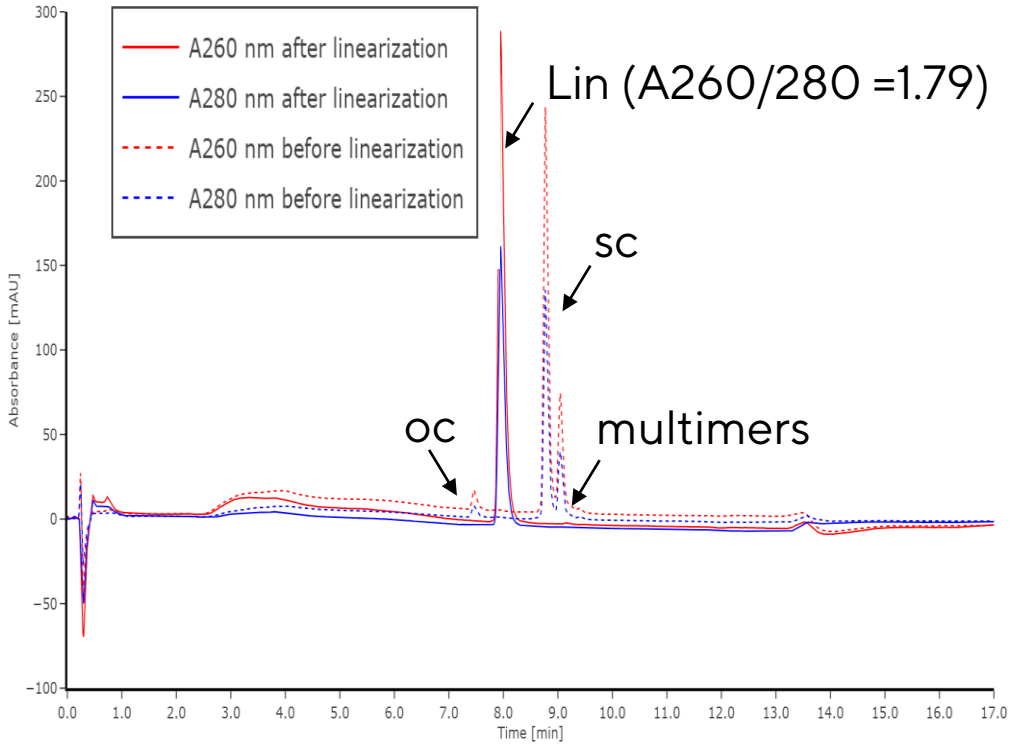
1: purified eGFP plasmid
2: GeneRuler 1 kb Plus

Step 2: pDNA Linearization Summary

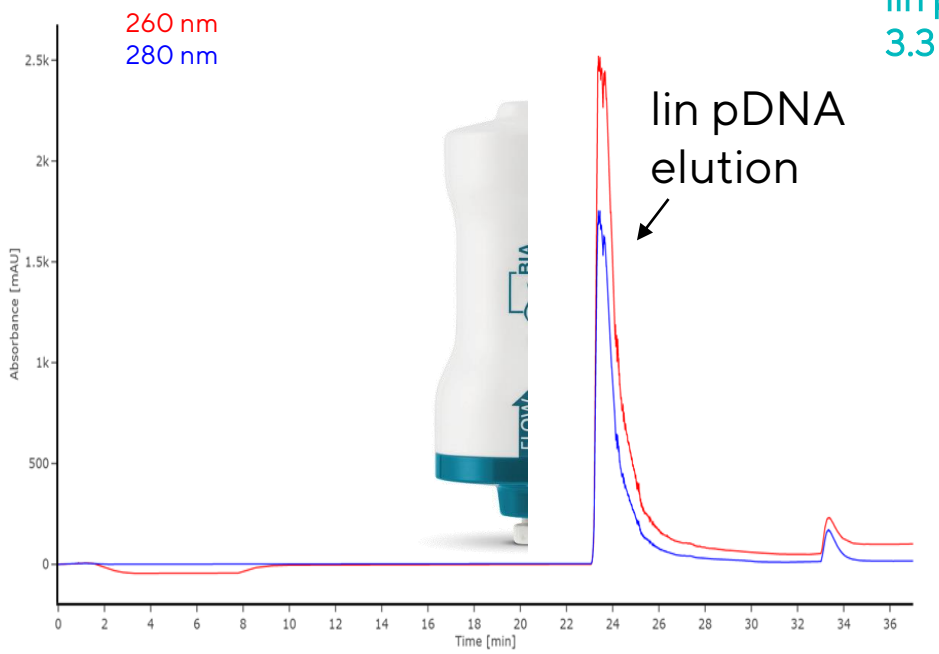


Purification of Linear pDNA Using CIMmultus™ C4 HLD Column

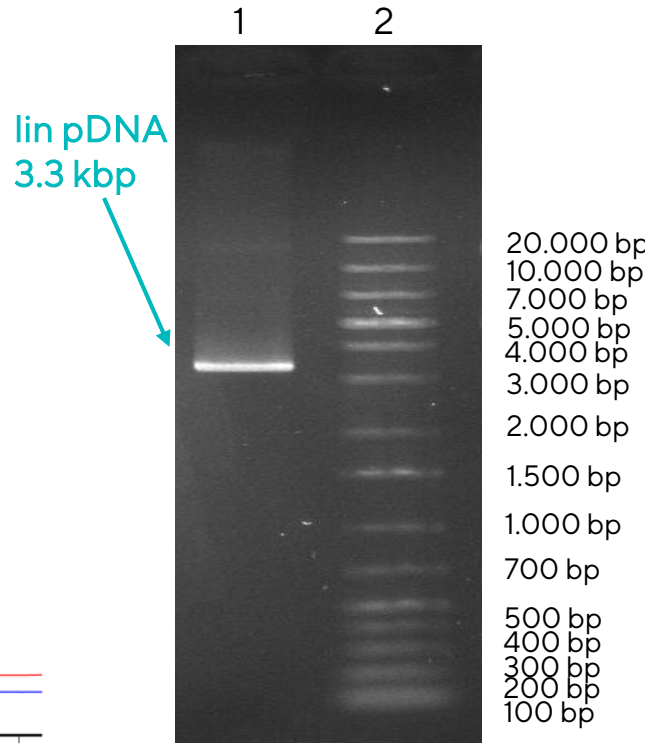
CIMac™ pDNA – analytics of linearization kinetics



Preparative CIMmultus™ C4 HLD



AGE

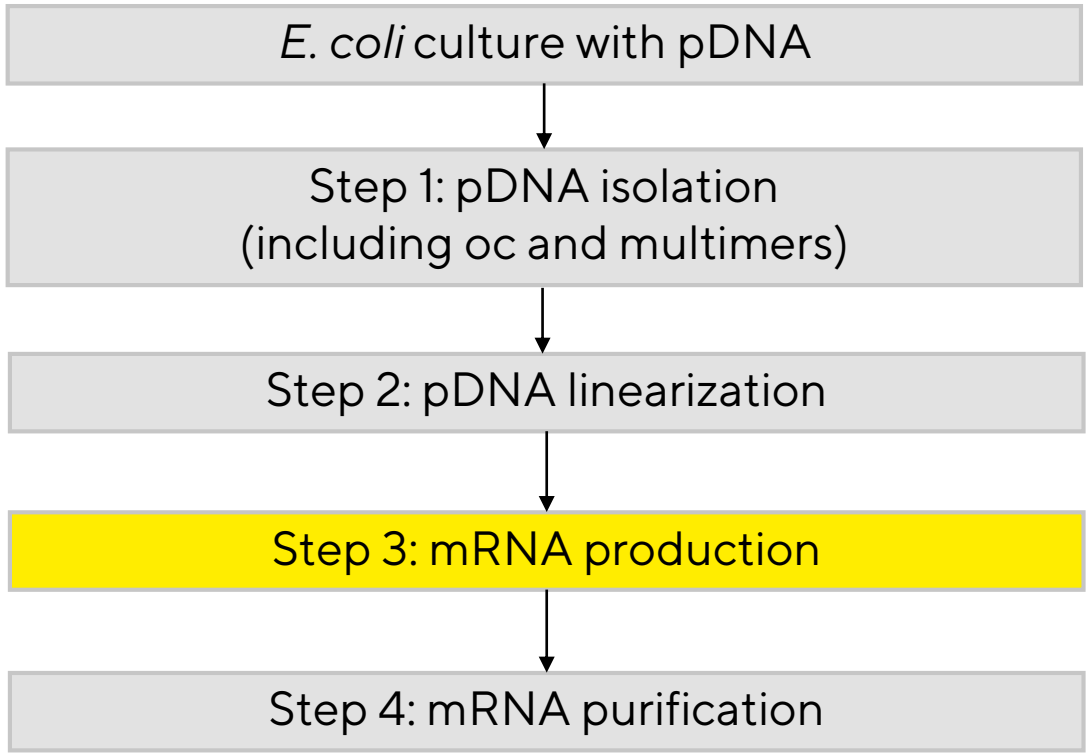


1: linear plasmid
2: GeneRuler 1 kb plus

Linearization kinetics is monitored by CIMac pDNA HPLC analytics. Baseline separation of lin isoform from oc/sc/multimer isoforms, 17 min run time.

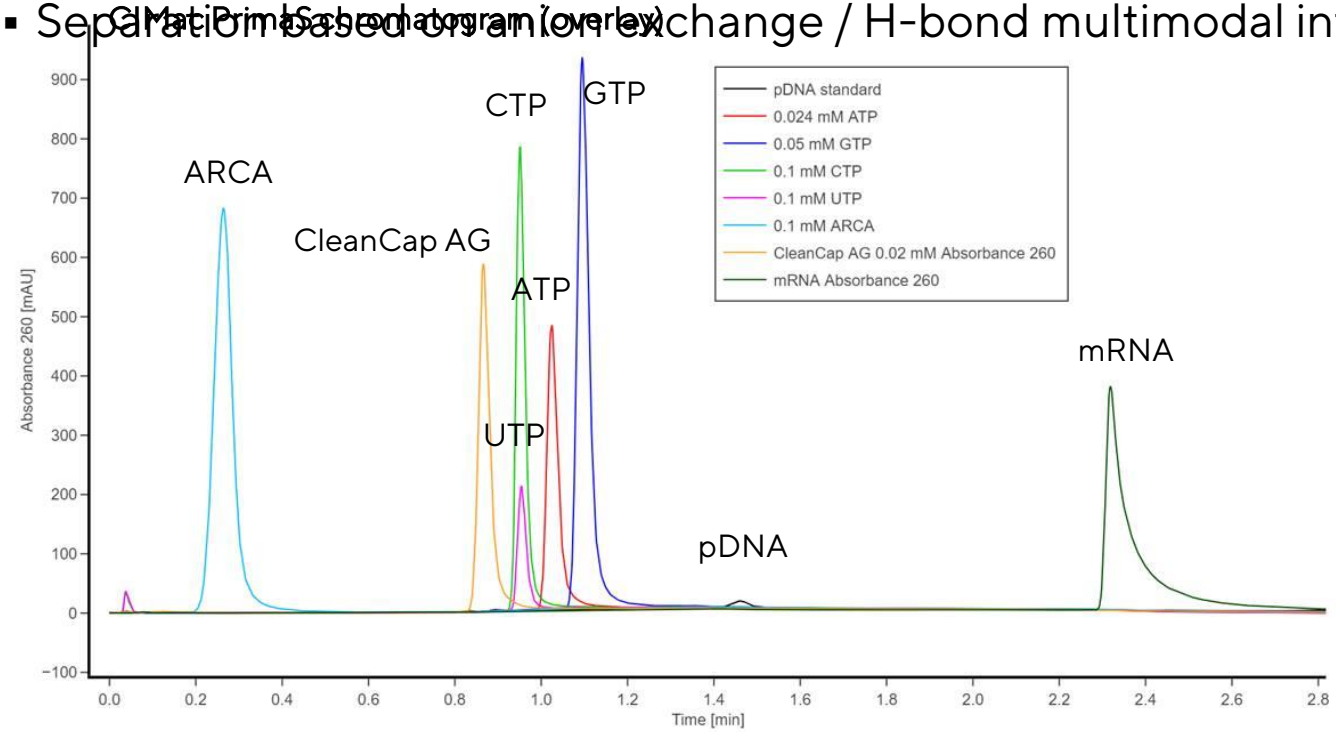
C4 HLD is used for purification of DNA from protein contaminants, followed by UF/DF in to IVT-compatible buffer using, e.g. Sartocon 10 kDa ECO Hydrosart.

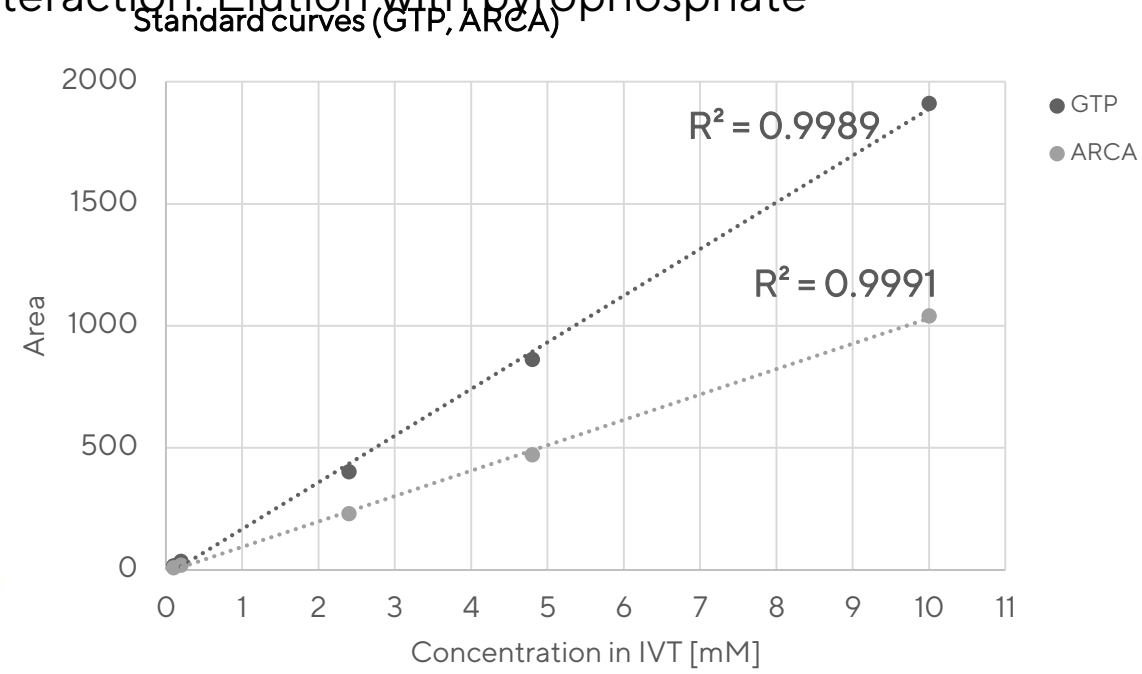
Step 3: IVT


E. coli culture with pDNA
Step 1: pDNA isolation
(including oc and multimers)
Step 2: pDNA linearization
Step 3: mRNA production
Step 4: mRNA purification

Building Up IVT Understanding With Analytics

- CIMac PrimaS separate and quantifies multiple individual IVT reaction components in a single, rapid assay
- Separation based on anion exchange / H-bond multimodal interaction. Elution with pyrophosphate

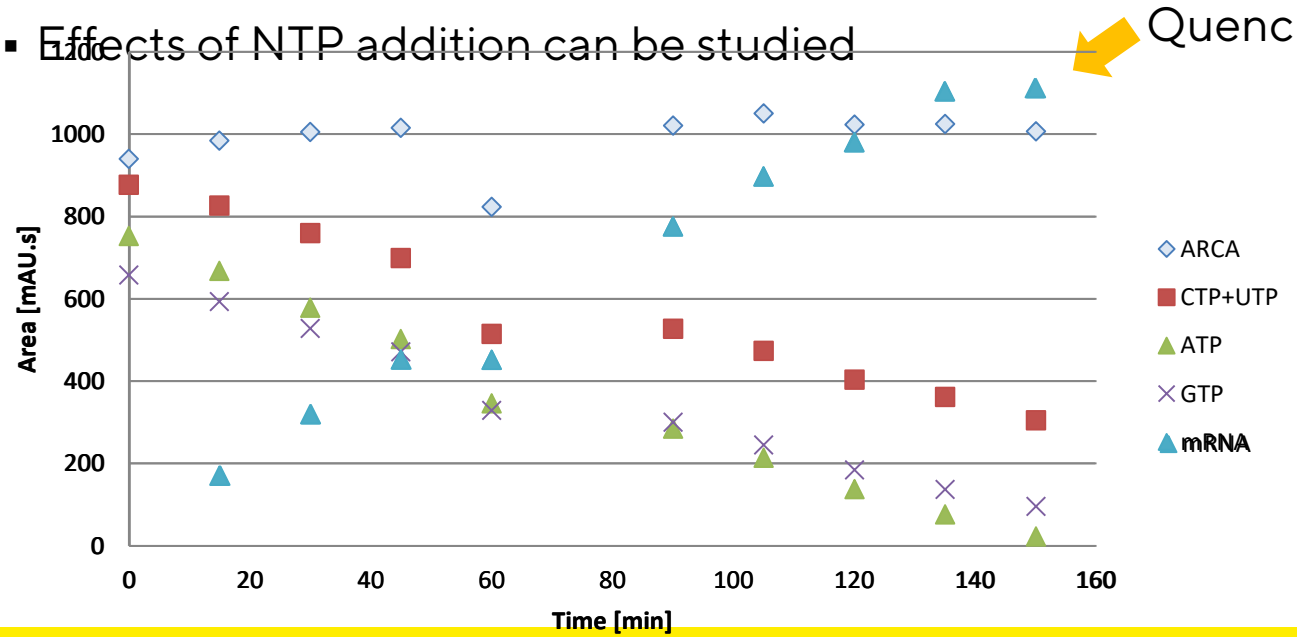


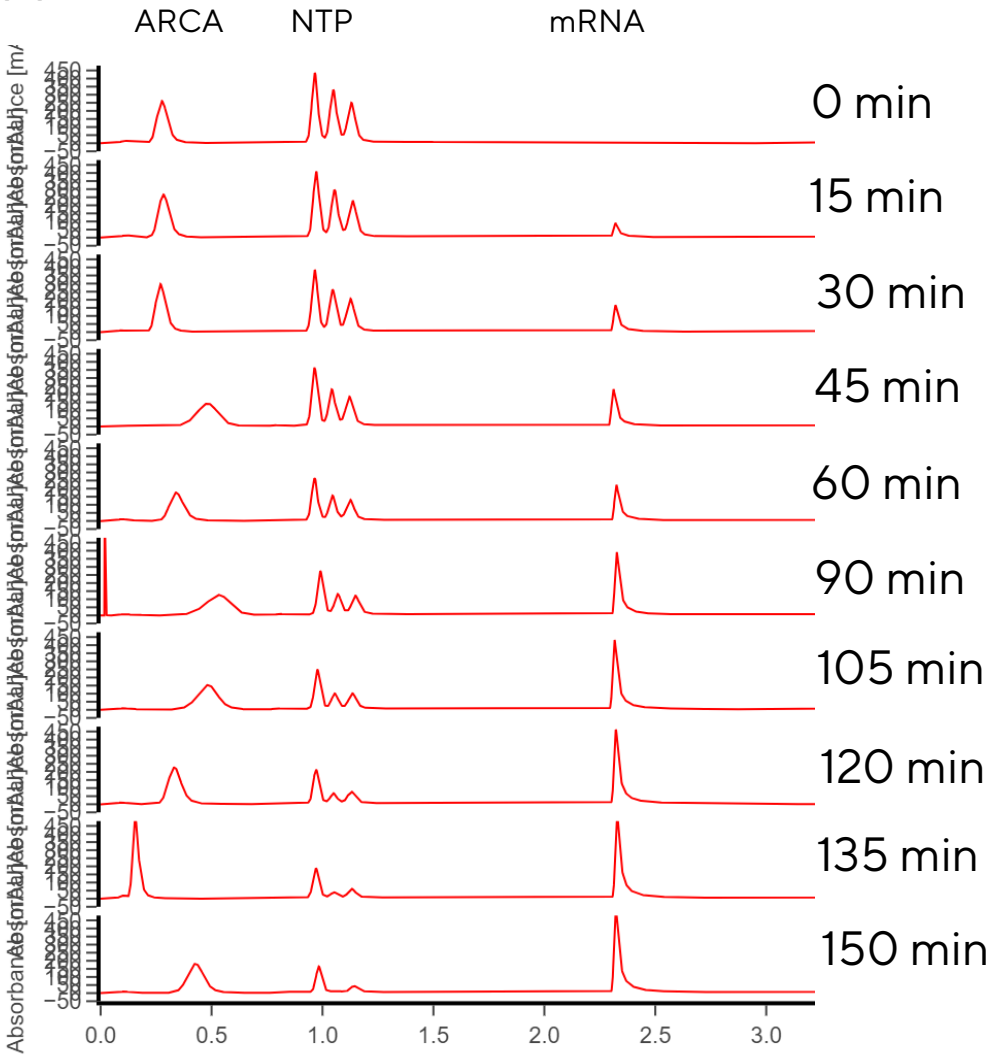


CIMac PrimaS (P.N. 110.5118-2), Buffers: MPA (50 mM HEPES pH 7), MPB (50 mM HEPES, 200 mM sodium pyrophosphate, pH 8.5), Method: 0–1 min (100 % MPA), 1–1.8 min (gradient to 20 % MPB), 1.8–2.5 min (gradient to 80 % MPB). Full method not shown. Flow rate 2 mL/min, PATfix™ HPLC system, UV absorbance at 260 nm, injection volume 25 µL.

Building Up IVT Understanding With Analytics

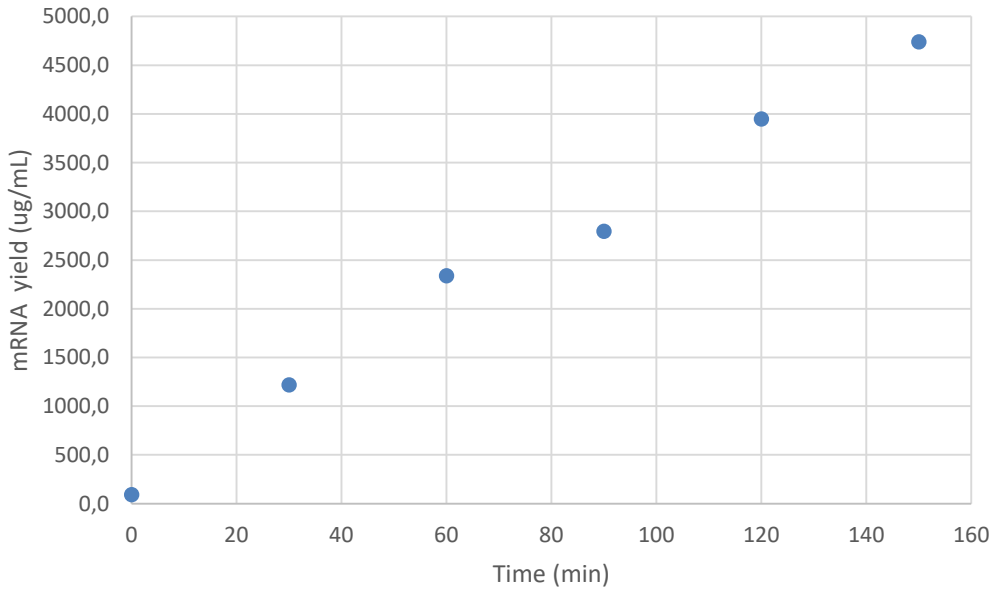
- The IVT reaction can be monitored at-line **by CIMac PrimaS**
- mRNA production kinetics is monitored. Productivity maximum can be identified, to prevent degradation.
- Consumption of nucleotides and concentration of capping reagent can simultaneously be monitored (multiple capping reagents)
- Effects of NTP addition can be studied

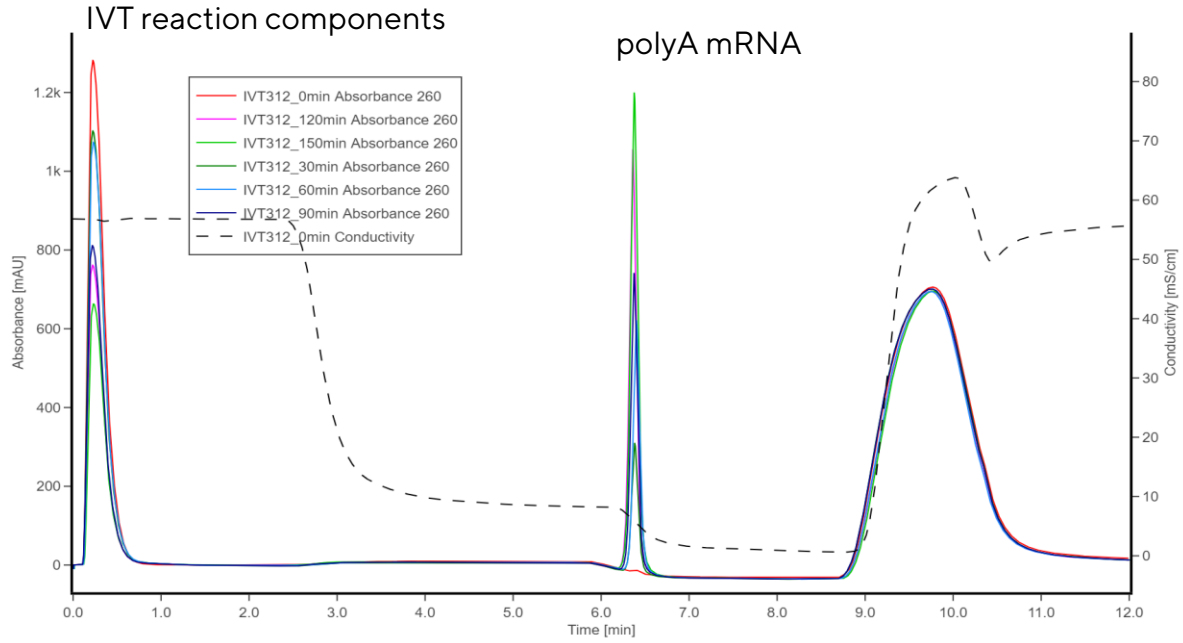




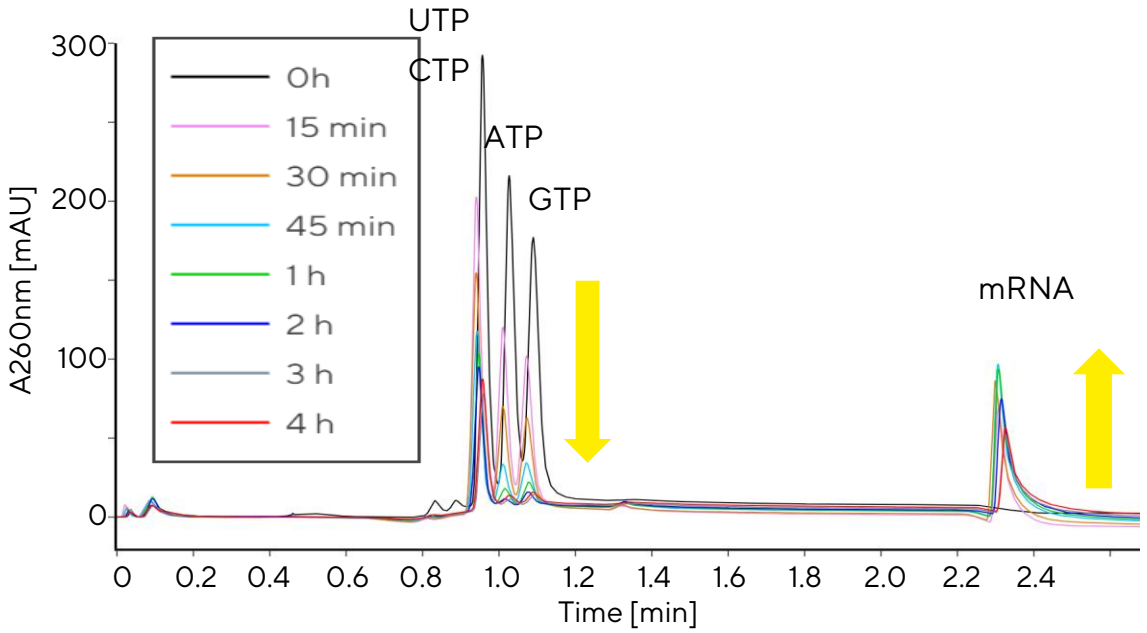
Building Up IVT Understanding With Analytics

- IVT reaction for constructs containing poly-A tail can also be monitored at-line by **CIMac OligodT**
- mRNA production kinetics is monitored
- Content measurement can be used to determine sizing of the chromatography column used for capture



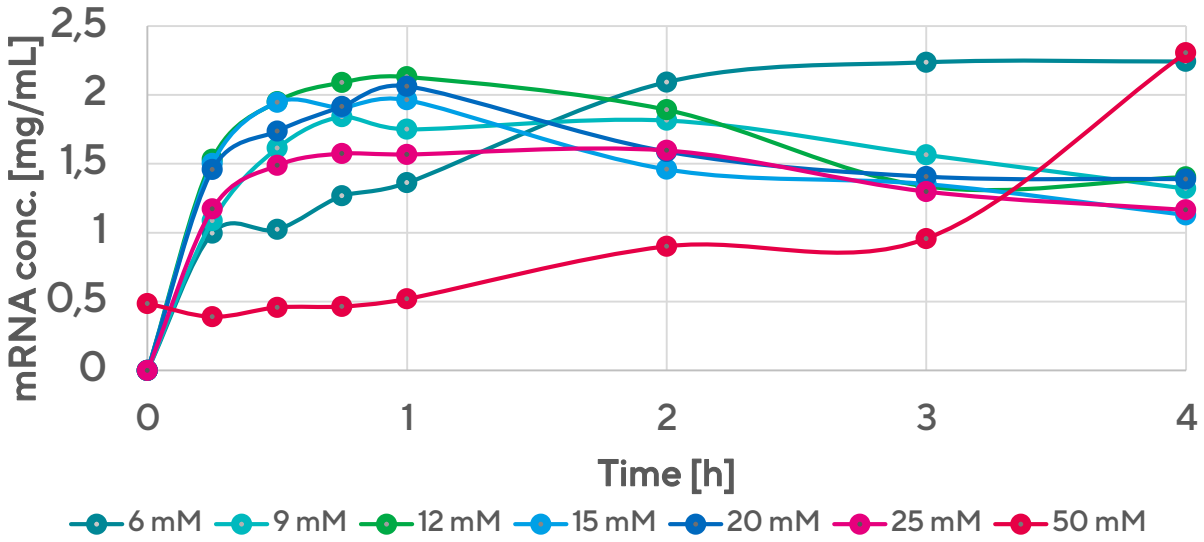


Building Up IVT Understanding With Analytics – optimization of IVT components

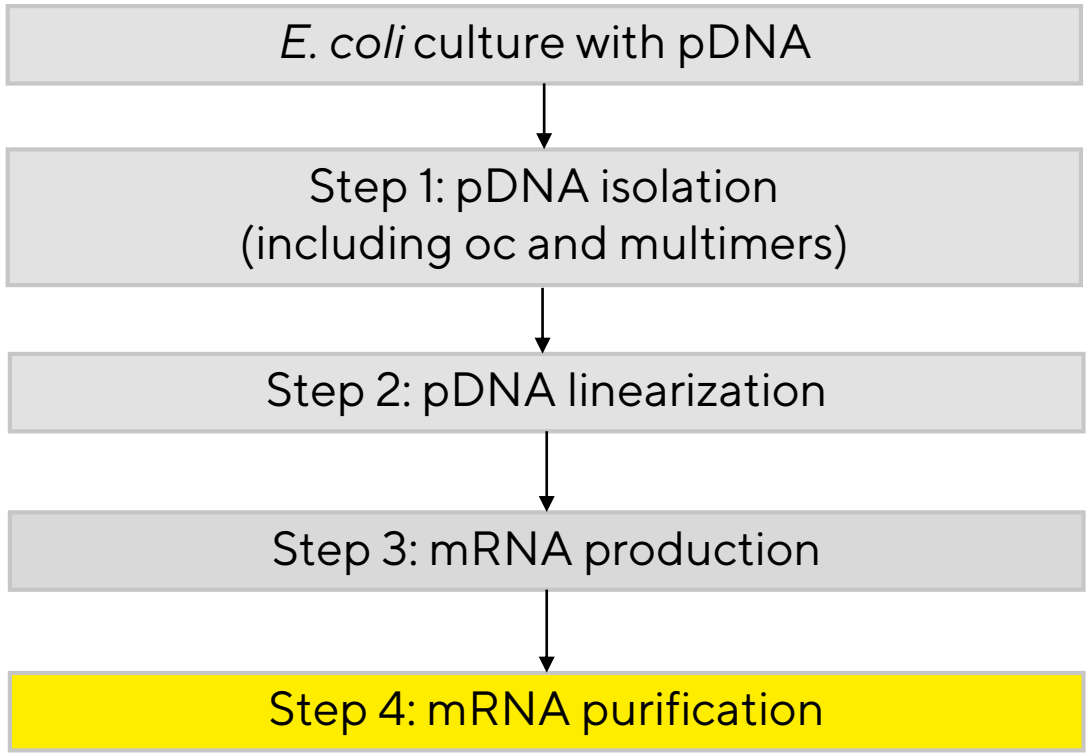


Sample: IVT reaction inactivated with EDTA and diluted in MPA (200-fold dilution). Conditions: 20 µg/mL linear pDNA, 500 U RNA polymerase per µg pDNA, 4 mM ATP, CTP, UTP and GTP each, 1U/ µL RNAse inhibitor, 1U/ mL pyrophosphatase, X mM Mg^{2+}

- The reaction can be analysed at regular short intervals (e.g. 10 min), rather than at selected end-point
- Reaction kinetics are modelled as a function of a specific component (shown for [Mg^{2+}] below)
- Kinetics of nucleotide consumption is monitored, as well as rate of production of mRNA



Low sample consumption (~250 ng RNA per analysis)


E. coli culture with pDNA
Step 1: pDNA isolation
(including oc and multimers)
Step 2: pDNA linearization
Step 3: mRNA production
Step 4: mRNA purification

Selecting the Optimal Ligand Chemistry

- Currently there is no single IVT platform for mRNA production
- Key elements of mRNA (5'-cap and poly-A tail) can be added in a one-pot reaction, or post-transcriptionally
- Purification solutions are needed to accommodate different processes

- Capture of polyadenylated mRNA directly from IVT mixture
- Affinity purification

Oligo dT18

- Capture of all mRNA directly from IVT mixture
- Anion-exchange & Hydrogen-Bonding mixed mo

PrimaS

Sartorius mRNA toolbox

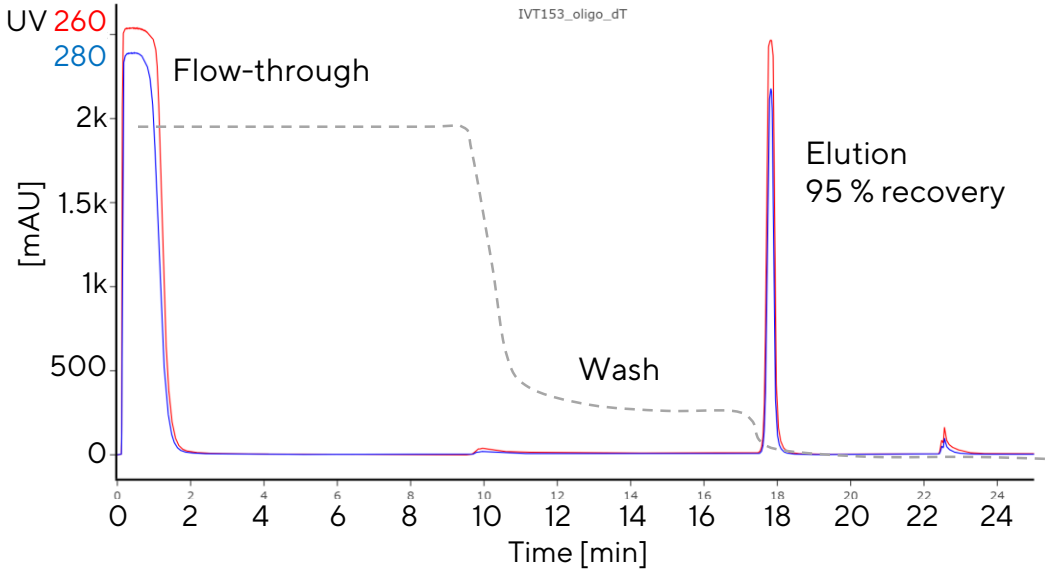
Two capture options cover different production scenarios – affinity, anion exchange

Multiple polishing options – targeted removal of residual impurities, can be selected as needed

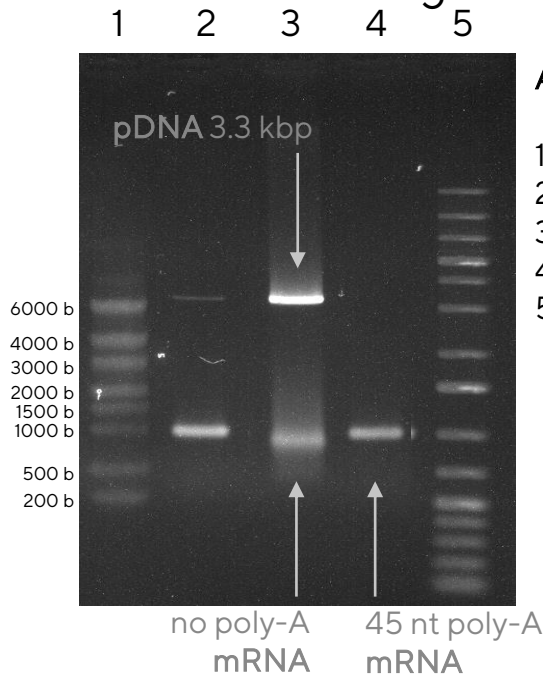
Adapts to upstream process, accommodates manufacturing requirements (e.g. high temperature, constraint on organic solvents, etc.)

Affinity Capture with Oligo dT18

- Pseudo-affinity capture for mRNA with poly-A tail based on hybridization on poly-dT
- Loading the sample at elevated salt concentration (e.g. 500 mM NaCl) reduces repulsion of negative charge on the nucleotide chains, and allows hybridization of base pairs (poly-A – dT). Elution in water or buffer in 2-3 column volumes. No need for buffer exchange.



Oligo dT purification of 950 nt mRNA with 45 nt poly-A tail. Load in 50 mM phosphate, 500 mM NaCl pH 7. Elution in 10 mM Tris.



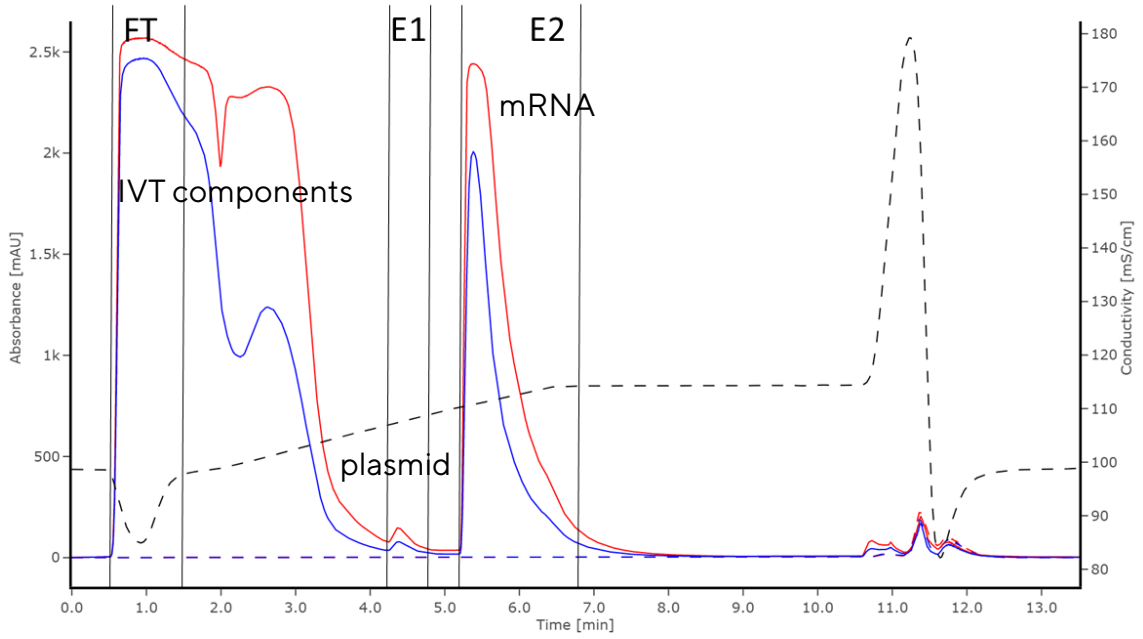
AGE

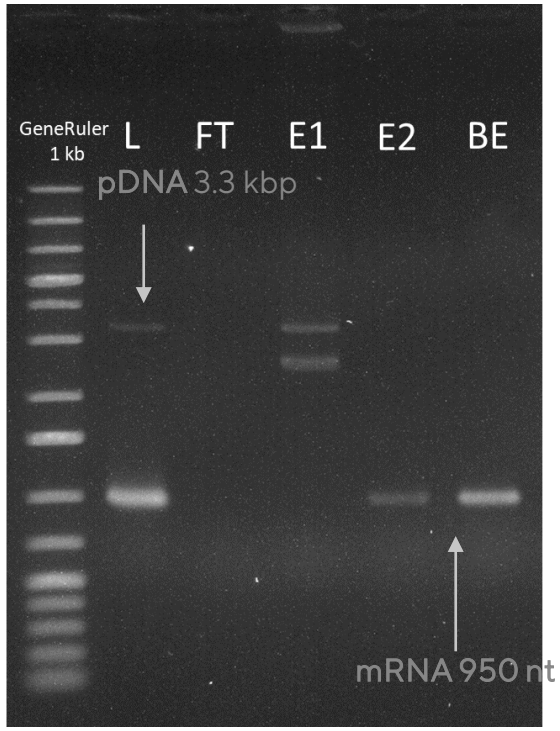
1 RiboRuler HR
2 IVTmix
3 Flow-through (concentrated
4 Elution
5 GeneRuler 1kb Plus

Capacity up to 3 mg/mL. High flow rate and short processing time.

Multimodal Capture of mRNA Without Poly-A Tail

- Multimodal anion exchanger elutes mRNA at ambient temperature.
- Elution at elevated pH (8-9). DNA elutes before ssRNA, achieving additional template clearance, if DNAse is not used in process



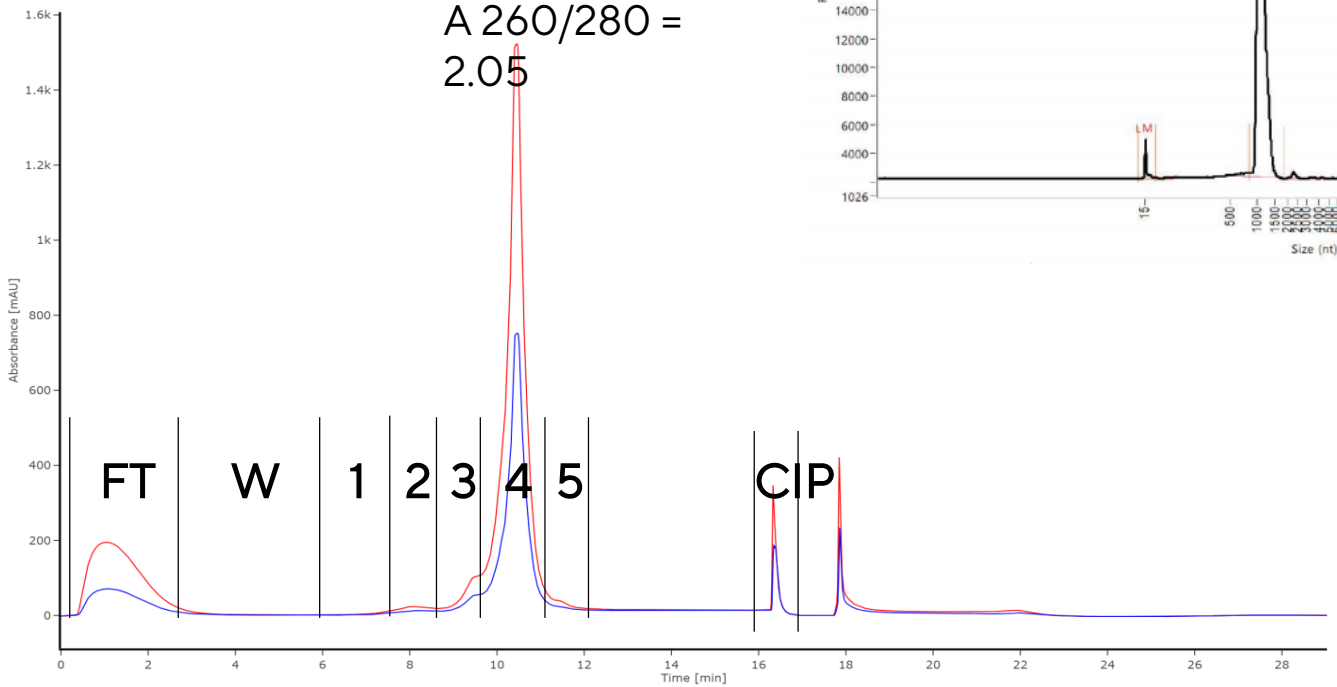


Sample: eGFP IVT mix. Column: CIMmultus PrimaS 1 mL MPA: 50 mM HEPES 1 M NaCl pH 7.5 MPB: 50 mM HEPES 1.2 M NaCl pH 10 MPC: 0.1 M NaOH + 2 M NaCl MPD: 1.5 M HEPES pH 7. Sample elutes into neutralizing solution, e.g. CH3 COOK pH 5.5.

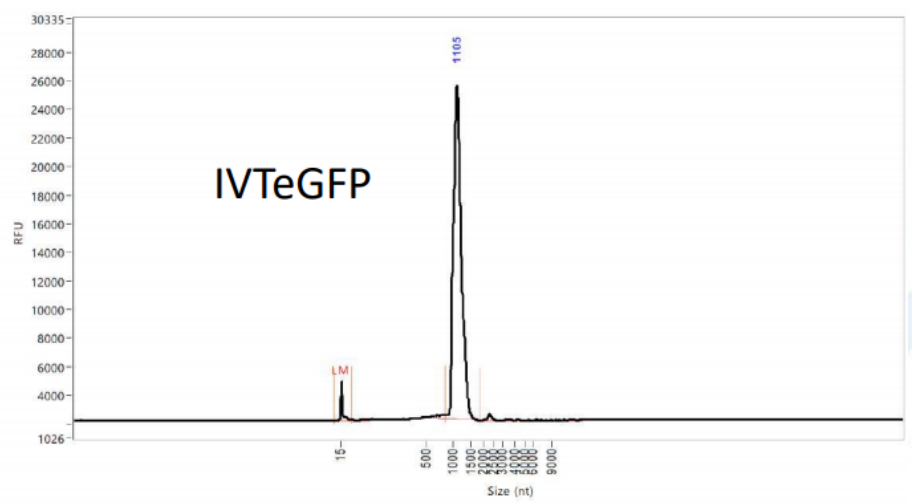
mRNA polishing – C4 HLD

C4

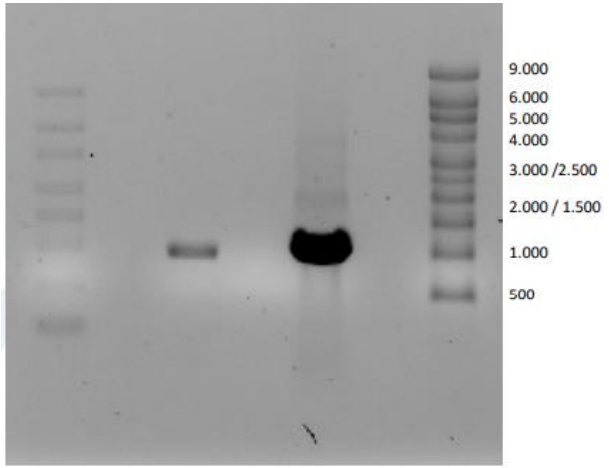
Elution mRNA
A 260/280 = 2.05



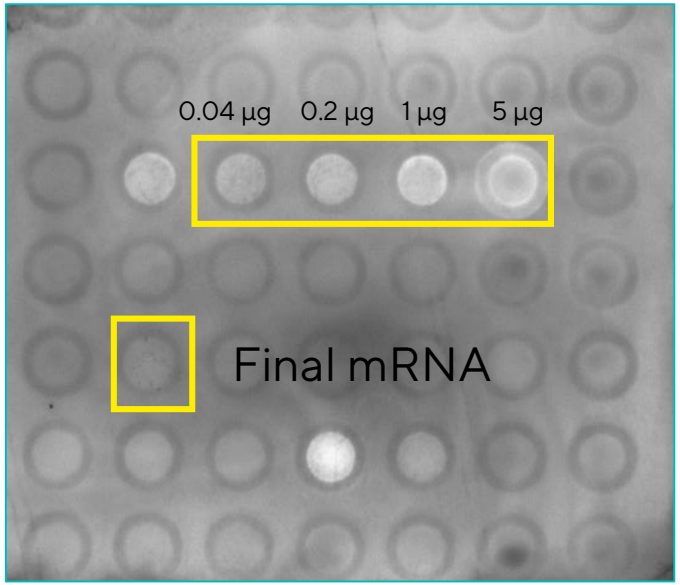
BioAnalyzer



Denaturing AGE

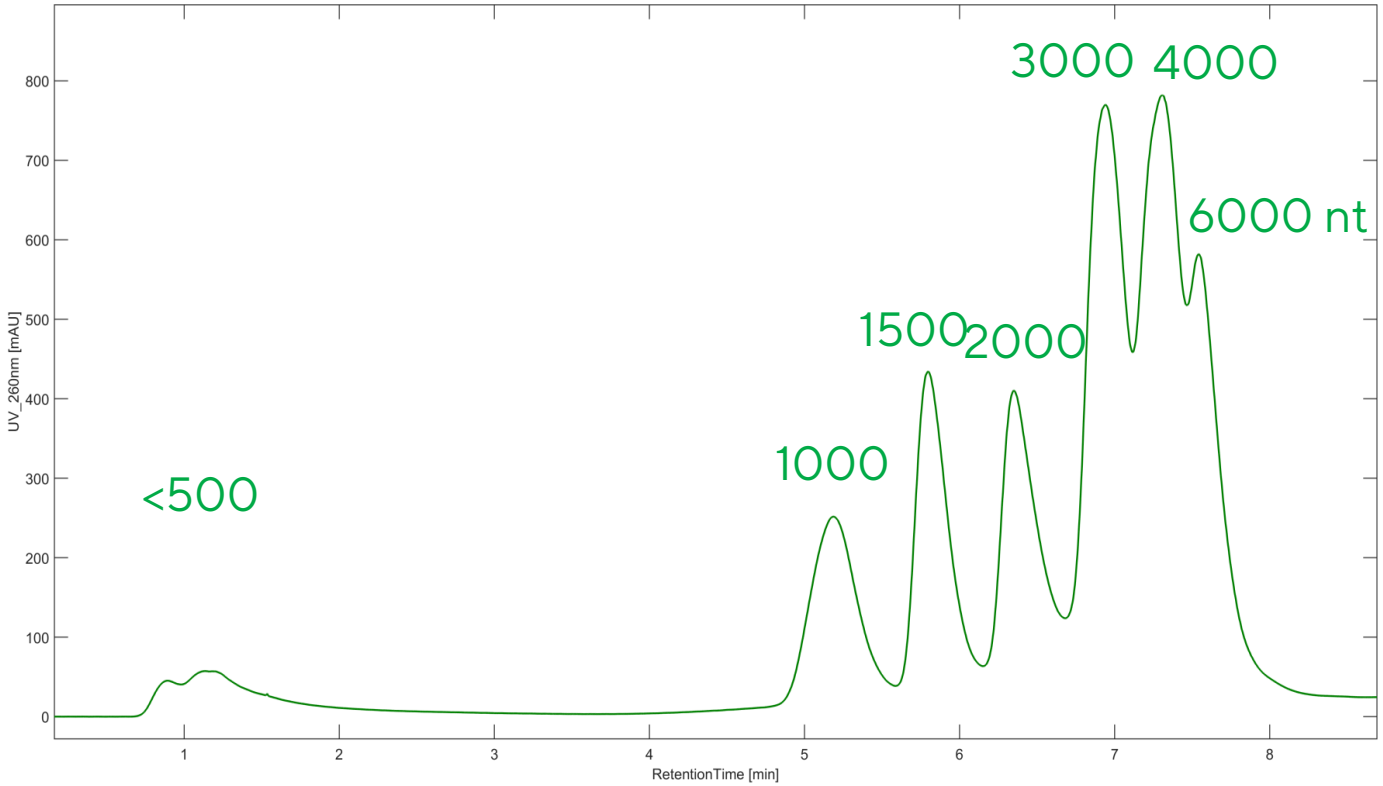


Dot-blot (J2, dsRNA standard MAGI)



- Hydrophobic interaction chromatography (HIC) removes residual proteins (in CIP), template DNA (in FT), fragments (pre-peaks in elution).
- Sample prepared in 0.55 M NaCl, in-line diluted into 2 M NaCl. Step elution in 0.4 M NaCl.

mRNA polishing – SDVB



- Styrene-divinyl benzene (SDVB) monolith can achieve separation of ssRNA by size. Requires reverse-phase mobile phases and elevated temperature (55-65°C)
- It also demonstrates selectivity for ssRNA/dsRNA/DNA

Column: CIM SDVB
MFA = 0.1 M TEAA, pH 7.0
MFB = 0.1 M TEAA, 25% ACN (v/v), pH 7.0
Column T = 55 °C
Sample: ssRNA ladder (RiboRuler High Range #SM1821)

SDVB_H_55C			
time	MFA	MFB	flow
Initial	65	35	0,4
2	65	35	0,4
13	30	70	0,4
13,1	10	90	0,4
16	10	90	0,4
17	65	35	0,4
18	65	35	0,4

mRNA workflow and Sartorius Solutions

Synthesis

Commercial phase (50 - 200 L)

Pro Mixer & BIOSTAT®

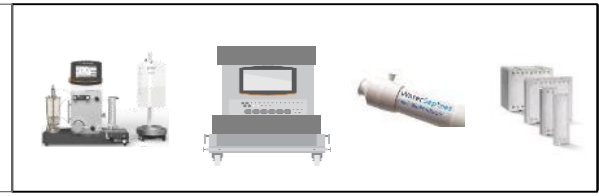
Sartorius Plasmid solutions

ambr®250 or 15

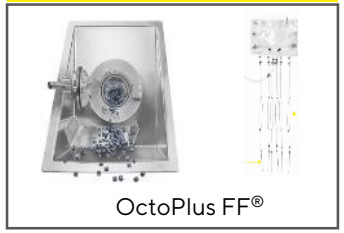
BIOSTAT® B

PD & Clinical phase (< 10L)

Harvest	UF/DF	Capture				Polish		Buffer Exchange after LNP*	Sterile Filtration
TFF system: Hydrosart® 30-300KD		Allegro SU BioSMB	Affinity-Oligo dT	Polishing – PrimaS™		BIA C4 – HLD	SDVB	Hollow Fiber or Hydrosart ®	Sartopore ® 2 XLG



Final Filling



OctoPlus FF®

HPLC and analytical column is also available for mRNA production. PD services can also be provided.

Pro Mixer

Flexsafe® Bags

Palletank® for Storage

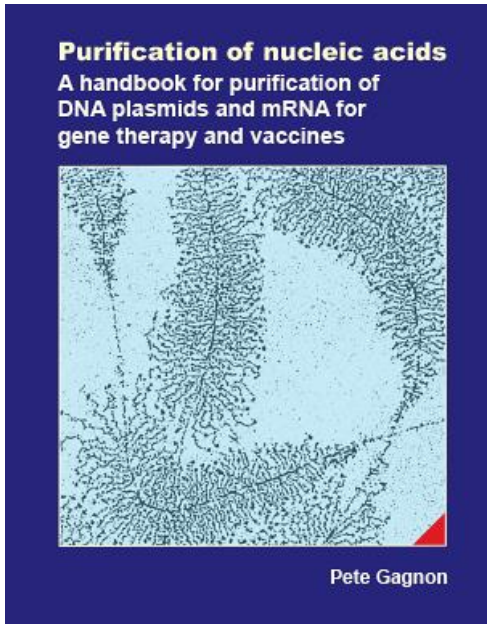
Buffer Preparation for LNP

* LNP: lipid nanoparticle

SARTORIUS

Take Home Messages

- The physical and chemical properties of mRNA present challenges for purification.
- Monoliths can provide a shear-free chromatographic medium with high capacity for mRNA
- Purification can be **scaled up with ease** due to flow-independent performance.
- Sartorius provides **a chromatography toolbox** including options for capture of mRNA, polishing, and for analytics, which can be tailored to construct and impurity profile
- **CIMmultus Oligo dT** and **PrimaS** monoliths provide capture of mRNA from IVT mixture whether the poly-A tail is added, or not
- **CIMac PrimaS** provides information on NTP, capping reagent, DNA template and RNA in a single, rapid assay
- **C4 HLD** is a proven column for nucleic acids (e.g. used for pDNA purification). Removal of fragments, double-stranded species, proteins.
- **SDVB** for polishing: Removal of dsRNA, size separation of ssRNA



Request your copy of the handbook via your Sartorius representative or at www.biaseparations.com

Thank you!

Rok Sekirnik
Head Process Development mRNA/pDNA
Rok.sekirnik@sartorius.com

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