Poll 1

Talk to our experts about the mRNA related processes at

Raman.Rani@Sartorius.com

Poll 2

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Simplifying Progress

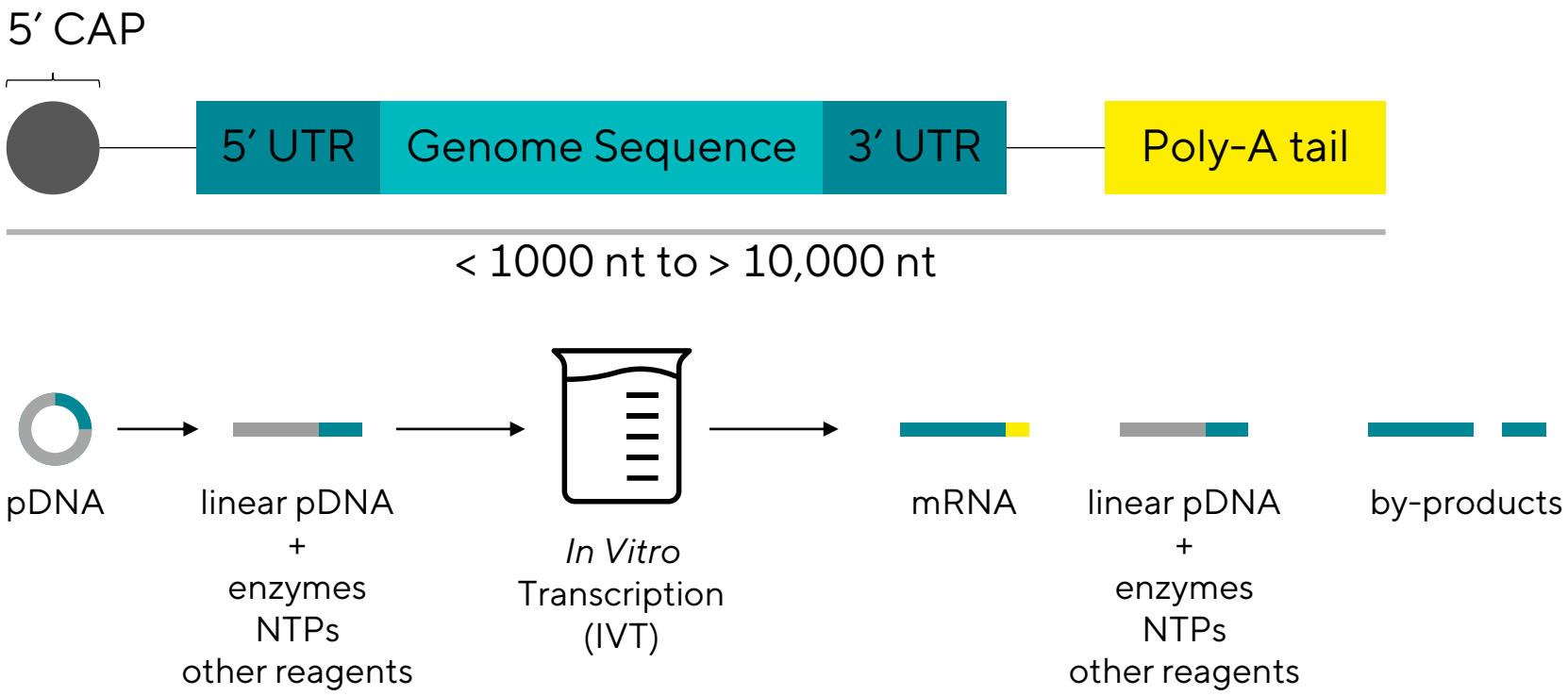
Accelerating mRNA process using scalable, flexible and reliable solutions

DCVMN, 12 April 2023

Agenda

- Introduction
- Upstream – *in vitro t*ranscription
- Downstream Solutions
- Formulation Development & Storage
- Data Analytics

Understanding mRNA Structure and Production



- Single-stranded RNA
- 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 understanding of IVT reactions is fundamental to maximise productivity and document the purity.

The Benefits of mRNA in Vaccines

Use the patient's cells as a manufacturing facility and patient creates its own vaccine.

mRNA technology…

01 **Reduce timeframes:** development and manufacturing (including variants update)

02 **Safety**: Cell free and virus free production.

03 **Footprint:** no seed train, smaller reactors…

mRNA : messenger Ribonucleic Acids

The Challenges of mRNA Technology

mRNA technology…

01 **Doses** is dependent of application (infectious diseases vs cancer)

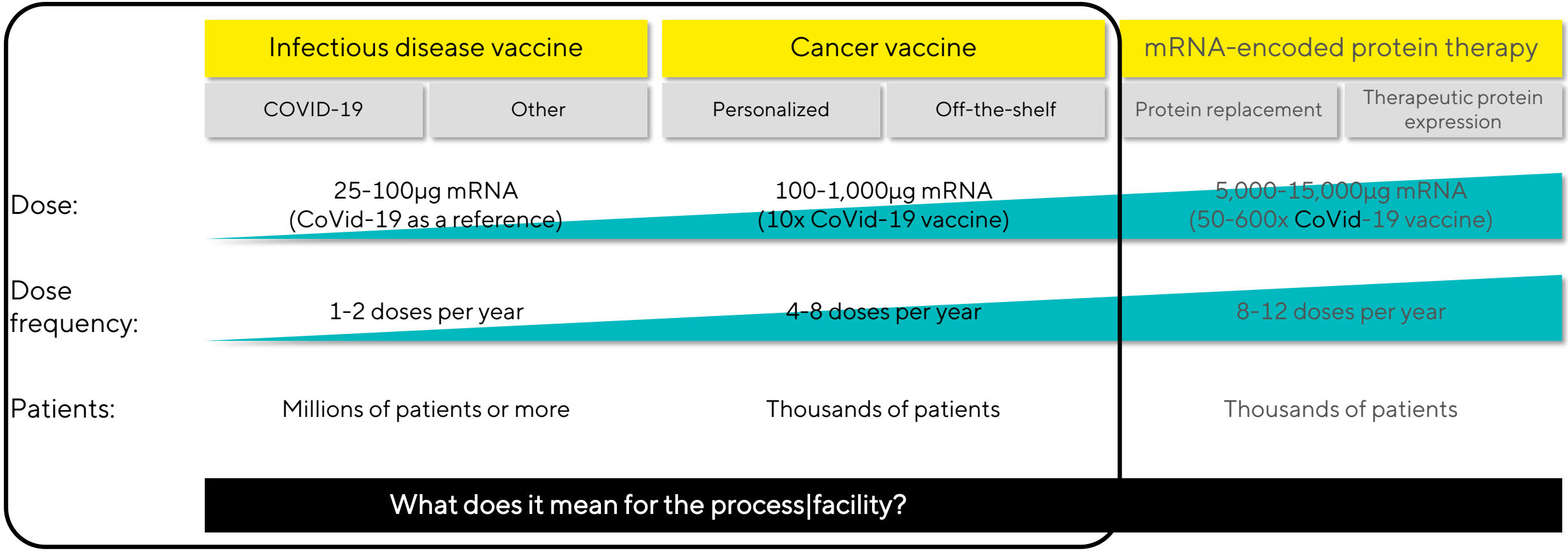
02 **Size, sequence and type** of construct.(i.e new constructs such as saRNA are very long).

03 **Cost per dose** due to raw material (enzymes, capping…)

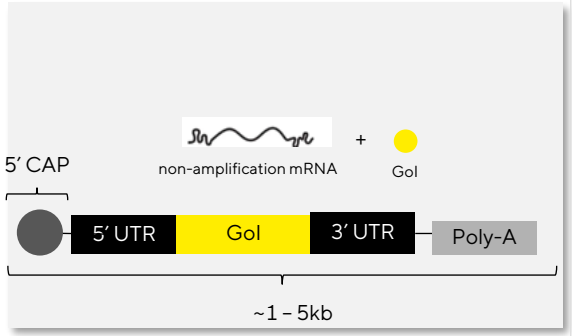
03 **New field, low expertise, no platform.**

mRNA : messenger Ribonucleic Acids

mRNA: doses challenge…



(m)RNA: various constructs to overcome some challenges BUT is challenging the process…



messenger RNA (mRNA)

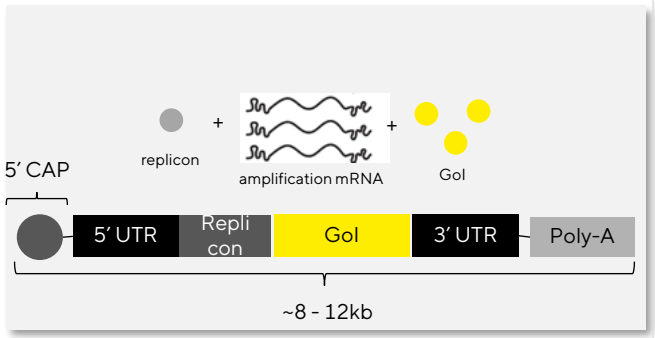
- Based on 'standard' cell mRNA

Pros

- ✓ Short transcript (easier to manufacture)
- ✓ Lots of human trial data

Cons

- ✗ Non-replicating
- ✗ One GoI per transcript



self-amplifying RNA (saRNA)

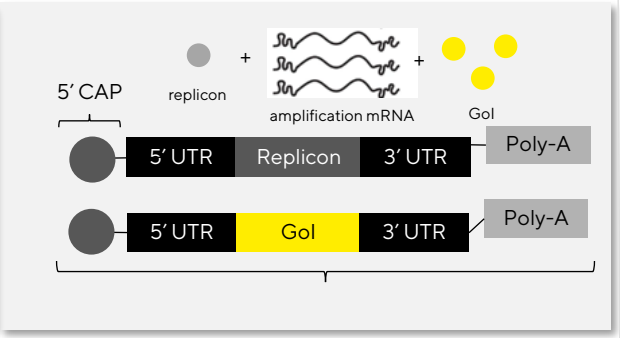
- Based on family of self-replicating alphavirus (Built-in RNA polymerase (viral replicon))

Pros

- ✓ Requires **lower dosage** for equivalent efficacy (non-vaccine applications)
- ✓ More durable expression than non-replicating mRNA

Cons

- ✗ Longer than traditional mRNA (difficult to manufacture)
- ✗ Viral origin - greater immunogenicity



trans-amplifying RNA (taRNA)

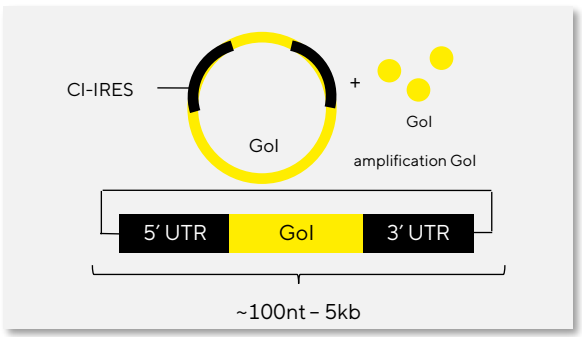
- Like saRNA, but split (trans) replicon/GoI

Pros

- ✓ Requires **lower dosage** for equivalent efficacy (non-vaccine applications)
- ✓ Shorter than saRNA (easier to manufacture)

Cons

- ✗ Requires delivery of two separate transcripts (replicon & GoI)
- ✗ Viral origin - greater immunogenicity



circular RNA (circRNA)

- synthetic form of RNA

Pros

- ✓ Durable expression & lower immunogenicity (closed configuration, non-vaccine applications)
- ✓ Lack of cap & polyA tail (easier manufacture)

Cons

- ✗ Lack of clinical validation (still in pre-clinical stage)
- ✗ Manufacturing scalability not established (esp for longer RNA sequences)

What does it mean for the process|facility?

Note: constructs & parts not to scale

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Key Drivers for mRNA Manufacturing

Current Technology

- Use plasmid for DNA template
- In vitro transcription
  - Enzymatic capping
  - Co-transcriptional capping
- LNP-Based Formulation
- Downstream needs to be adjusted to fit different capping methods

Pain Point

- High-cost raw materials (especially enzymes).
- No standardized platform approach
- DNase/RNase contamination

Speed to Clinic | Market

- Robust scalable processes
- Standardization of the process
- Process simplification
- Skilled professionals

Process Improvement

- Increase manufacturing yield
- Reduce cost per dose
- CQA & Analytical tools
- In house capacity expansion

Flexible Processes

- Multiple product options
- Modular and mobile
- Single use technology
- CDMO service

mRNA Workflow and Sartorius Solution

pDNA





Seed Expansion and Production → Alkaline Cell Lysis → Clarification → Concentration | Diafiltration → Capture → Polish → Concentration | Diafiltration → Final Filtration

Analytics

Synthesis

Pro Mixer & BIOSTAT®


Commercial phase (50 - 200 L)

BIOSTAT® B

PD & Clinical phase (< 10L)

ambr®250 or 15

Sartorius Plasmid solutions

* LNP: lipid nanoparticle

mRNA

IVT → UF/DF → Capture → Polish → Buffer Exchange after LNP* → Sterile Filtration

TFF system: Hydrosart® 30-300KD | Resolute Flowdrive/BioSMB | Oligo dT/ Prima S | C4 – HLD/SDVB | Hollow Fiber or Hydrosart® | Sartopore® 2 XLG





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

Final Filling


OctoPlus FF®

Poll 3

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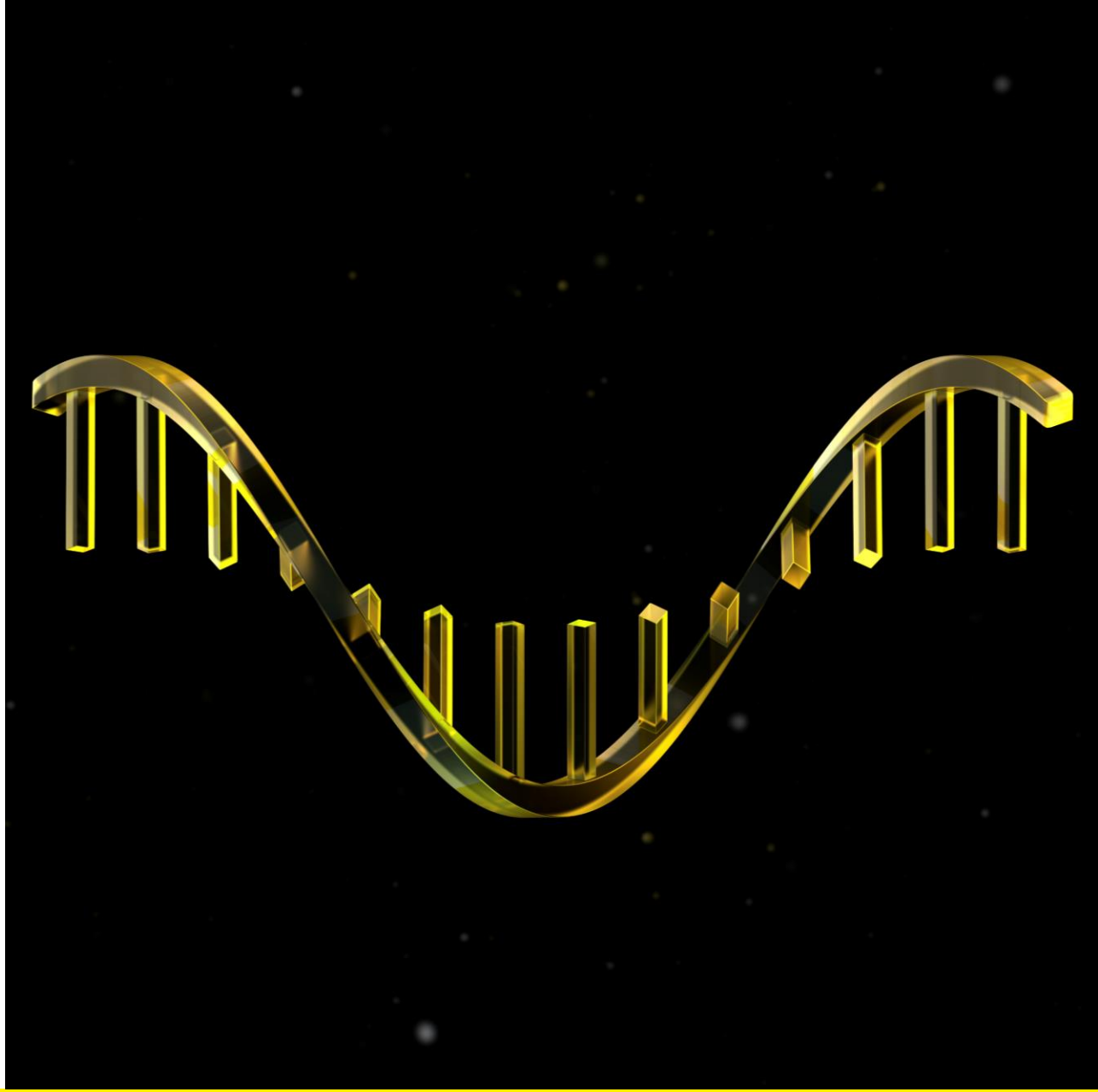


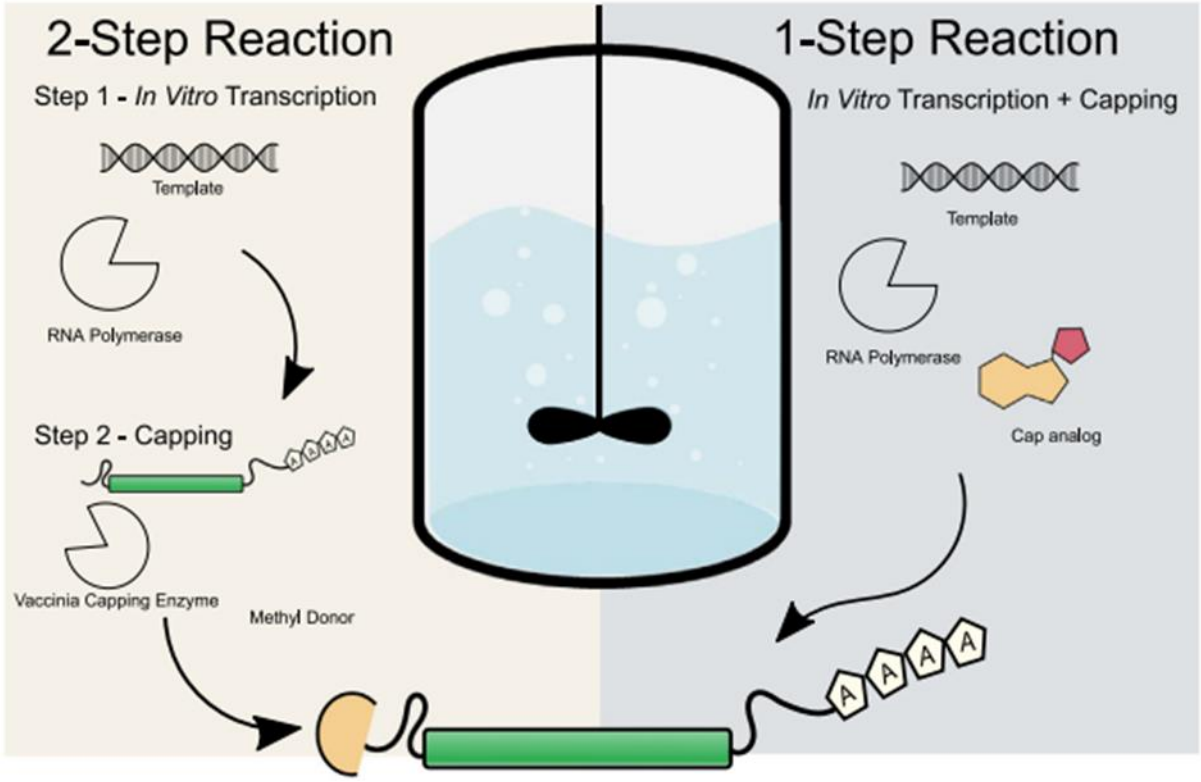
Unique Challenges of mRNA IVT

Pain points	No standard efficient IVT reaction protocol available	High cost and weak supply chain of raw materials	Lack of dedicated tools supporting PD of mRNA	DNase and RNase contamination / instability of mRNA	No dedicated analytical methods for this new modality
Needs	High-throughput	mRNA expertise	Single-use technology	Analytical solutions	

*LNP: Lipid nano particle

In Vitro Transcription Reaction – Production of mRNA from DNA Template

- Reaction time is typically 2-3 hours for batch processes.
- High yield (low reaction volumes)
- IVT is a multi-component reaction :
  - Plasmid (dsDNA)
  - RNA polymerase (e.g. T7)
  - NTPs (optional modified NTPs)
  - Capping reagent (optional)
  - $MgCl_2$
  - Pyrophosphatase (optional)
  - RNAse inhibitor
  - Spermidine
  - DTT
  - Buffer

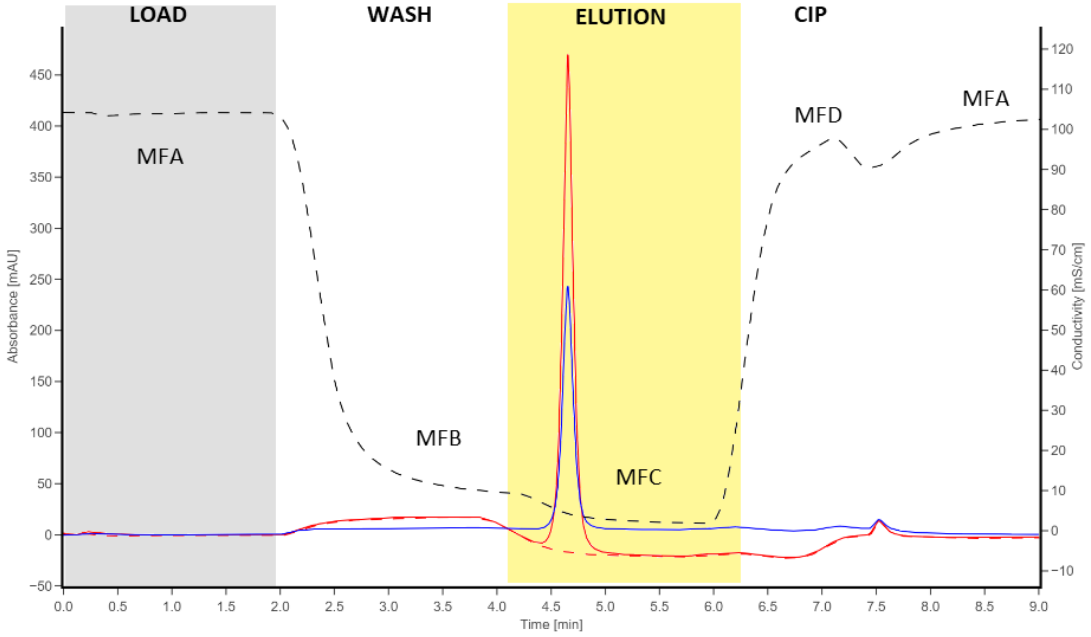


Rosa, S. S., et al., Vaccine. 2021 Apr 15; 39(16): 2190–2200.

Monitoring of IVT: Two Paradigms for Rapid At-Line Analytics

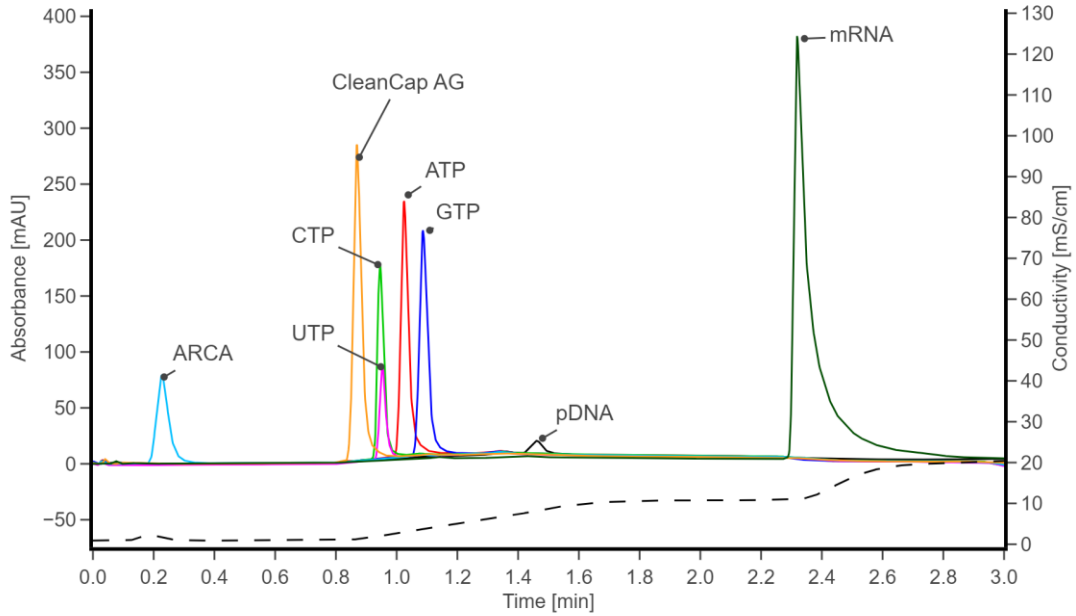
CIMac Oligo dT - affinity

- One-parameter-at-a-time, faster than Ribogreen
- ‘Protein A – mAb’ paradigm for mRNA
- Titre of polyadenylated mRNA throughout process

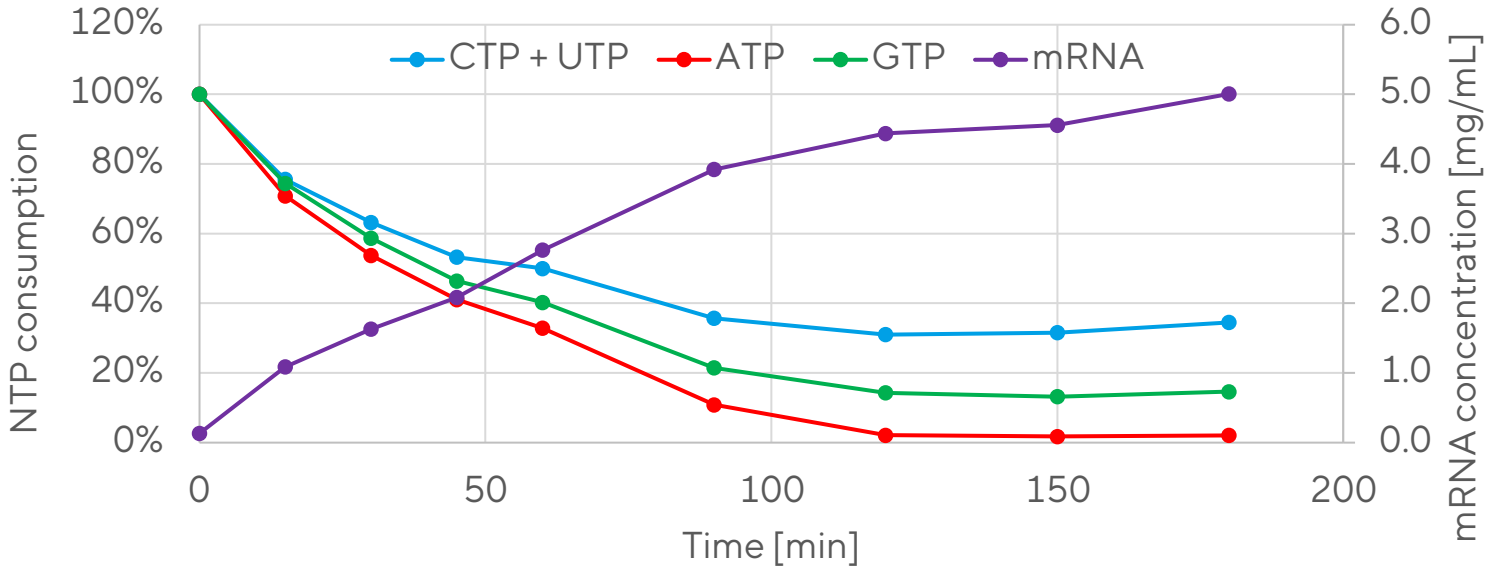


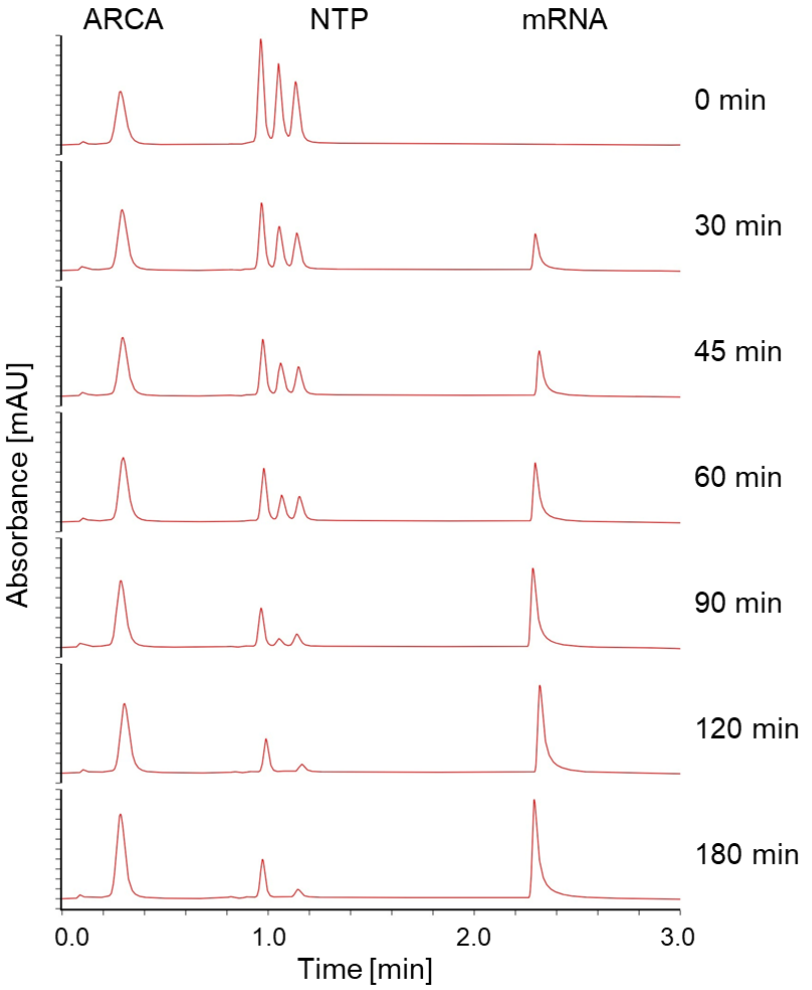
CIMac PrimaS - multimodal

- Multi-parameter method
- New paradigm for mRNA
- NTP, capping, RNA content. Applies to all RNA modalities



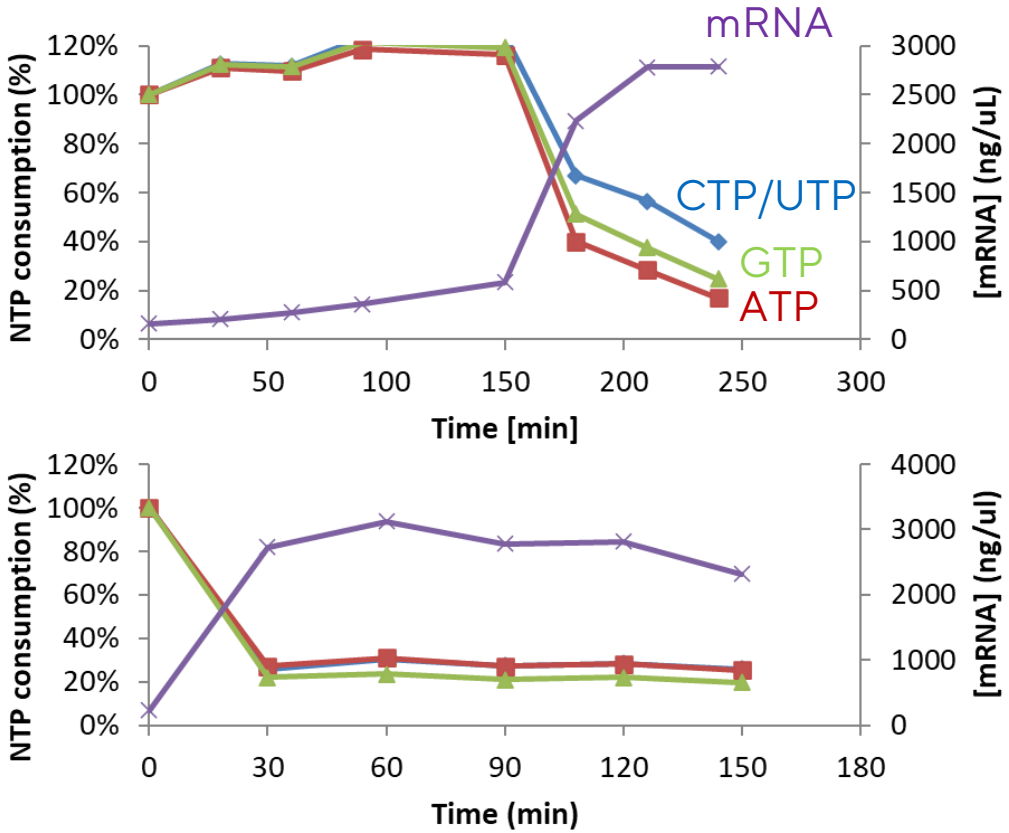
- The IVT reaction can be monitored at-line by **CIMac PrimaS**
- mRNA production kinetics is monitored. Productivity maximum can be identified, to prevent degradation.
- Consumption of nucleotides and concentration of capping reagent can simultaneously be studied
- Effects of feed addition can be studied

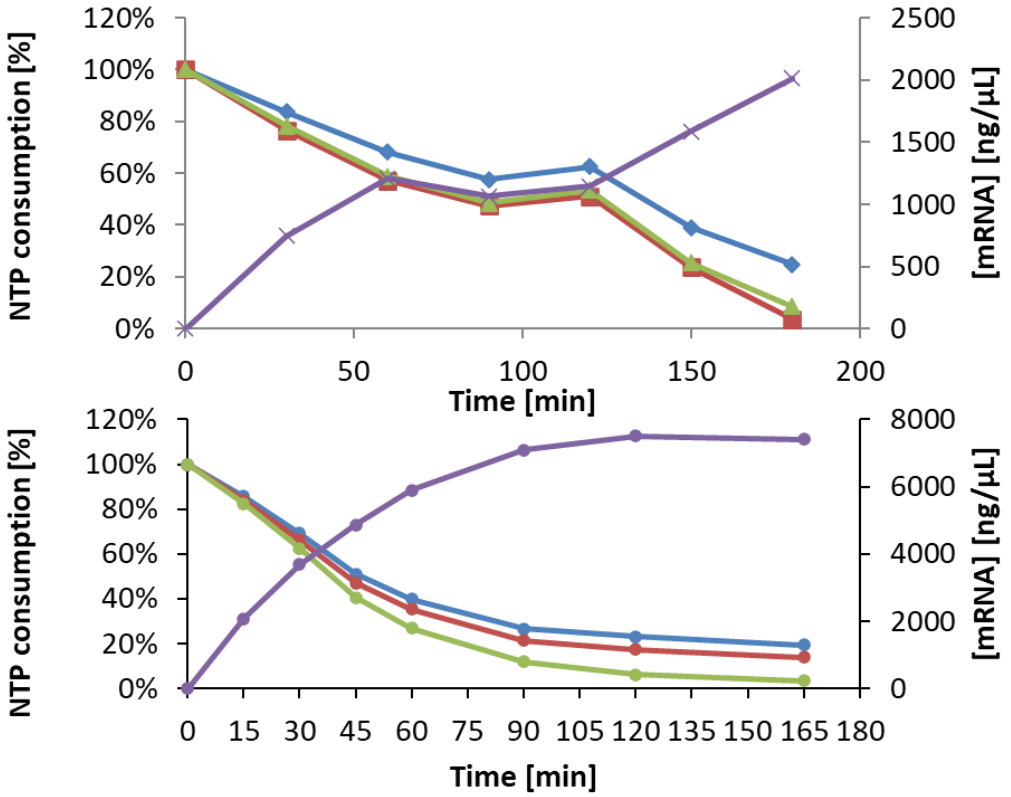




Pregeljc, D. et al. "Increasing yield of in vitro transcription reaction with at-line high pressure liquid chromatography monitoring." *Biotechnology and Bioengineering* (2023, 3, 737-747)

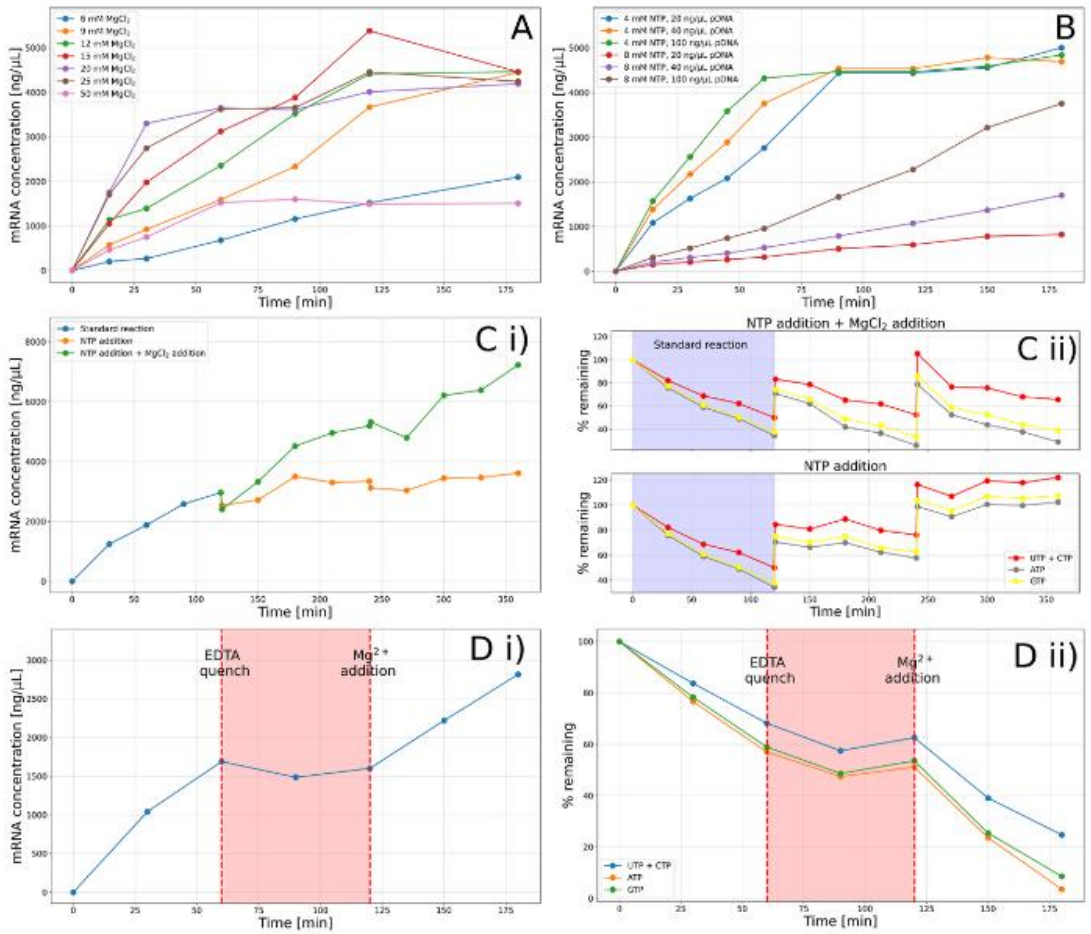
CIMac PrimaS Provides Tight Control Over IVT Reaction





Pregeljc, D. et al. "Increasing yield of in vitro transcription reaction with at-line high pressure liquid chromatography monitoring." *Biotechnology and Bioengineering* (2023, 3, 737-747)

Optimization of IVT reaction



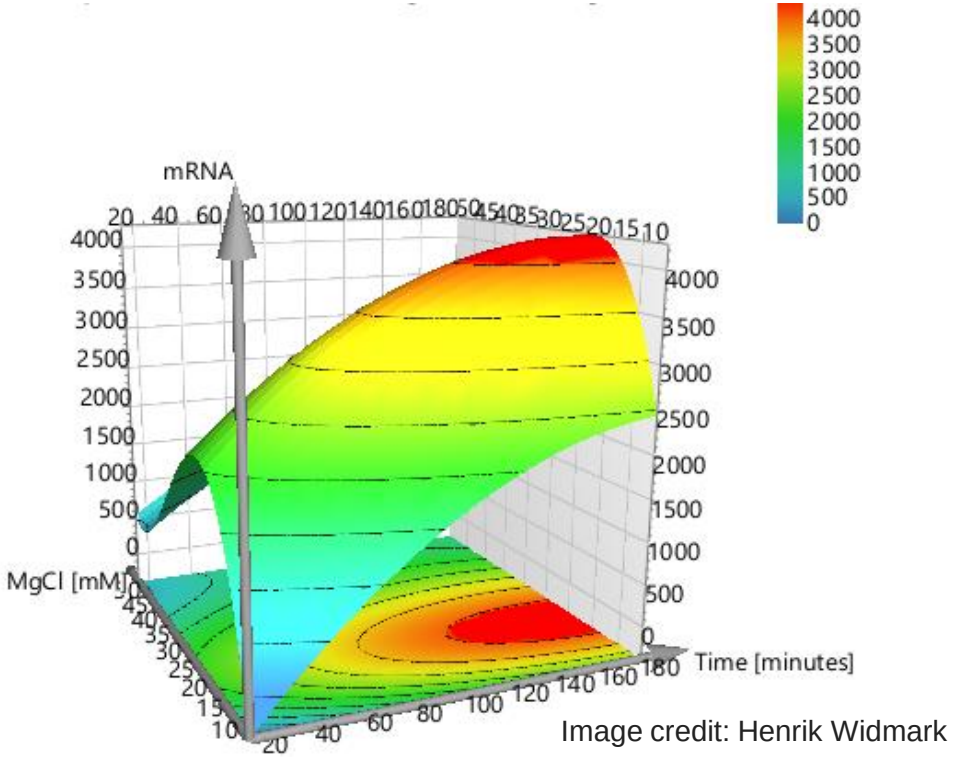
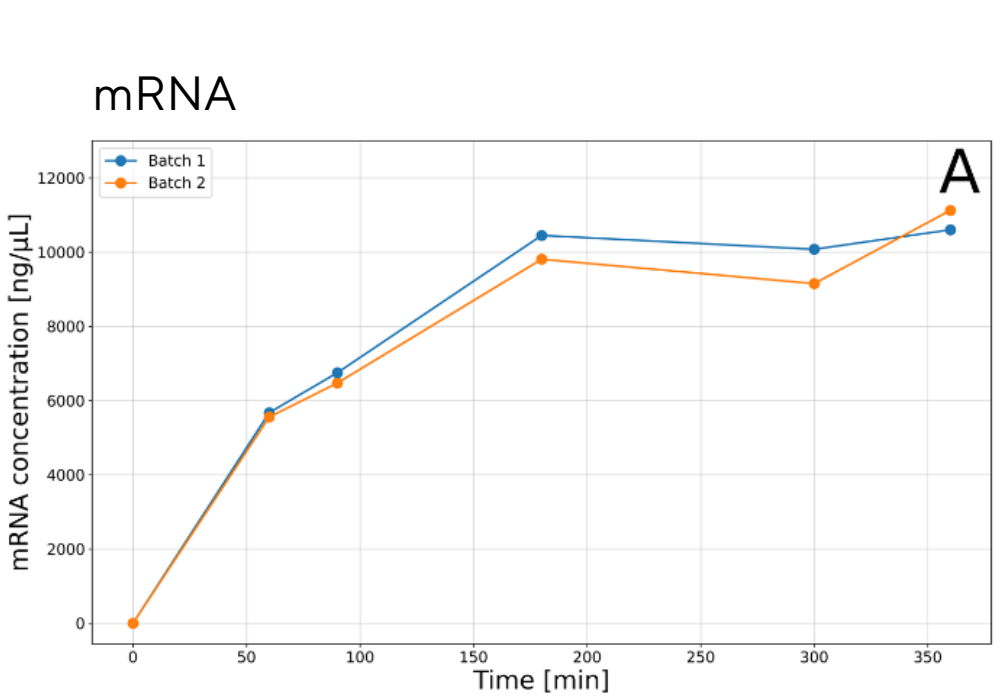


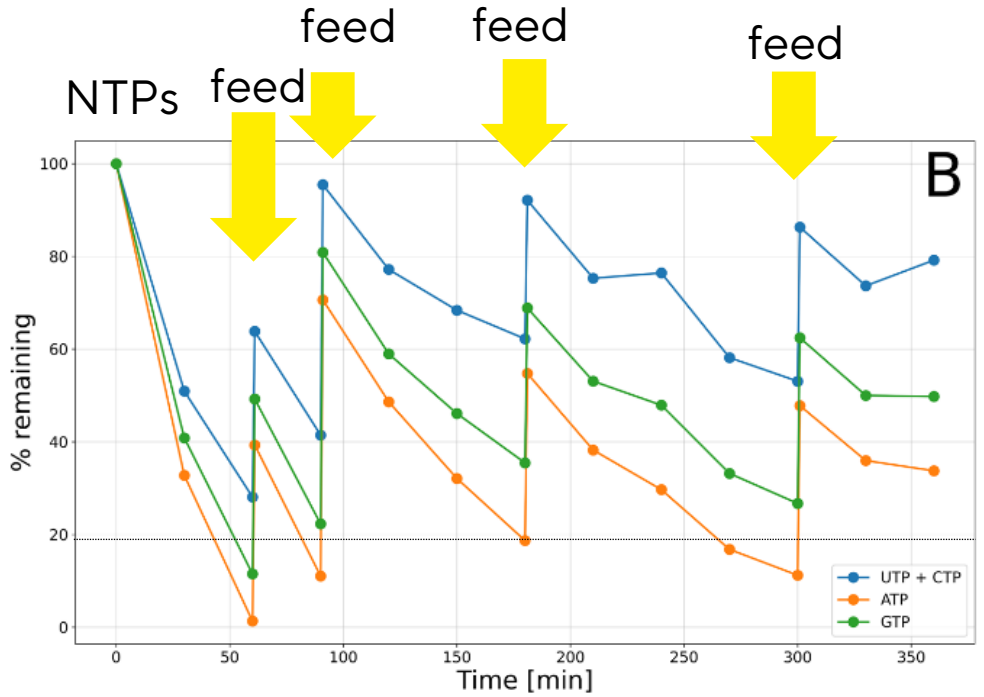
Image credit: Henrik Widmark

- Methodological platform for studying individual or combinatorial effects of IVT variables on mRNA production and NTP consumption
- Combined with MODDE® software for DOE and data analysis to derive deeper process understanding

Pregeljc, D. et al. "Increasing yield of in vitro transcription reaction with at-line high pressure liquid chromatography monitoring." *Biotechnology and Bioengineering* (2023, 3, 737-747)

Batch to Fed-Batch – Monitoring NTPs and mRNA



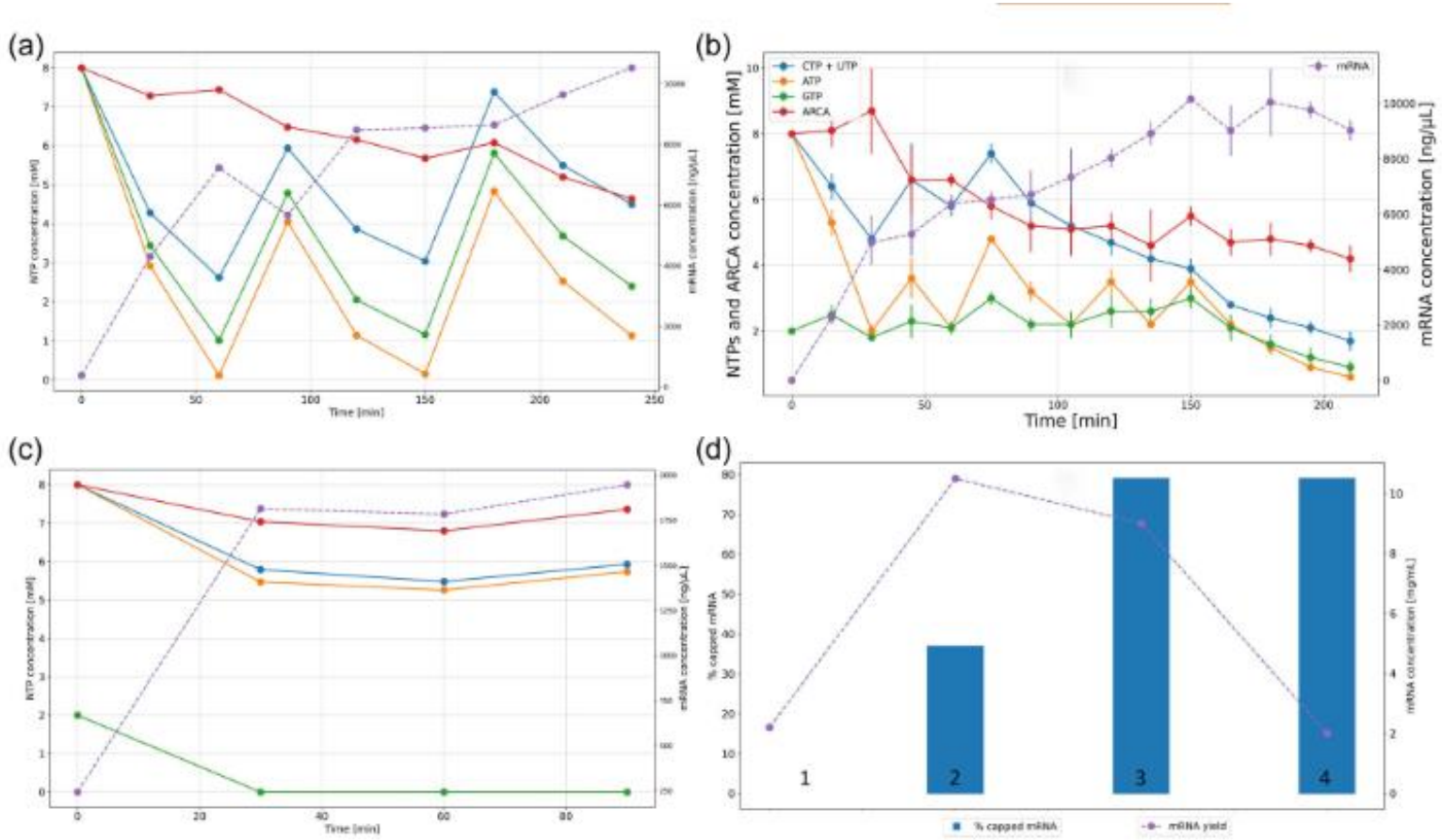


- PATFix Monitoring of depletion of NTPs; react with feed addition
- Control scale-up of IVT reaction
- Control tech transfers
- Calculate kinetics of NTP consumption → transform to continuous feeding (e.g. AMBR250)

Pregeljc, D. et al. "Increasing yield of in vitro transcription reaction with at-line high pressure liquid chromatography monitoring." *Biotechnology and Bioengineering* (2023, 3, 737-747)

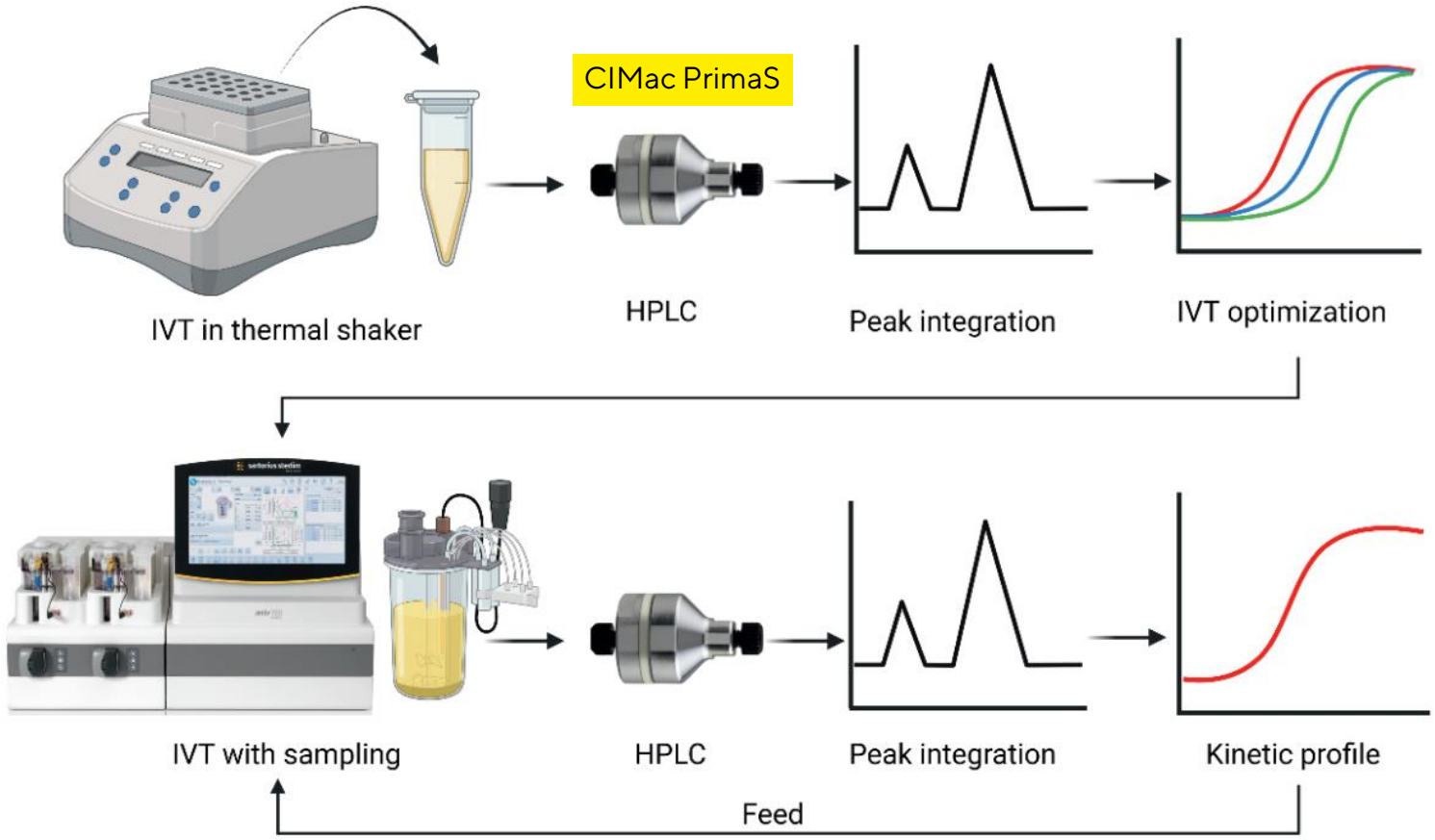
Combining Fed-Batch With Co-transcriptional Capping to Reduce CoGs

- Combine **fed-batch** and **co-transcriptional capping** paradigms to decrease CoGs by lowering T7 / plasmid requirements
- Feed GTP to keep constant 2 mM concentration
- High capping efficiency (80%) at high mRNA yield (10 g/L) demonstrated with ARCA
- Principle applies to all co-transcriptional cap analogues



Pregeljc, D. et al. "Increasing yield of in vitro transcription reaction with at-line high pressure liquid chromatography monitoring." *Biotechnology and Bioengineering* (2023, 3, 737-747)

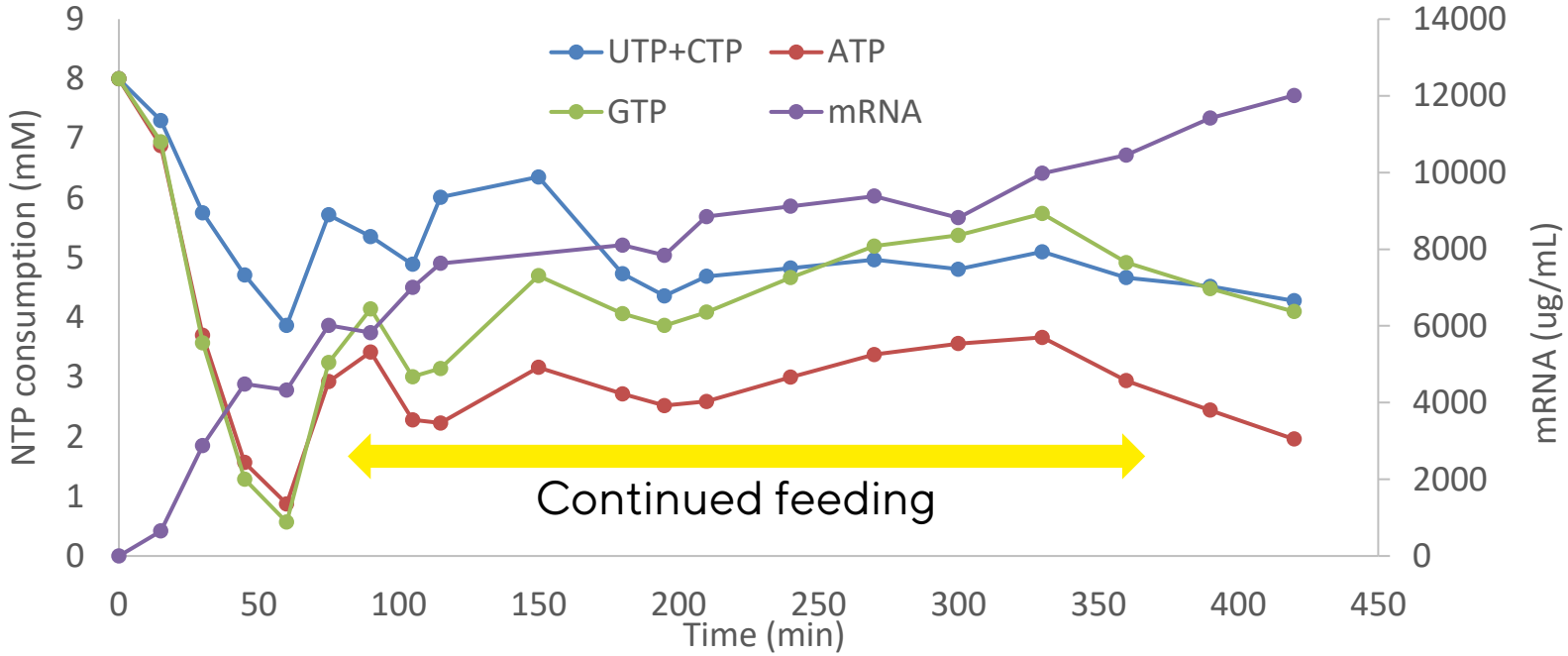
Towards Continuous Production of mRNA



- Determination of optimal IVT conditions and characterisation of reaction kinetics in thermal shaker.
- NTP feeding strategy designed and tested in thermal shaker and transferred to automated scale-up Ambr 250 system.

Skok, J, Megušar, P. et al. "Gram-Scale mRNA Production Using a 250-mL Single-Use Bioreactor." *Chemie Ingenieur Technik* (2022) 94 1928-1935

Multi-gram mRNA Production in 250 mL Single-Use Bioreactor



- At-line monitoring of NTP in reaction container.
- 2 g mRNA produced from 100 mL starting volume
- cost reduction of up to 50 % per mg of mRNA produced compared to batch mode production by better utilization of enzymes and plasmid.

Skok, J, Megušar, P. et al. "Gram-Scale mRNA Production Using a 250-mL Single-Use Bioreactor." *Chemie Ingenieur Technik* (2022) 94 1928-1935

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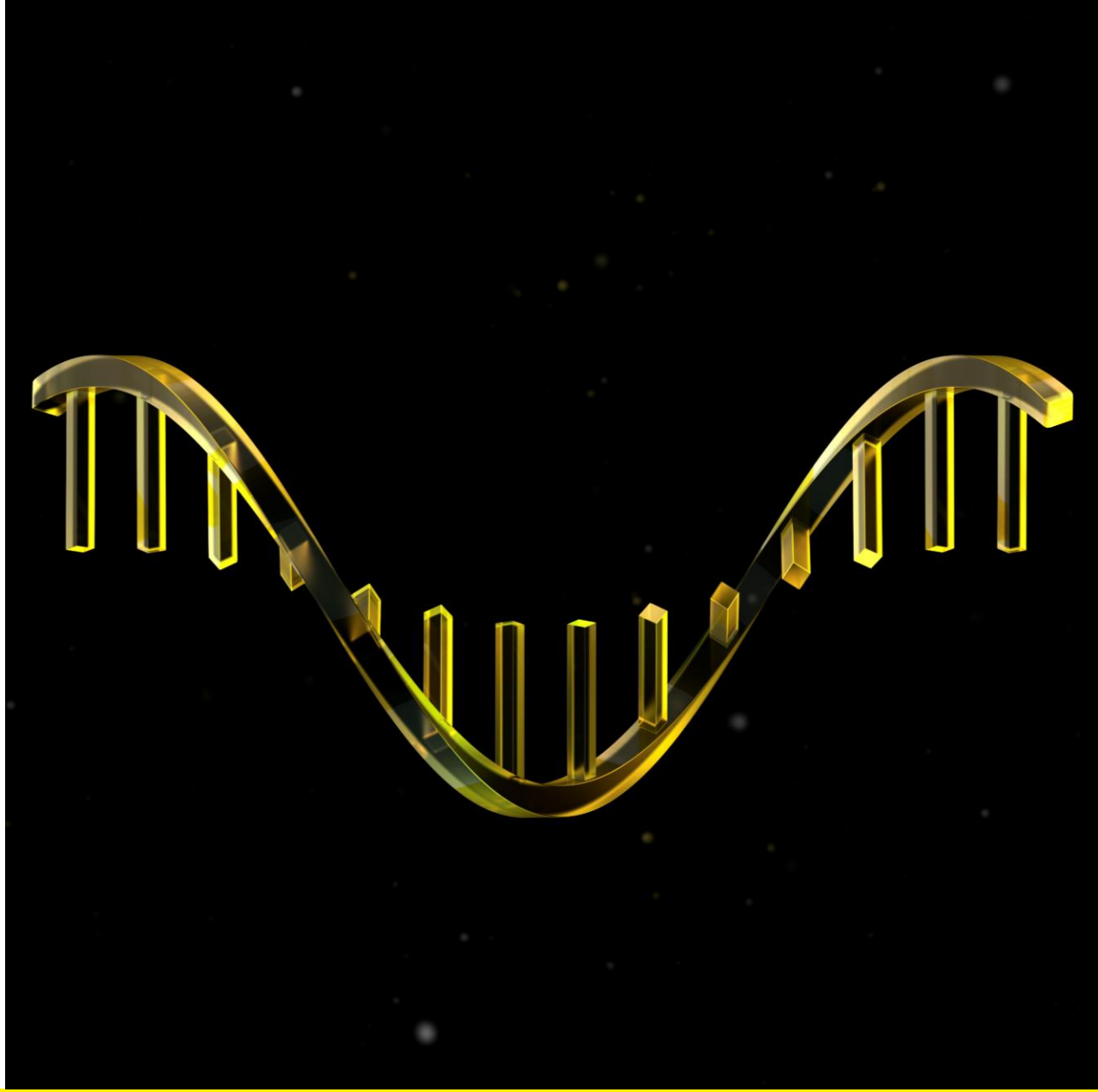
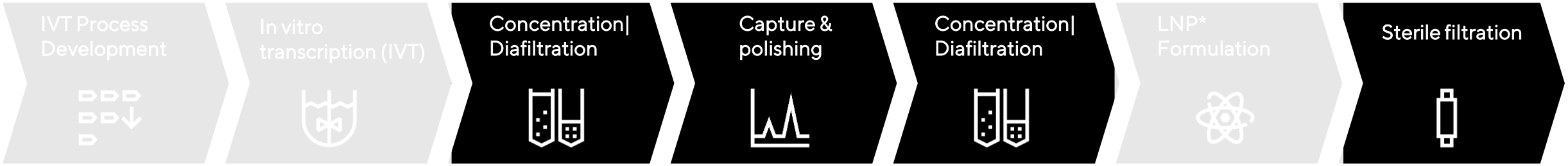


Unique Challenges of mRNA Downstream Processing

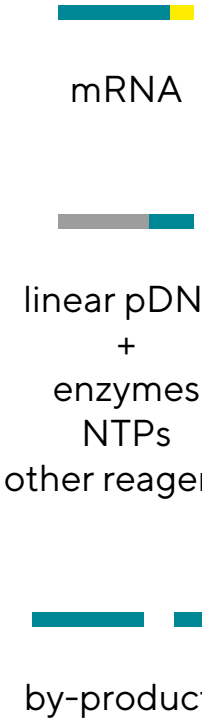


Pain points	IVT protocol is still evolving, impacting the DSP	There is no established DSP platform that can be applied to all mRNA	Reagents and raw materials are poorly defined and bring contaminants	Impurities are very similar to the target and may interact with it	Many technologies used in labs are not scalable	mRNA are large molecules, shear sensitive and relatively unstable
Needs	Monolithic technology	mRNA expertise	Single-use technology	Proven Scalability	Toolbox of filters	

*LNP: Lipid nano particle

What are we Separating mRNA from?

- IVT reaction components (DNA template, enzymes, NTPs, capping and/or other reagents)
- Contaminants in the raw materials:
  - In the plasmid: E.coli proteins, DNA, and RNA.
  - In the enzymes: fragments, host DNA, RNA, 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.



Chromatography

Toolbox for mRNA Purification and Analytics

Capture from IVT

Oligo dT18

Affinity

Polyadenylated mRNA capture

Elution in water

PrimaS

Multimodal

Capture all RNA

Elution in pH gradient

Polishing

SDVB

Ion-pair RP

dsRNA removal

Room temperature, low pressure

C4 HLD

Hydrophobic

Aqueous conditions

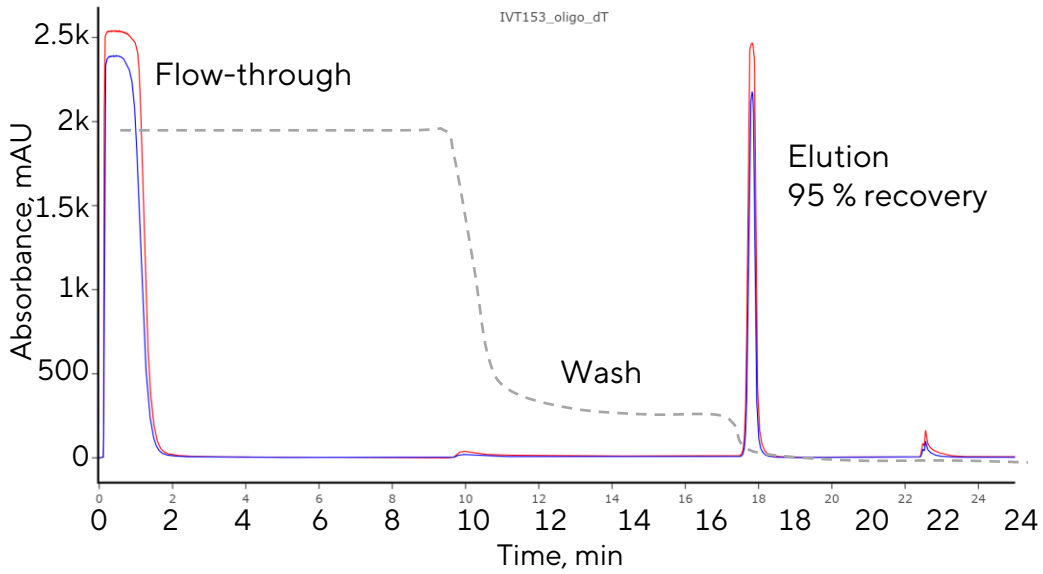
Analytics

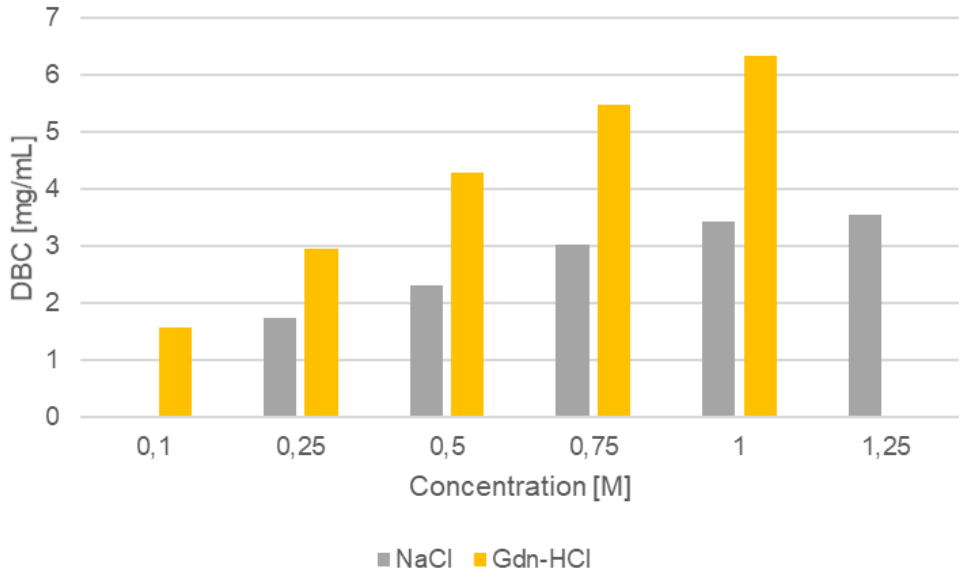
PATfix™

Advantages of a Toolbox

- Capture mRNA from IVT without need for UF | DF
- Poly-A specific capture with affinity Oligo dT18
- Multimodal capture for RNA without poly-A tail
- Solutions are ready to deploy and screen with new projects or customer wishes.
- Sanitise and re-use CIM® monoliths

Affinity Capture of Polyadenylated mRNA by CIMmultus Oligo dT18





- IVT > add NaCl > Load on CIMmultus Oligo dT (no concentration, no buffer exchange, no TFF)
- Binding in moderate NaCl concentrations (250 mM – 1.5 M) leads to DBC of 3-4 mg/mL, in Gdn up to 6 mg/mL
- Elution in low concentration buffer or in water

Mencin, N., Štepec, D. et al., Development and scale-up of oligo-dT monolithic chromatographic column for mRNA capture, Separation and Purification Technology, 304 (2023)

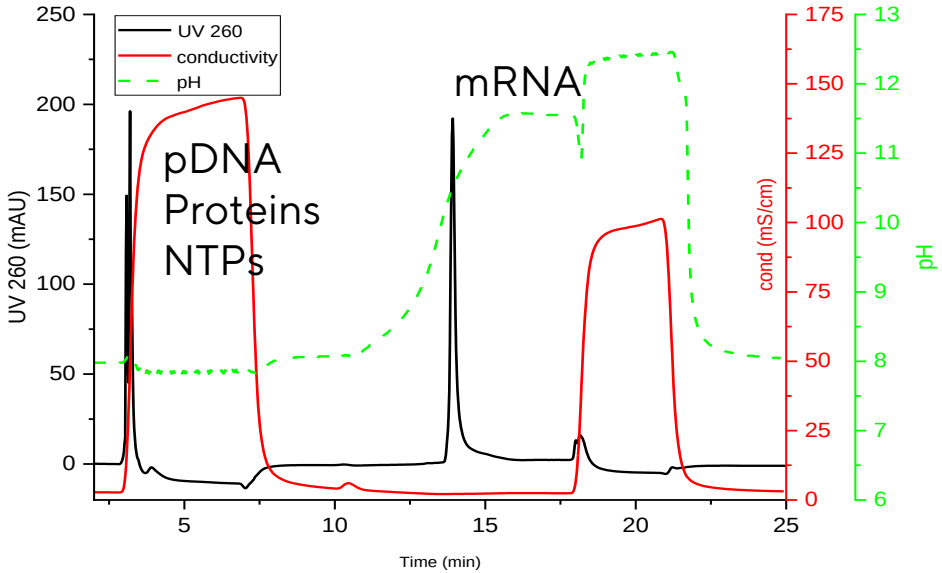
Korenč, M. et al, Chromatographic purification with CIMmultus™ Oligo dT increases mRNA stability, Cell & Gene Therapy Insights 2021; 7(9), 1207–1216

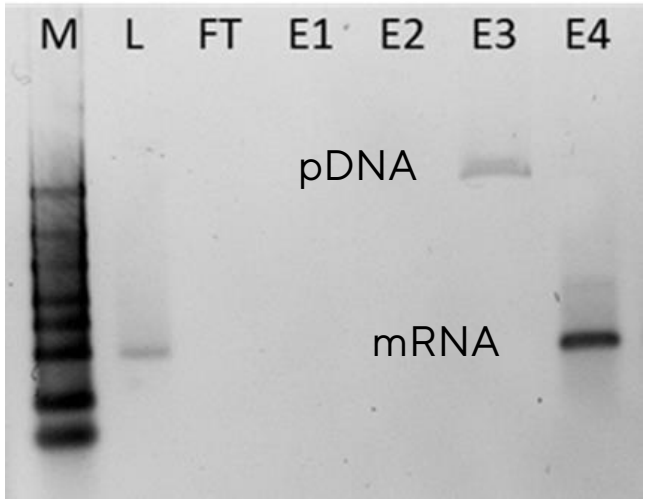
Capture of mRNA Without Poly-A Tail Using PrimaS

- Elution at pH gradient (or step) can separate mRNA from IVT components.
- Capture of mRNA without poly A tail, ability to remove protein, nucleotides, plasmid
- Robust, IVT is applied to the column after initial dilution with loading buffer.

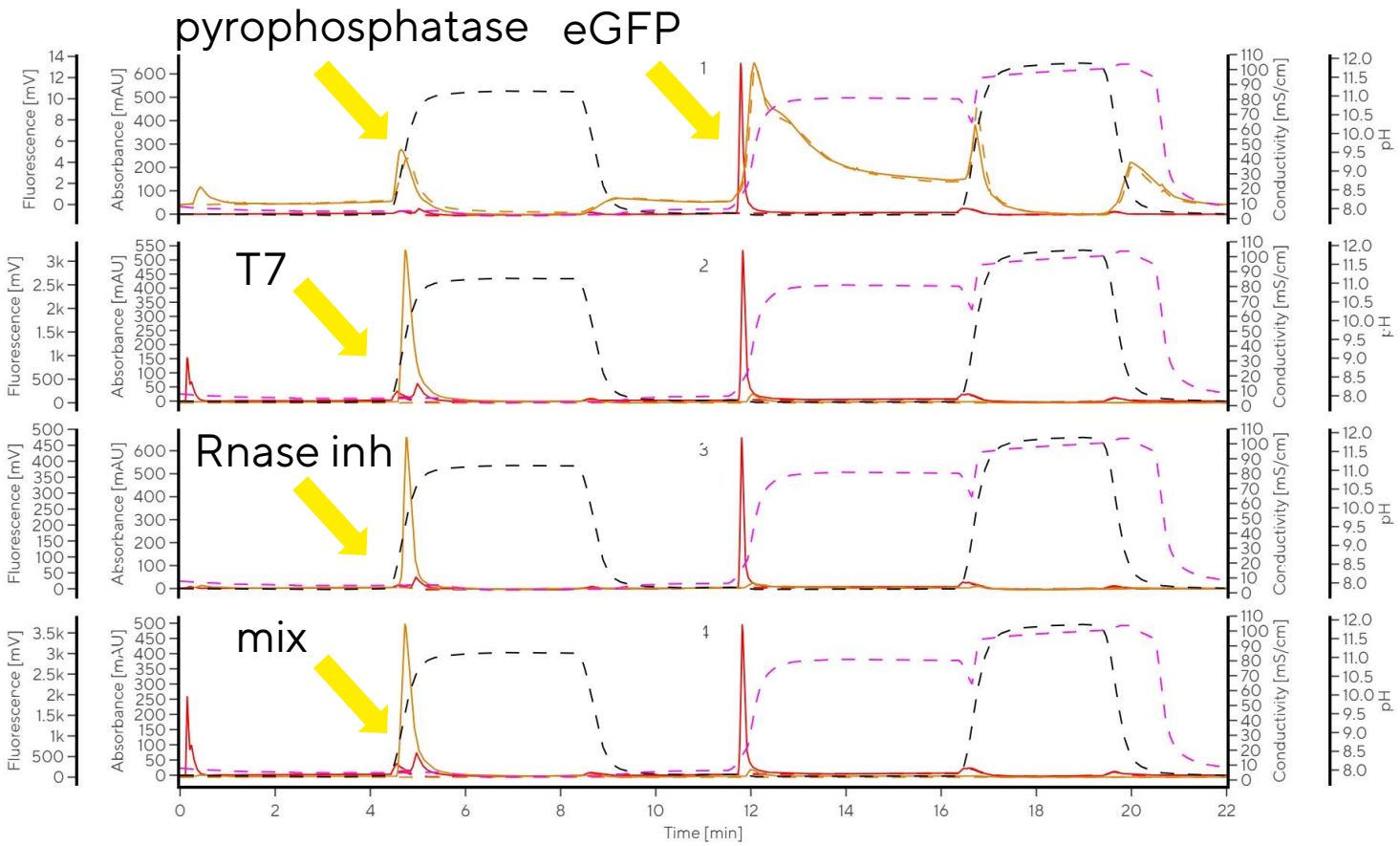
Pyrophosphate gradient elution

pH gradient elution





FLD + UV to monitor protein clearance by PrimaS



Pyrophosphatase + mRNA eGFP

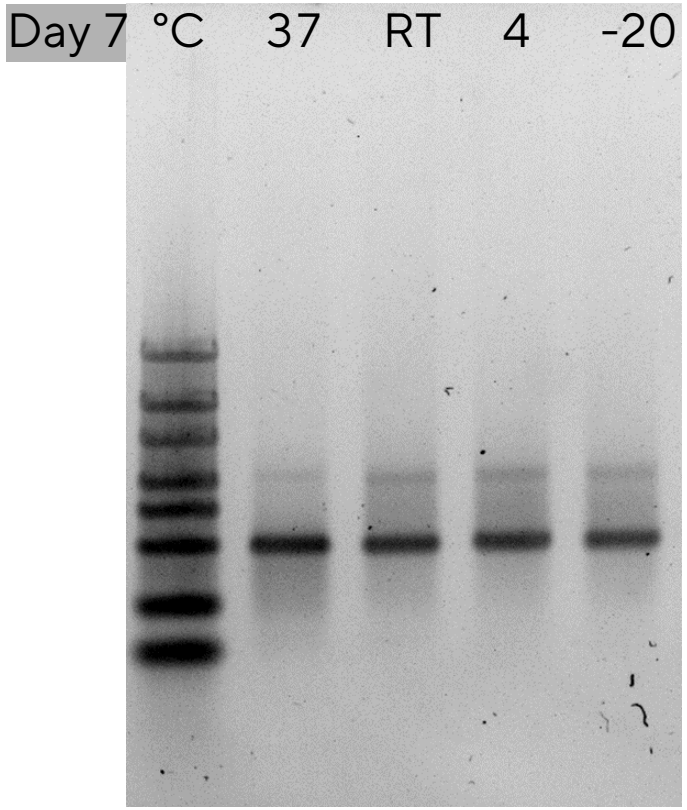
T7 polymerase + mRNA eGFP

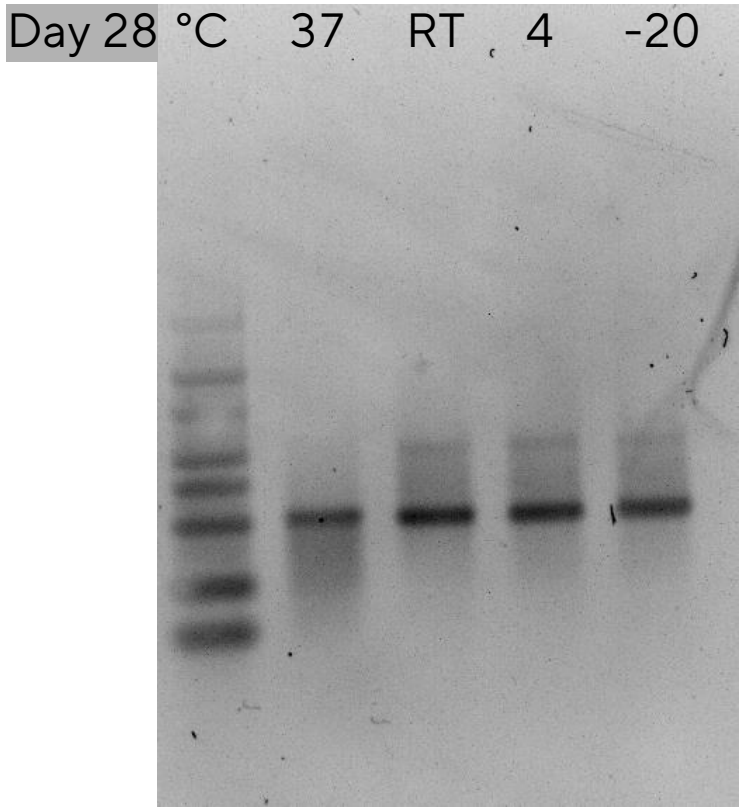
RNase inhibitor + mRNA eGFP

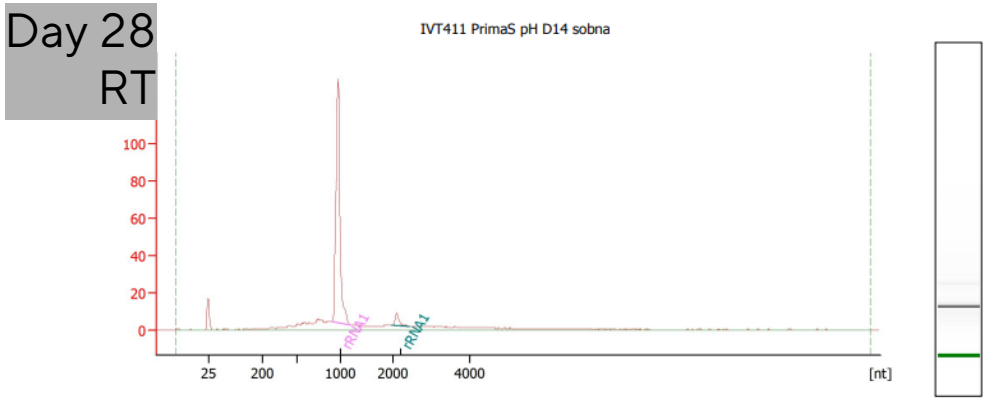
All proteins + mRNA eGFP

FLD detection demonstrates protein removal in high-salt wash (UV signal too low to detect)
mRNA elutes in pH gradient

pH gradient elution from PrimaS does not impact mRNA stability



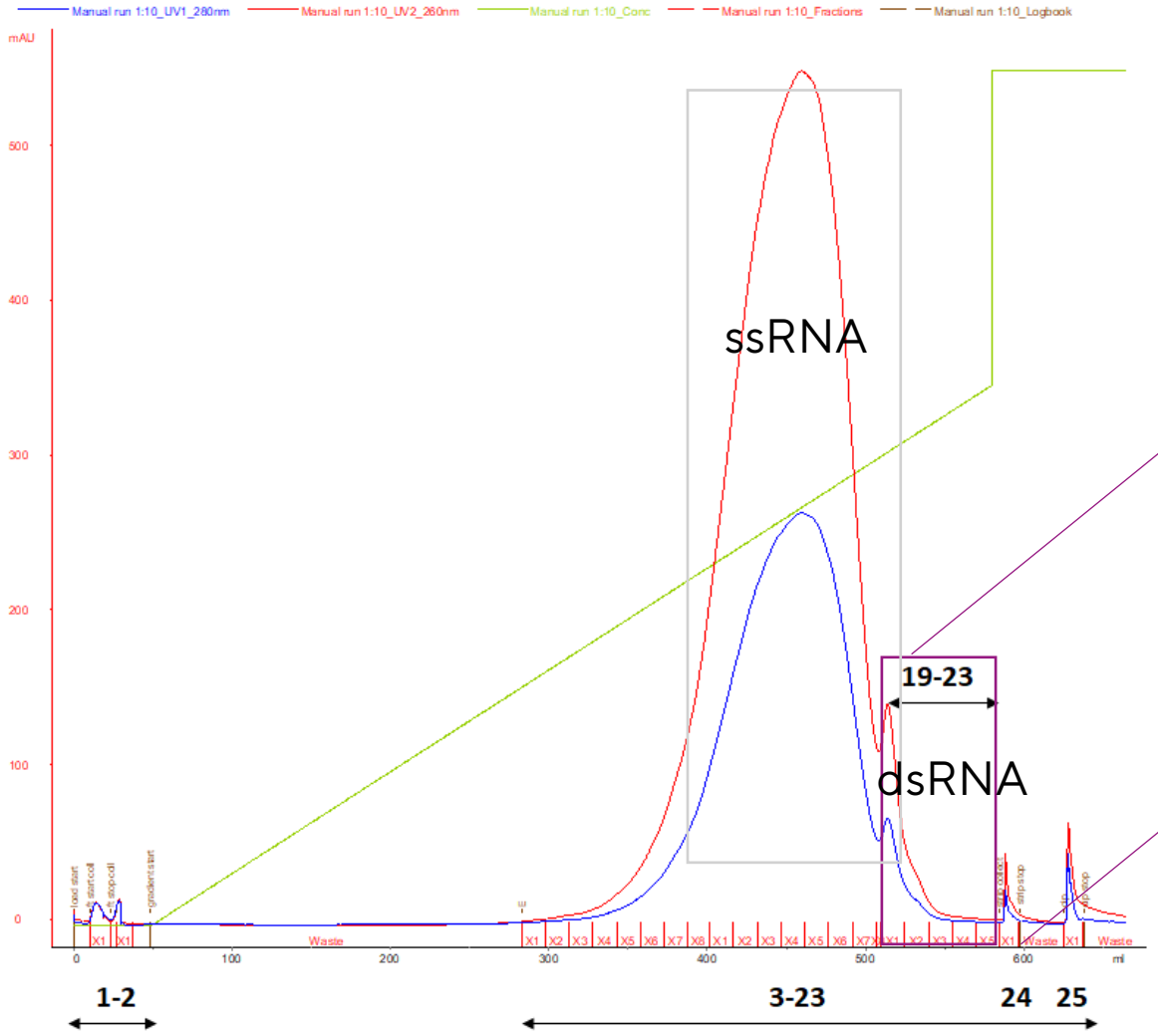


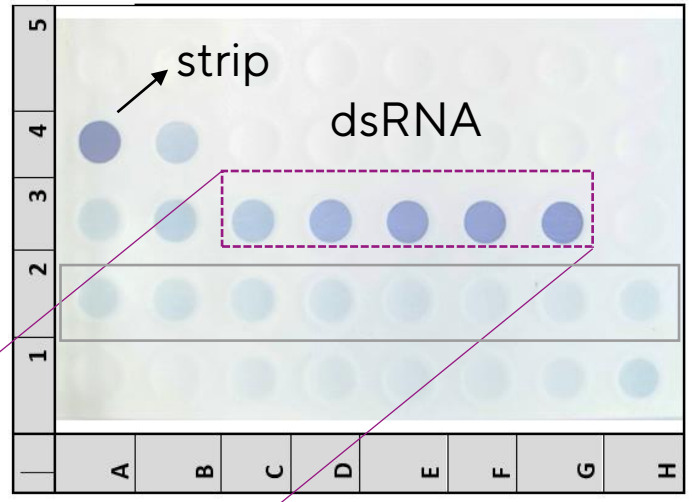


- mRNA stable for at least 28 days at room temperature after PrimaS capture from IVT

Eluate neutralisation required for mRNA stability.

Removal of dsRNA by Reverse-Phase Chromatography (SDVB)





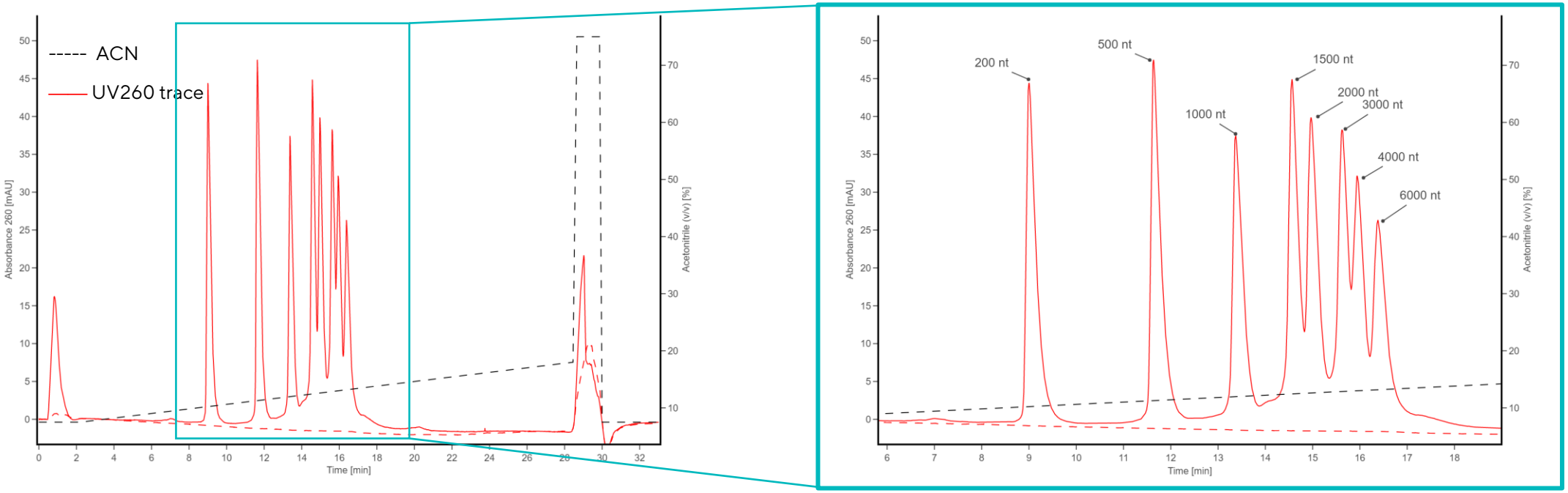
ssRNA (main elution)

- CIMmultus Oligo dT-purified Cas9 mRNA (4000 nt)
- Loaded onto CIMmultus SDVB 8 mL
- Room temperature separation 7.5→18% ACN in 50 mM TEAA, pH=7.0
- Removal of dsRNA demonstrated by J2-dot-blot

mRNA Size Separation Using CIMac SDVB Column

CIMac SDVB

- Separates ssRNA by size, example below shows RiboRuler High range RNA ladder
- Enables monitoring of shorter transcripts removal, tracking of degradation products during stability studies



CIMac SDVB with 2µm channels, Buffers: MPA (50 mM TEAA + 7.5 % ACN), MPB (50 mM TEAA + 18 % ACN), Method: 0–0.5 min (100 % MPA), 0.5–11 min (gradient to 100 % MPB). Full method not shown. Flow rate 1 mL/min, PATfix™ system, UV absorbance at 260 nm, column thermostat at 60 °C.

Concentration | Diafiltration | Sterile Filtration

DCVMN

UFDF for development and manufacturing of mRNA

Sartoflow® Smart TFF System

Ambr® Crossflow
(5 mL – 500 mL screening)

Hydrosart® 100/300 KD Sartocon Slice

Hollow Fiber TTF modules (up to 750kD) Green line

Multi-Use TFF Systems

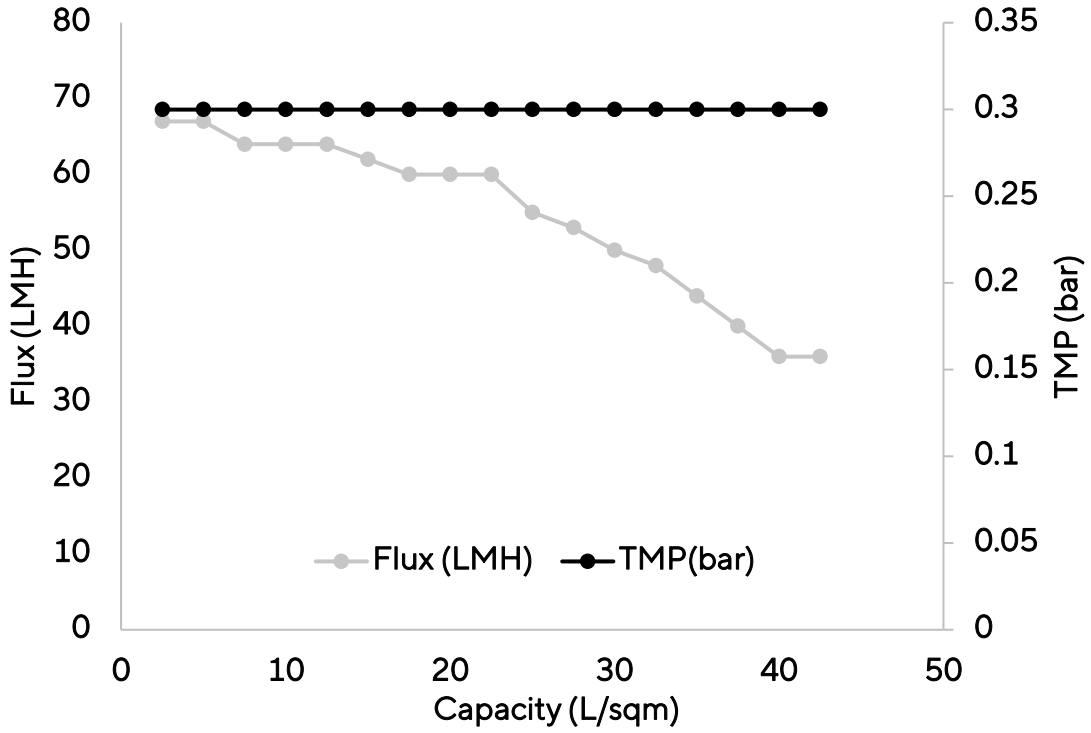
Sartocon Self-contained modules and assemblies

Single-Use TFF Systems

Select the filters and crossflow devices to get the best recovery from a comprehensive toolbox of scalable solutions:

- Purification to get rid of DNA fragments, residual enzymes such as Polymerase (99 KDa), Dnase (74 kDa), Pyrophosphatase (19 KDa) and other impurities.
- Hydrosart, a state-of-the-art crossflow membrane designed for low adsorption of mRNA and LNP, available in a 50cm² and scalable format
- Hollow fiber modules for gentle TFF and maximized mRNA yield in a 52cm² scalable format
- Run your experiments using the Sartoflow® Smart benchtop system designed for process development, including a low shear membrane pump for highest mRNA recovery
- Mitigate risk of contamination and RNAse degradation with Sartocon® Self Contained Units, hollow fibers and Maxicaps filters which can be delivered gamma sterile, facilitating closed processing
- Facilitate closed processing by using pre-assembled transfer sets including filters, tubes and connectors

UFDF for mRNA and LNP



Hydrosart 300 kDa performance data for LNP

- For LNP no change in Particle size were observed
- Cassettes are tested up to 10 kb size mRNA

Parameters	Value
Device	Hollow fiber | Hydrosart (100 | 300 kDa)
TMP	< 0.5 bar
Trial loading (L/sqm)	Up to 70 L/m^2
Volumetric concentration factor	8X
Diafiltration volume	Up to 12 DV

Sterile Filtration for mRNA and LNPs

Screening

Sartoscale 25 device

PES

Sartopore® 2/XLG/Platinum

SU Assemblies

Filter transfer sets & Maxicap MR

A toolbox of filters to find the best balance between filtration capacity and recovery

- Broadest range of PES membrane for high mRNA and LNP recovery
- **PES** filters performance better than other MOC
- Low non-specific binding which results in high recovery
- Simplify process development with the use of scalable options
- Off-the-shelf standardized pre-designed solutions
- Ensure process automation and control by using single-use systems

Poll 4

Talk to our experts about the mRNA related processes at

Raman.Rani@Sartorius.com

Poll 5

Talk to our experts about the mRNA related processes at

Raman.Rani@ Sartorius.com

Poll 6

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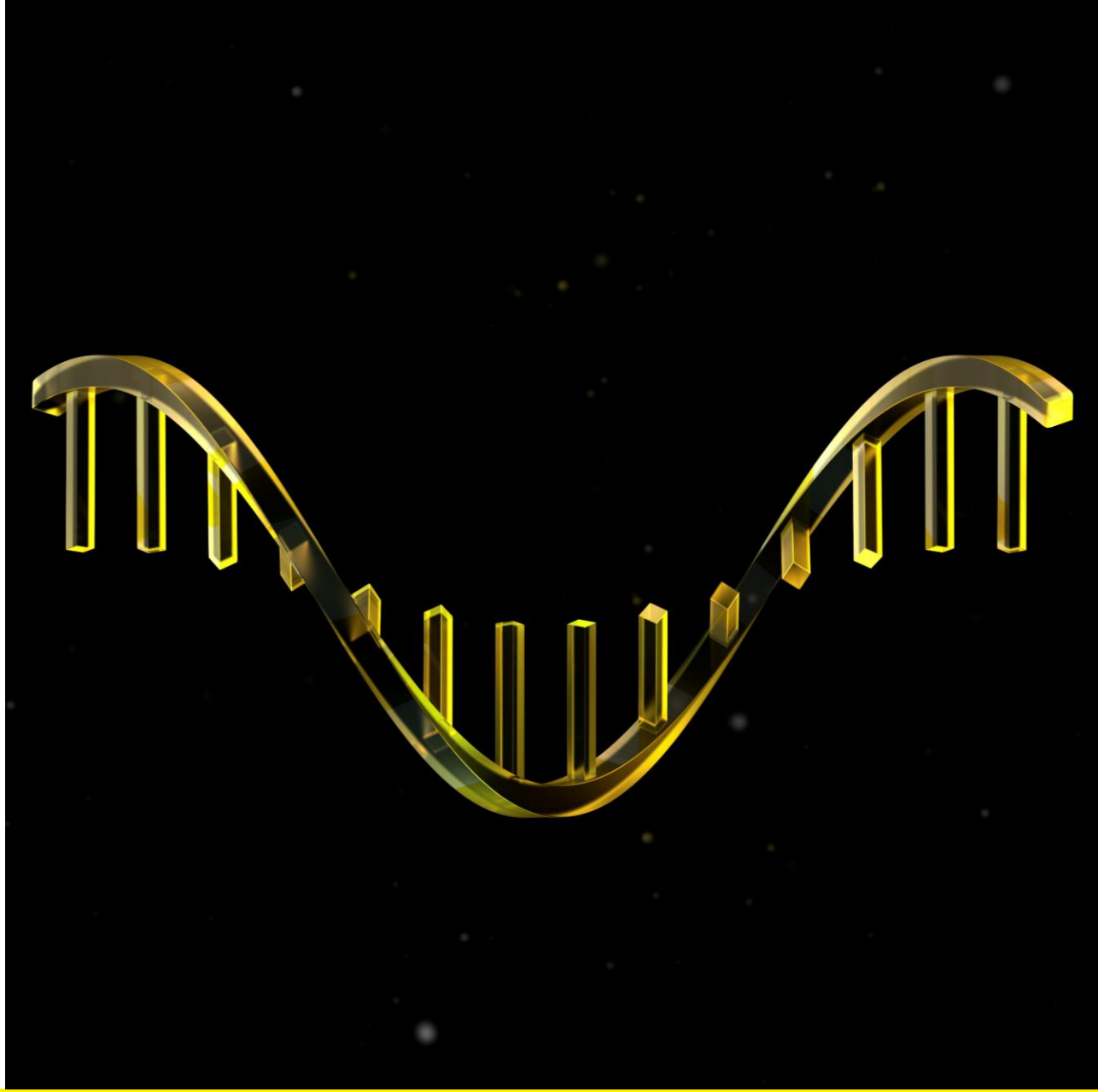
Agenda

- Introduction
- Upstream – *in vitro t*ranscription
- Downstream Solutions
- **Formulation Development & Storage**
- Data Analytics



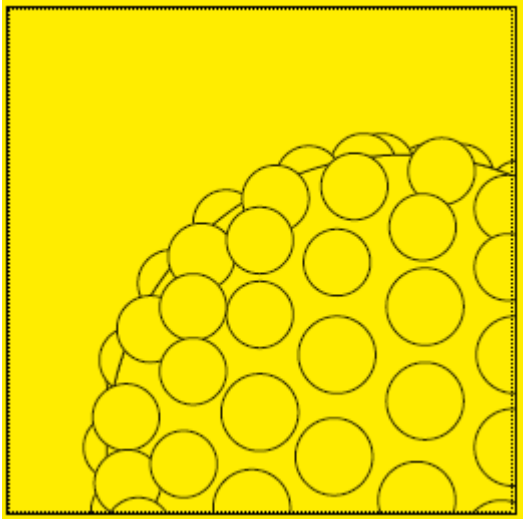
Unique Challenges of LNP Development, Storage and Shipping of mRNA

Pain points	LNPs are fragile and shear sensitive	mRNA is relatively unstable and easily degraded by RNAse	Performance differs from small-scale to late-stage	Current LNPs are stored at extremely low temperatures
Needs	Toolbox of filters	Single-use technology	Proven Scalability	Controlled Freeze|Thaw

*LNP: Lipid nano particle

SARTORIUS

Delivering via Lipid Nano Particles (LNP)



- Lipids composition of LNP may vary to optimize cellular uptake, endosomal escape, mRNA payload
- LNP size varies from 50 – 150 nm

Challenges

- Aggregation and Stability
- Control of lipid type, source and quality

The typical liposome for a mRNA vaccine contains 4 lipids

1x Cationic lipid

1x Helper lipid

1x Cholesterol

1x PEG lipid

Cationic/ionizable lipids

e.g. DOTMA/DOTAP or proprietary lipid

- Nucleic acid complexation
- Membrane fusion

"Stealth" PEG lipids

- PEG 2000
- Hydrophilic surface
- Steric hindrance

Structural helper lipids

e.g. DSPC, DPPC

- Bilayer support

Cholesterol

- Integrity
- Endosomal release

Reference : Rein Verbeke, et.al , Three decades of messenger RNA vaccine development, August 2019, Nano Today 28(Pt 1):100766

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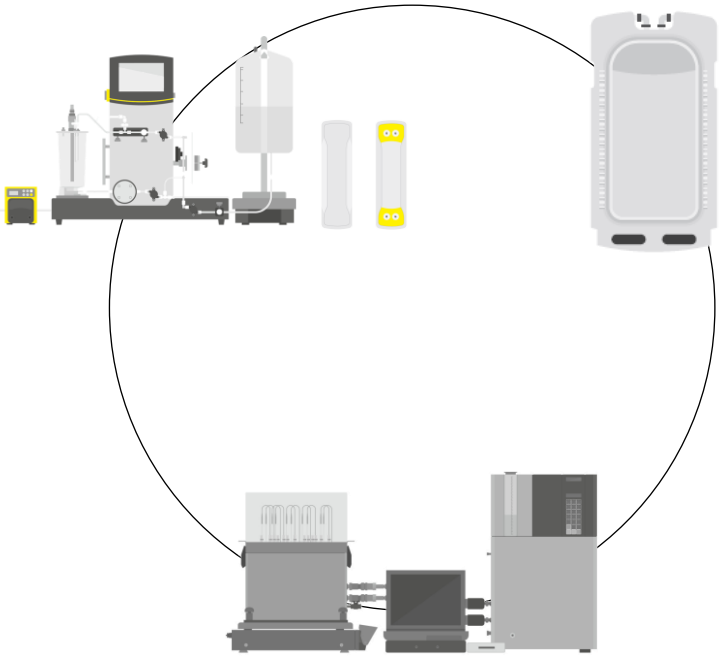
Simplify Formulation and Filling Development of mRNA

Speed up your LNP development with high throughput screening TFF and controlled Freeze/Thaw system

☑ Screening and identification of CPPs and CQAs

☑ Set Design Space



LNP formulation and filling development mRNA package

TFF systems & consumables:

- Sartoflow® Smart TFF System
- Hydrosart® 100/300KD Sartocon® slice 50 TFF Filters
- Hollow Fiber TFF (100KD/300KD)

Filters:

- Sartopore® 2/XLG Sterile Filters

Software:

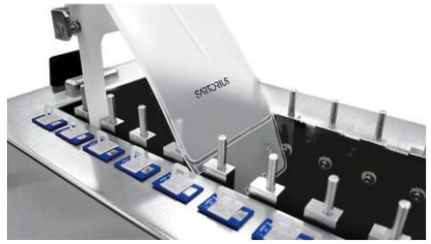
- MODDE® DOE Software

Freeze/Thaw System:

- Celsius® S^3 Benchtop Freezer

Controlled Freeze/Thaw Characterization for mRNA Formulation

Celsius® S3 Benchtop Freezer



Celsius® Pak Bags

Ensure stability of drug product and drug substance during storage by optimizing the freeze & thaw steps:

- Benefit from an end-to-end integrated approach for faster and more efficient testing and validation of freeze & thaw, assuring quality at scale
- Investigate optimal process conditions using the Celsius S3, generate a consistent samples library to investigate product stability, storage and shipping process steps
- Enables freeze/thaw characterization in 30–100 mL single-use bags with the same product contact material from lab-scale to commercial-scale Celsius® platforms
- Automated operation and data collection, combined with accurate temperature monitoring, at well-defined last-point-to-freeze location

Late-Stage mRNA Storage and Shipment with Controlled Freeze|Thaw

Celsius® CFT Freeze and Thaw Solution

Celsius® FT Plate Freezer

Celsius® Pak Bags

Celsius® FS Filling Station

Celsius® SSM Shipper

Celsius® FFTp SU Container

Biosealer® TC Sterile Disconnection

Biowelder® TC Sterile Connection

Ensure safe and simple preservation and transfer of frozen mRNA and LNPs :

- Control your process to minimize the adverse effects of the cryoconcentration in your LNP with a controlled-rate freeze and thaw system with Celsius® CFT
- Ensure consistent freeze|thaw performances in single-use bags with same product contact material from 1L up to 100L. One film for all to facilitate process validation with Celsius® CFT
- Celsius® FFTp - pre-assembled and ready to fill single-use containers to leverage existing infrastructure, fully qualified down to -80°C
- Mitigate the risk of contamination and RNase degradation with gamma-irradiated single-use bags
- Sterility Assurance during connection and disconnection, thanks to effective solutions adapted to your process requirements
- Confidence® Validation Services to support your DNAse|RNAse mitigation strategy.

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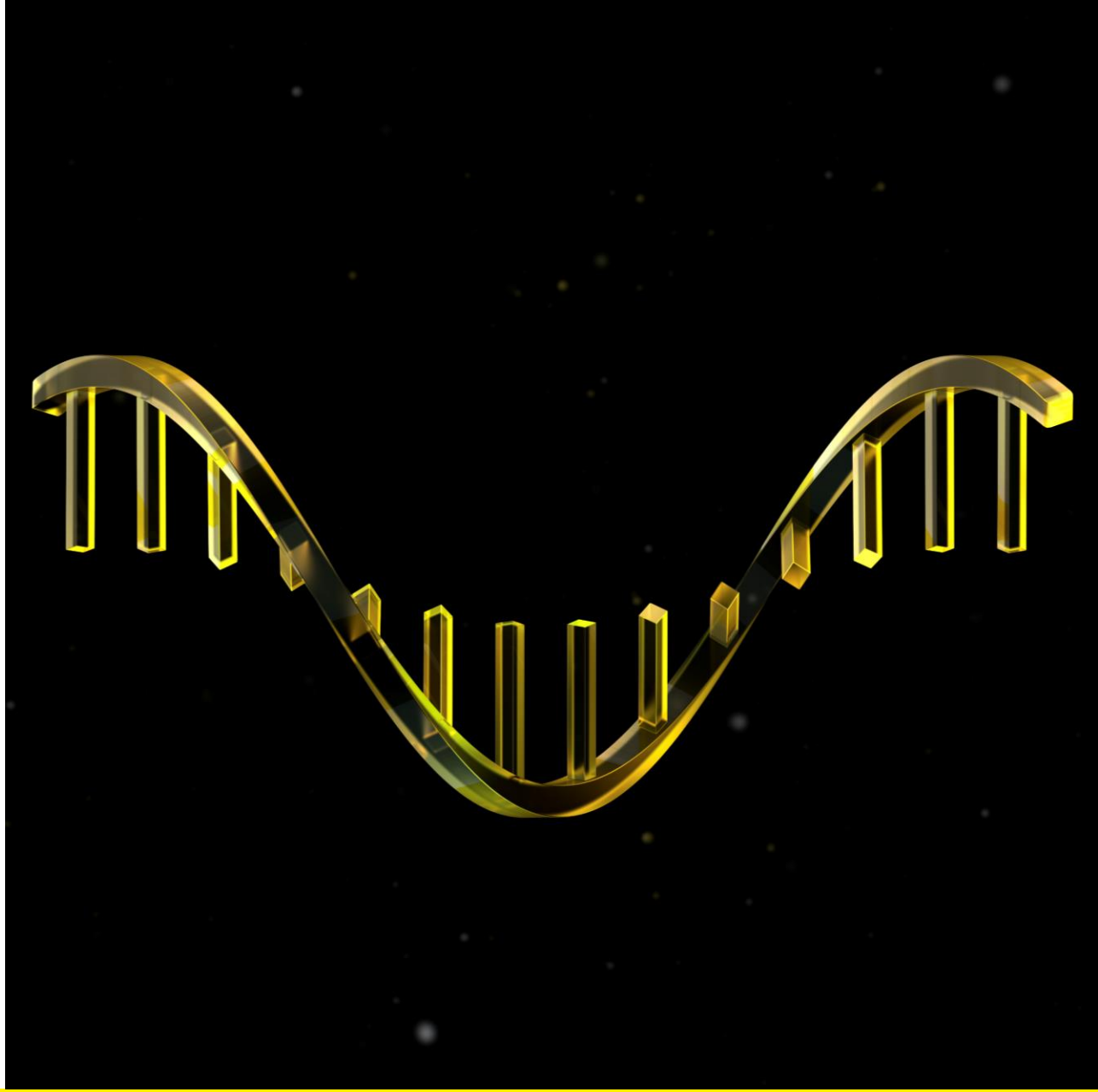
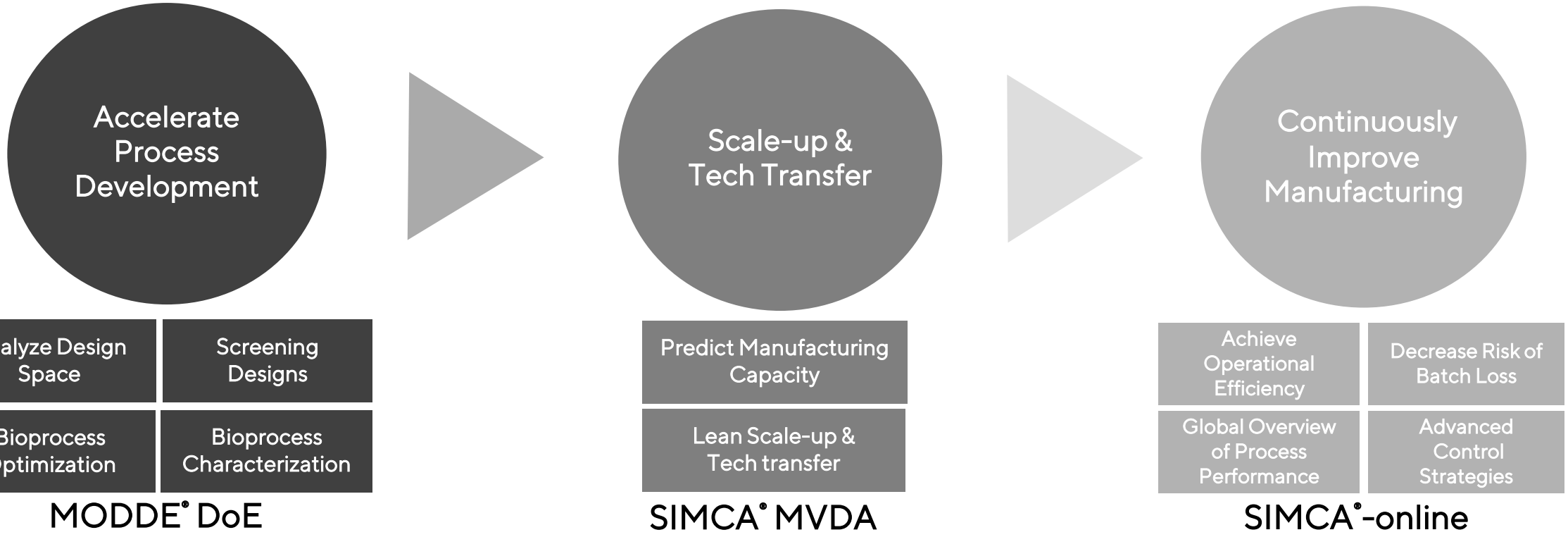


Maximize Process Understanding & Control Using Data Analytics

Systematically approach process development studies and reduce the numbers of experiments run

Accelerate scale-up and de-risk tech transfer while ensuring process robustness

Monitor and control manufacturing processes to deliver high quality product



Sartorius Solutions Simplify Progress And Fast-Track Time-to-Market

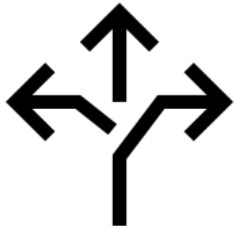
Expertise

Combining high-throughput with innovative analytics and supportive experts enable you to quickly develop in-house expertise

Innovation

Only innovative solutions dedicated to mRNA applications can eliminate some of your challenges and support your next generation process

Scalability



Develop the process with the end in mind to ensure successful implementation at late-stage from a cost, process and supply perspective

Thank you!

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