

Optimize Your Process From pDNA Template
to LNP Encapsulation With the Help of a
Single Analytical Platform

The Winding Road from E.coli to LNPs, February 15th, Boston, MA

SARTORIUS

Agenda

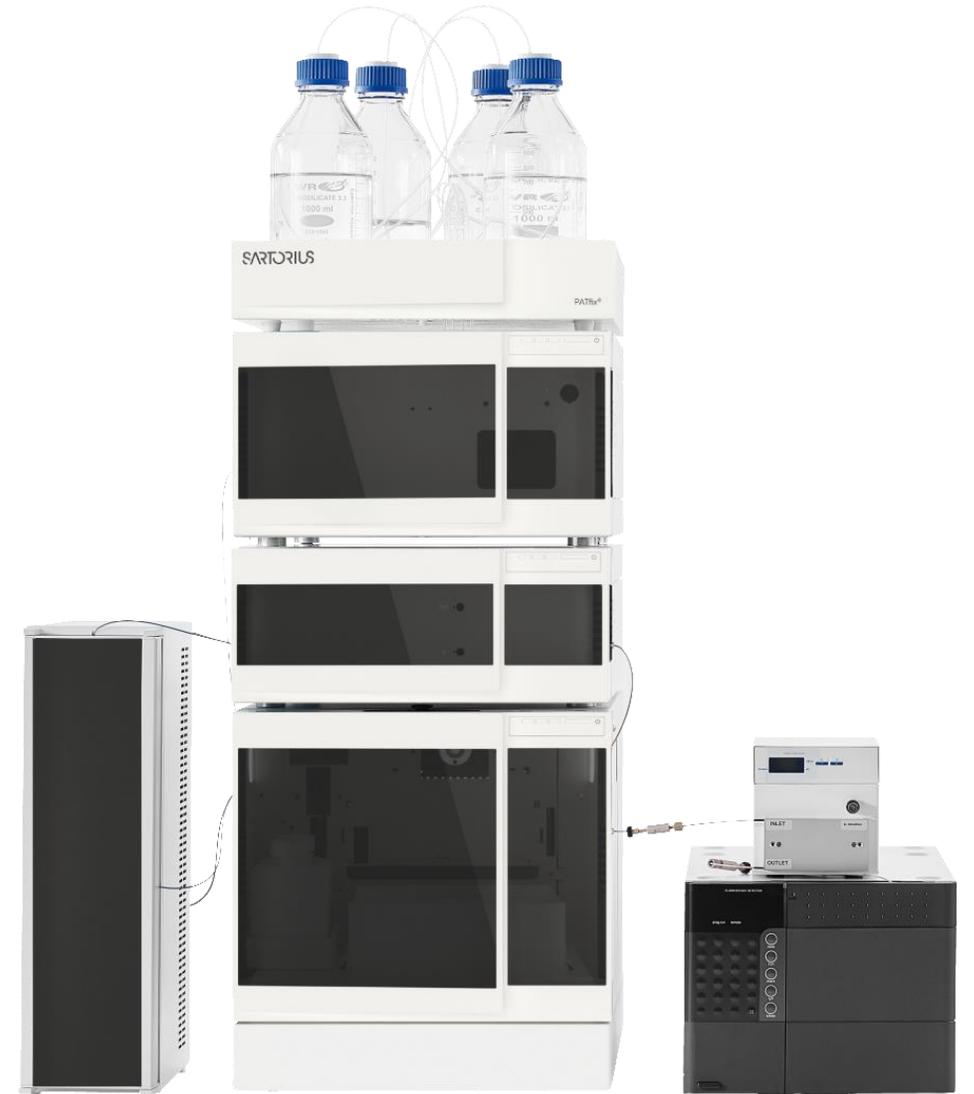
About mRNA and nucleic acids

pDNA linearization monitoring using PATfix

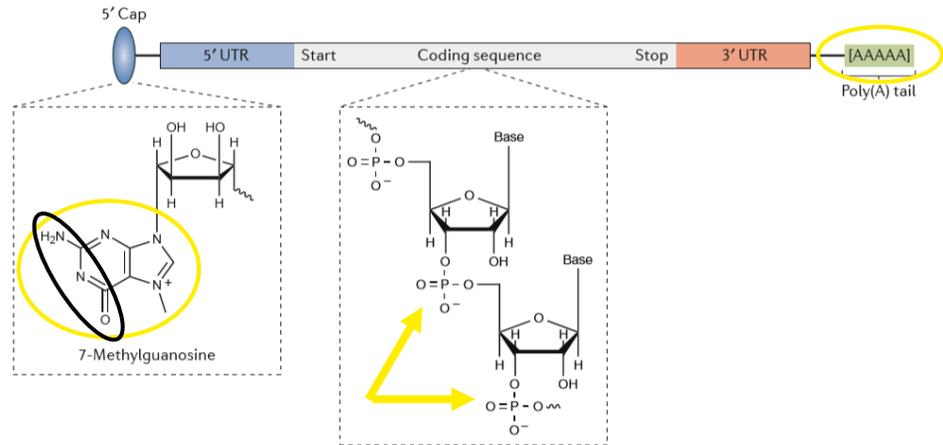
In vitro transcription yield and productivity optimisation using PATfix

Monitoring capping efficiency

New method for encapsulation efficiency



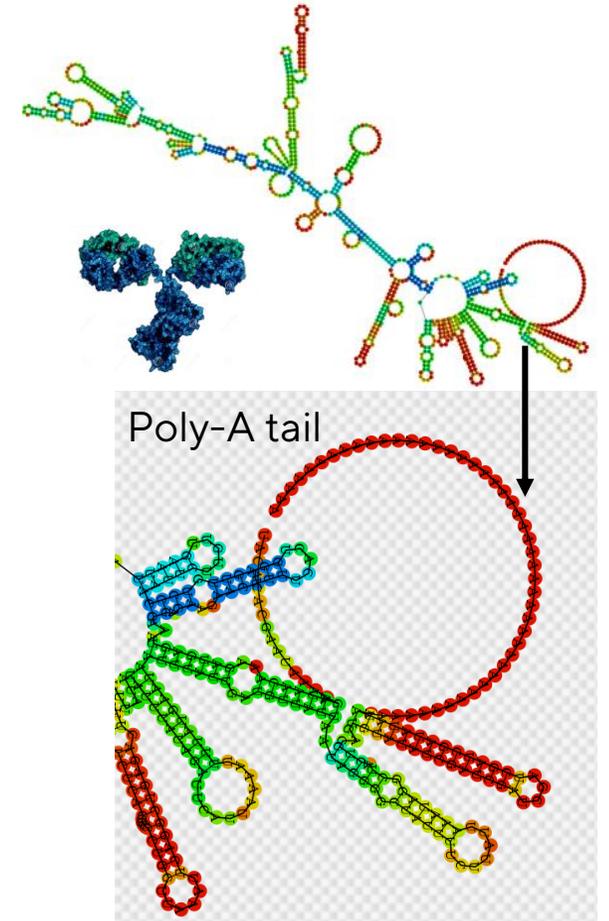
Structure and Properties of mRNA



mRNA structure: Hajj, Khalid A., and Kathryn A. Whitehead. "Tools for translation: non-viral materials for therapeutic mRNA delivery." *Nature Reviews Materials* 2.10 (2017): 17056.

- Negative charge of the phosphate backbone
- Base hydrophobicity/aromatic character
- Poly-A tail
- Base hydrogen bonding

- mRNA much bigger than proteins (e.g. IgG)

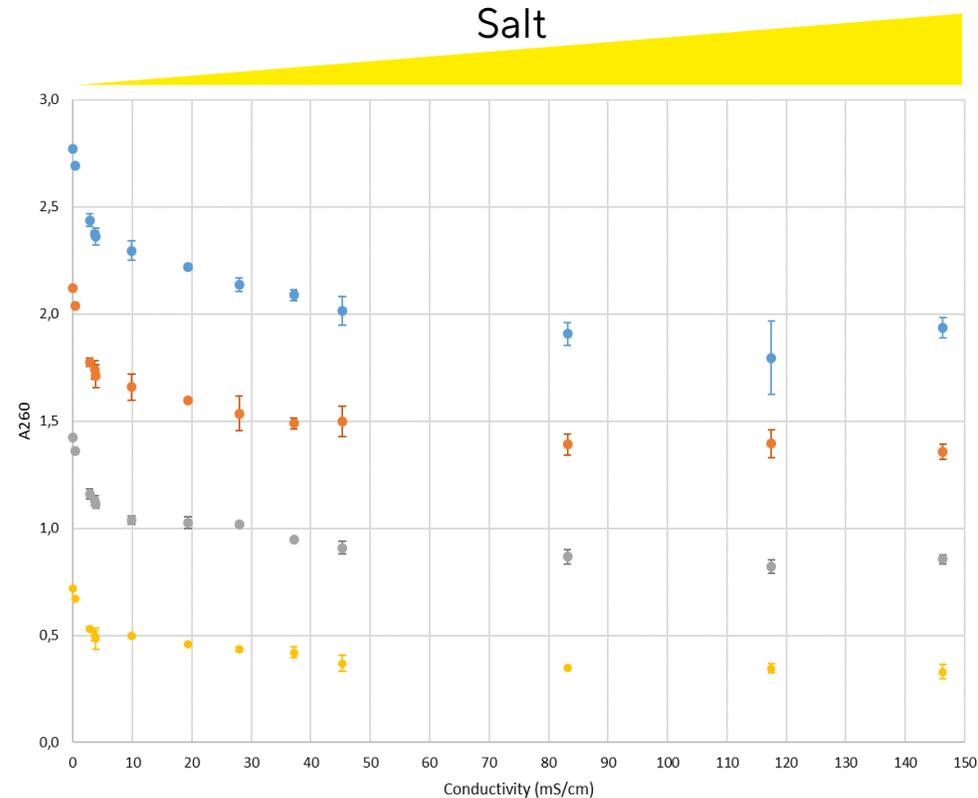


- mRNA is base-paired
BUT
- The structure is dynamic

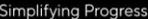
eGFP construct with 45 nt poly-A tail, minimum free energy prediction, poorly understood

Quantification is Affected by Matrix and Other Nucleic Acid Species

- UV absorbance of nucleic acids is affected by the buffer (salt concentration and pH)
- Dyes have off-target binding that skew results
- Both contribute to erroneous readouts that can lead to wrong conclusions
- Biochromatography – separation before quantification



Matrix effects on UV determination of mRNA content - Sartorius BIA Separations

Matrix effects on UV determination of mRNA content

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Higher conductivity results in lower A₂₆₀ measurements of pure mRNA samples

Deriving a formula for more accurate determination of mRNA quantity

Microvolume spectrophotometers are commonly used as quick and easy method to measure concentration and purity of nucleic acids. DSP process for purification of mRNA includes unit operations with salt concentrations up to 2.75 M (HIC) or up to 1.25 M (Oligo dT) during load and low salt concentrations during elution (Table 1).

Spectrophotometric measurement of absorbance at 260 nm (abbreviated A₂₆₀) is used for calculation of mRNA concentration using modified Beer-Lambert Law: $c = A_{260} / \epsilon \cdot d$. Microvolume spectrophotometers use averaged factors $\epsilon = 40$ ng/μL for ssRNA, $\epsilon = 50$ ng/μL for dsDNA and 33 ng/μL for ssDNA. Effect of matrix conductivity on UV absorbance has been reported [Wilfinger et al., 1997], but not yet explored for mRNA purification process-relevant matrices. A₂₆₀ variations due to matrix conductivity can lead to errors in mass balance calculations, particularly when load and elution conductivities differ significantly. We tested the effect of process-relevant NaCl concentrations on A₂₆₀ absorbance experimentally and derived a mathematical model to correct for effect of matrix conductivity on A₂₆₀.

mRNA purification step	Commonly used matrices
Oligo dT column load	50 mM Na phosphate + 1.25 M NaCl, pH 7.4
elution	dsH ₂ O
C4 HLD column load	50 mM Tris + 2.75 M NaCl, pH 7.2
elution	50 mM Tris, pH 7.2
Storage buffer	1 mM Na citrate, pH 6.0 or dsH ₂ O

Table 1. Typical process used for Oligo dT and C4 HLD purification of mRNA

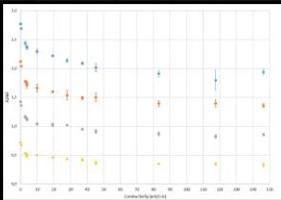


Figure 1. A₂₆₀ measurements for mRNA in matrices with different conductivities. mRNA was diluted to nominal concentration 25 ng/μL (yellow), 50 ng/μL (grey), 75 ng/μL (orange) and 100 ng/μL (blue) in matrices with different conductivities. Average value of triplicate measurements is plotted for each sample, error bars denote SD.

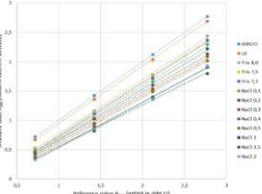


Figure 2. Comparison of A₂₆₀ measurements in dsH₂O and matrices with different conductivities. A₂₆₀ value for four nominal mRNA concentrations (25, 50, 75 and 100 ng/μL) were measured in dsH₂O (used as a reference (x-axis)). Measured A₂₆₀ values for mRNA in tested matrices are plotted on y-axis.

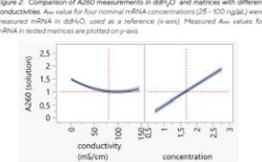


Figure 3. Model response curves for UV absorption at 260 nm. The concentration response curves were generated based on Equation 1. Conductivity response curve (left) reveals a negative effect of matrix conductivity on mRNA UV absorbance, while the effect of concentration on A₂₆₀ is linear, as expected based on Beer-Lambert law.

Factor name	Estimate	Standard error
t	-0.0252	0.0495
c	0.0051	0.0005
m	0.8044	0.0235
D	5.6E-05	1.0E-05

Equation 1:

$$A_{260}(dsH_2O) - A_{260}(matrix) = c \cdot x + d \cdot x^2 + t$$

Table 2. Formula for determination of A₂₆₀ value with conductivity correction. The formula was derived on the basis of experimental set to exclude the effect of conductivity on absorbance measurements at 260 nm. A₂₆₀ value for mRNA in a matrix and conductivity (k in mS/cm) for the matrix are required for more accurate determination of mRNA content.

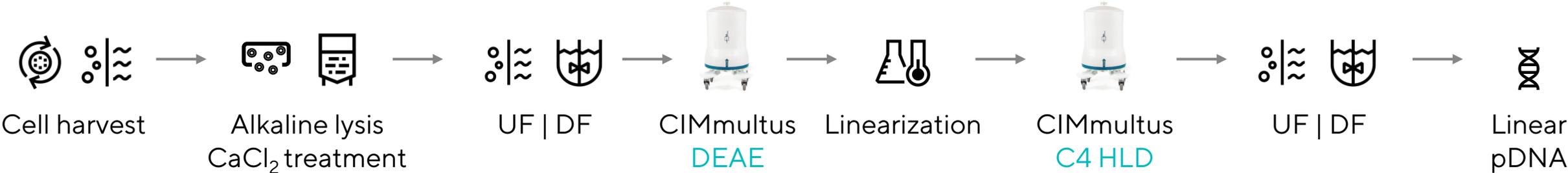
Conclusions

- DSP process for purification of mRNA includes matrices with a wide range of NaCl concentration.
- Matrix conductivity affects absorbance at 260 nm, potentially leading to errors in mass balance calculations.
- Higher matrix conductivity results in lower absorbance values. Variations >30% can be observed in matrices containing 0-1 M NaCl.
- A mathematical model is proposed to correct A₂₆₀ measurements for effect of matrix conductivity.

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mRNA Drug Substance Production Workflow

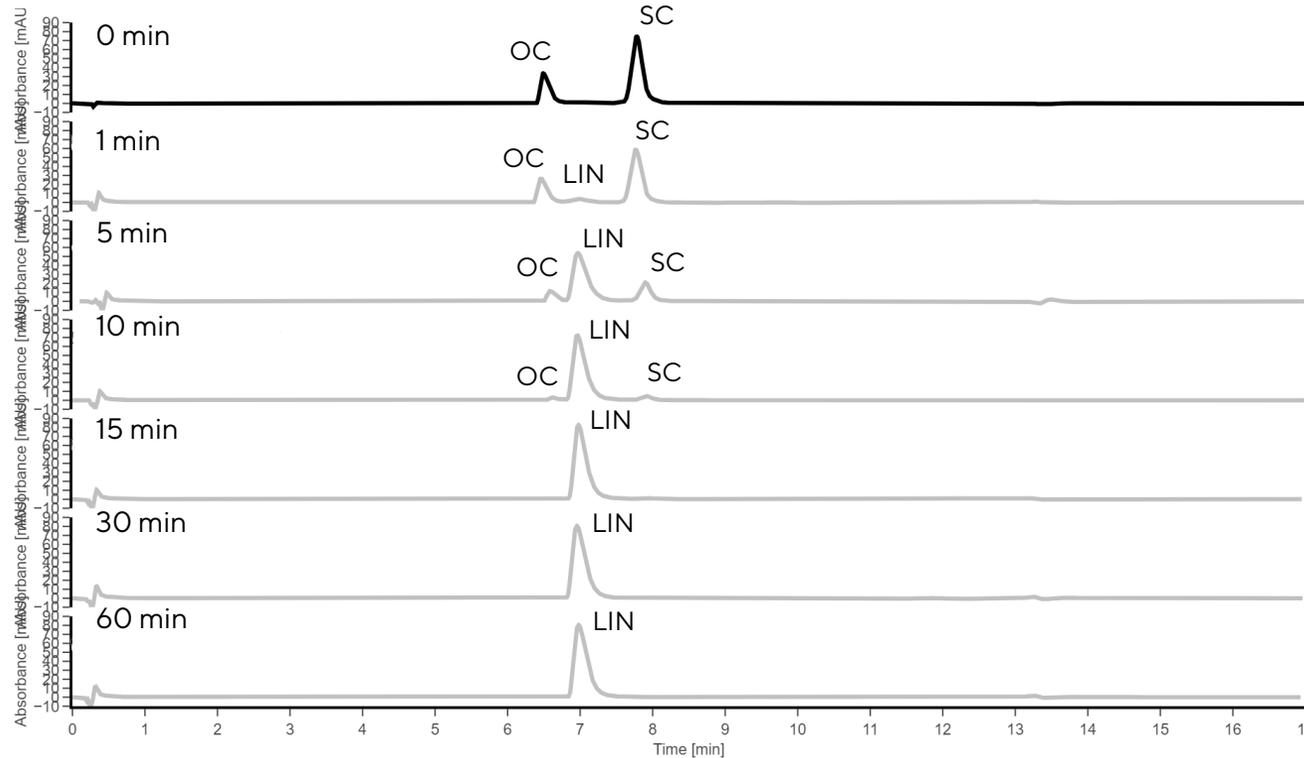
Analytical workflow (PATfix pDNA; CIMac pDNA)



Analytical workflow (PATfix mRNA; CIMac PrimaS, CIMac Oligo dT, CIMac SDVB)

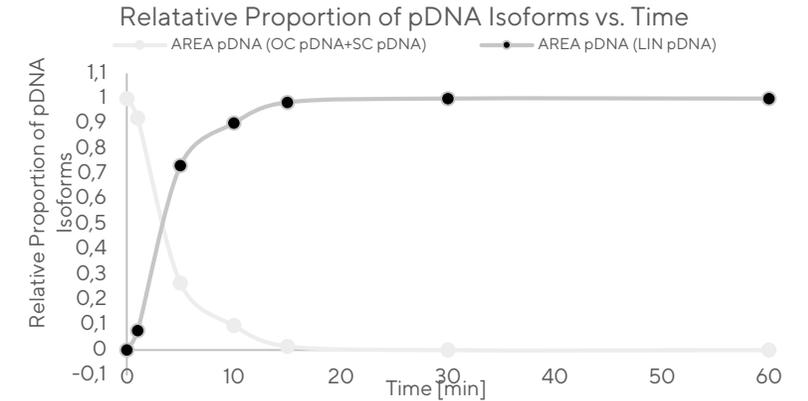
PATfix™ pDNA Platform For pDNA Linearization Control

PATfix pDNA monitoring of linearization reaction

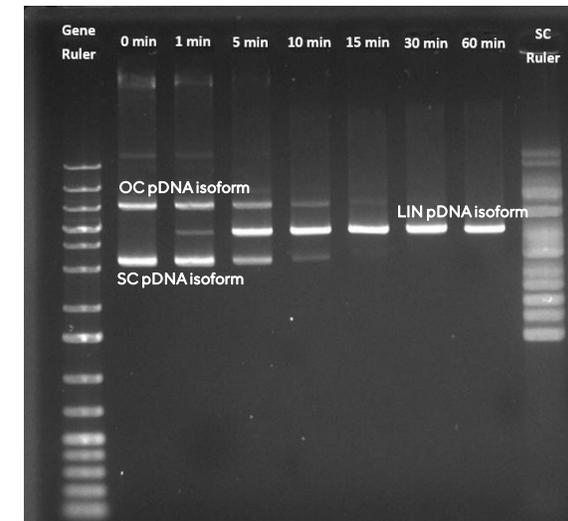


- PATfix™ pDNA platform enables us to study kinetics of pDNA linearization reaction
- Information of remaining OC & SC isoform and possible other contaminants

Kinetics of reaction

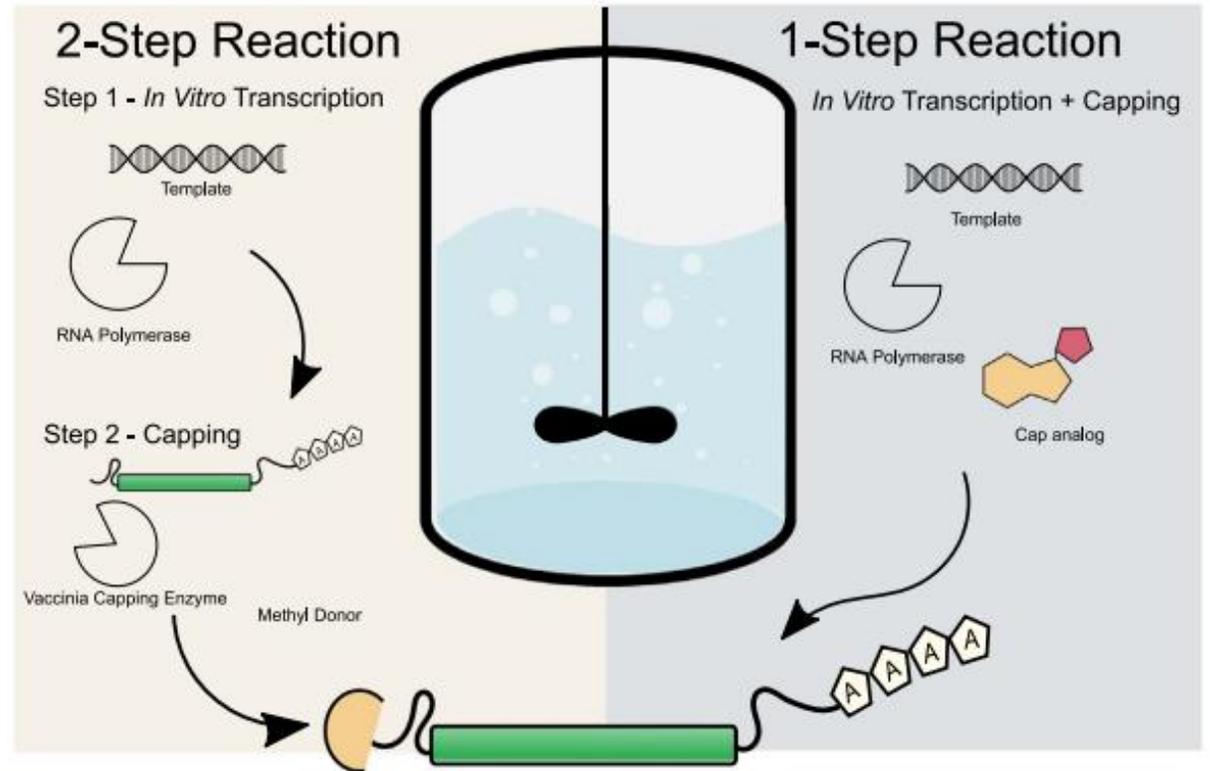


Agarose gel electrophoresis



In Vitro Transcription Reaction – Very Fast 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)
 - MgCl₂
 - Pyrophosphatase (optional)
 - RNAse inhibitor
 - Spermidine
 - DTT
 - Buffer

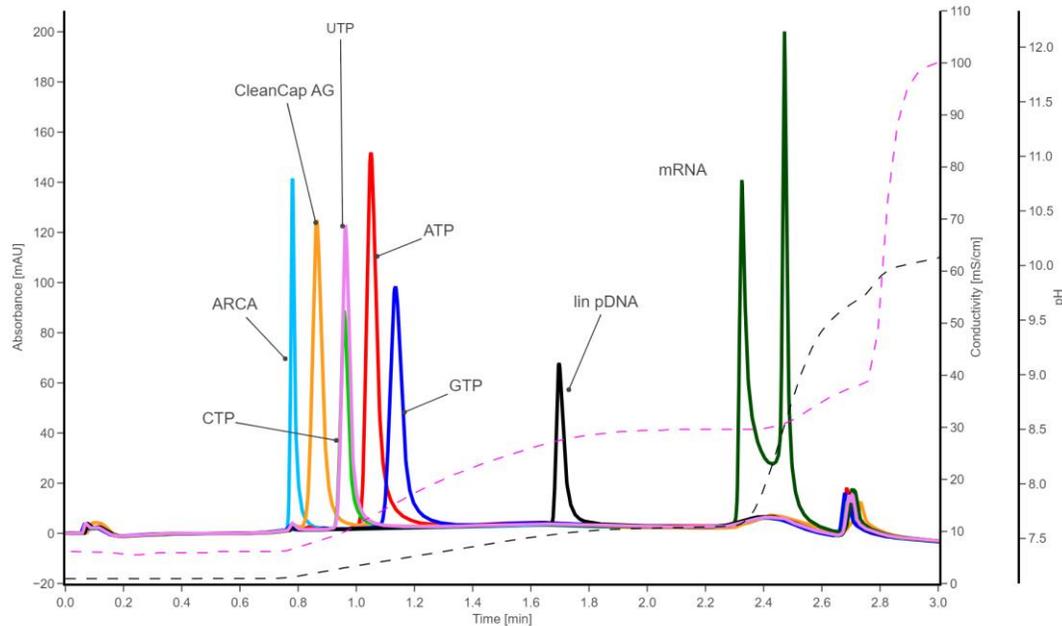


Rosa, S. S., et al., *Vaccine*. 2021 Apr 15; 39(16): 2190-2200.

mRNA Analytics: Monitoring IVT - Paradigms For Rapid At-Line Analytics

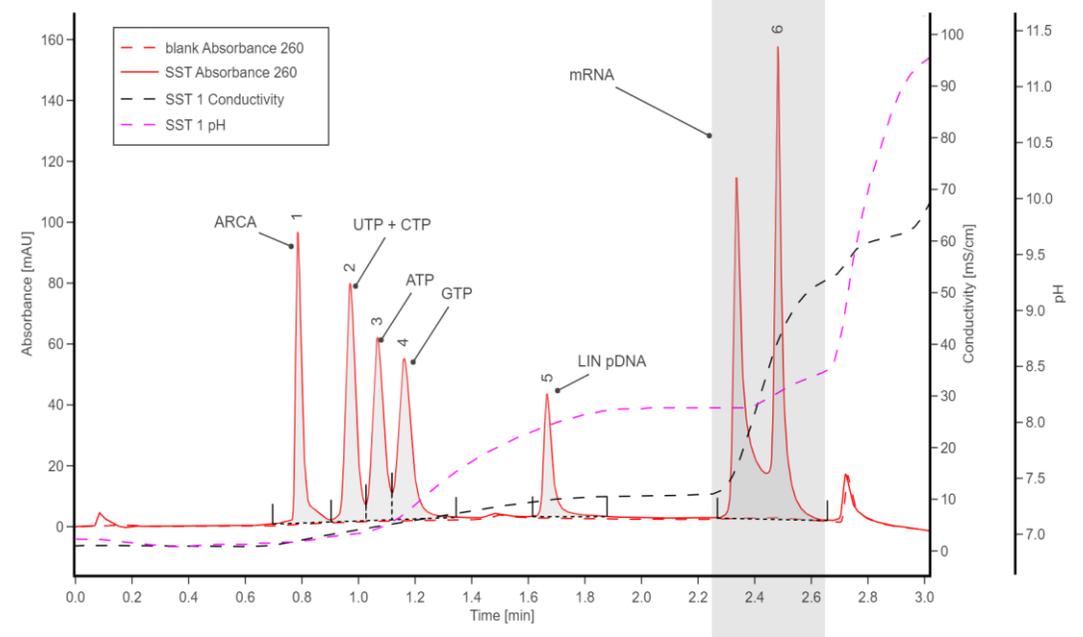
CIMac PrimaS - multimodal

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

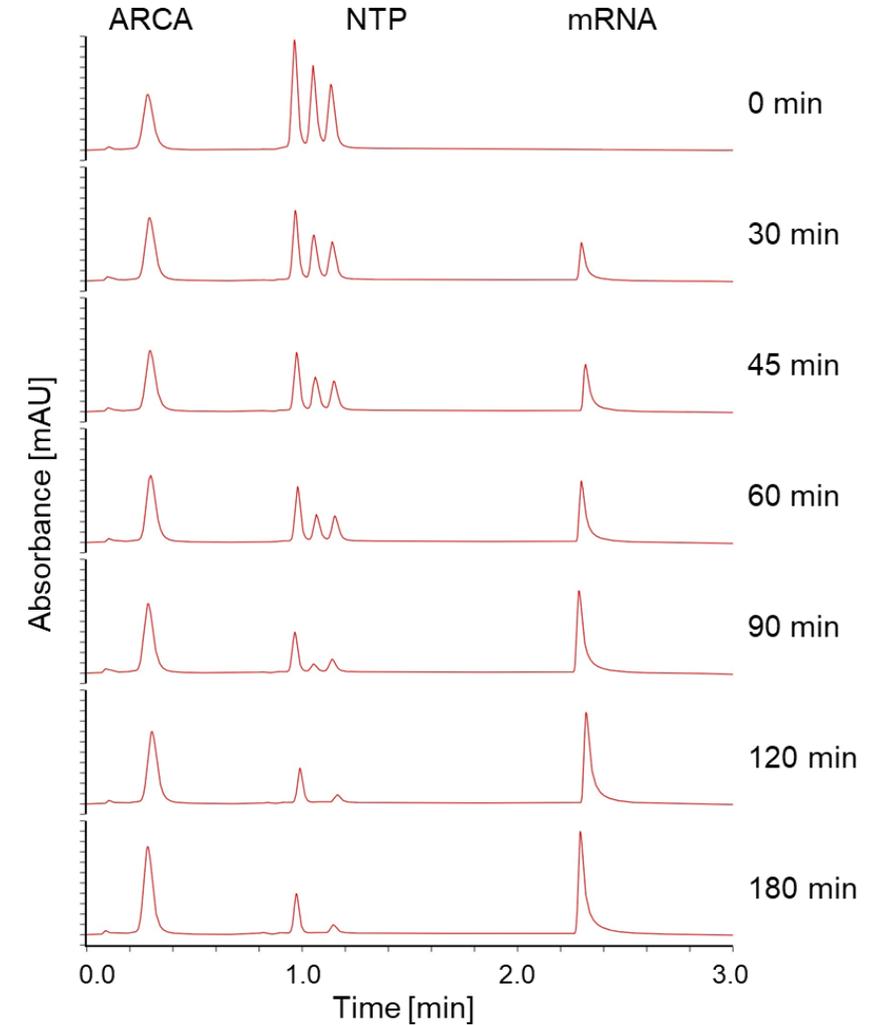
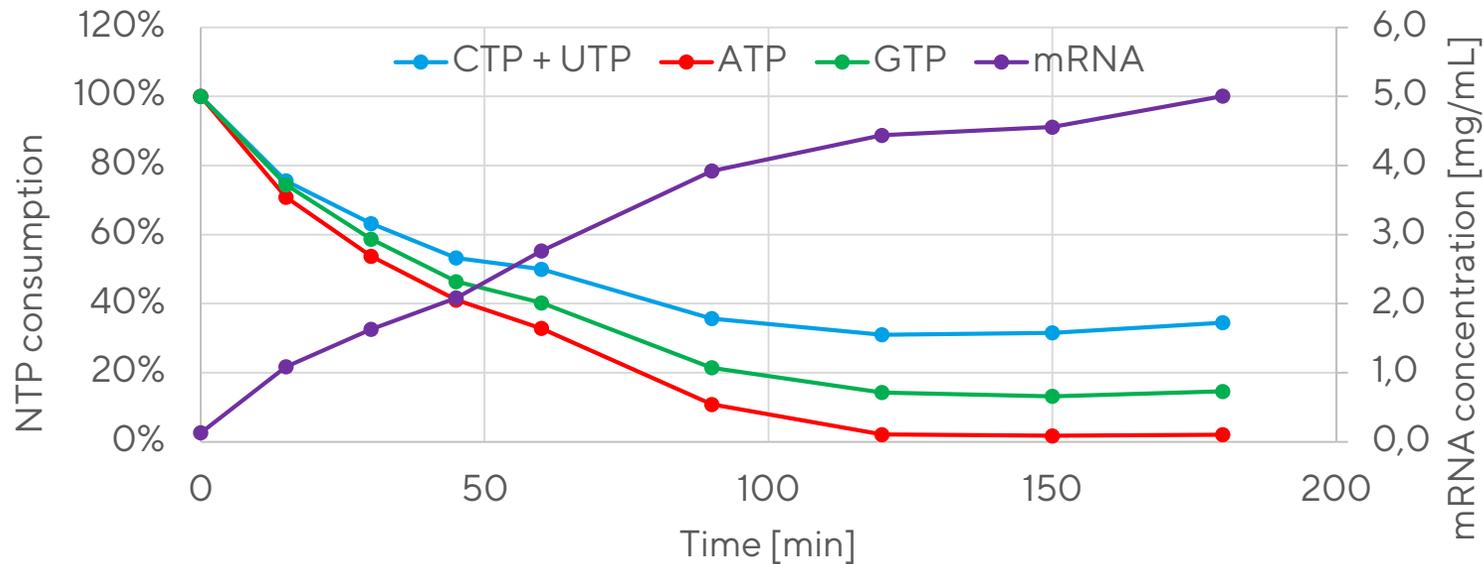


PATfix mRNA platform

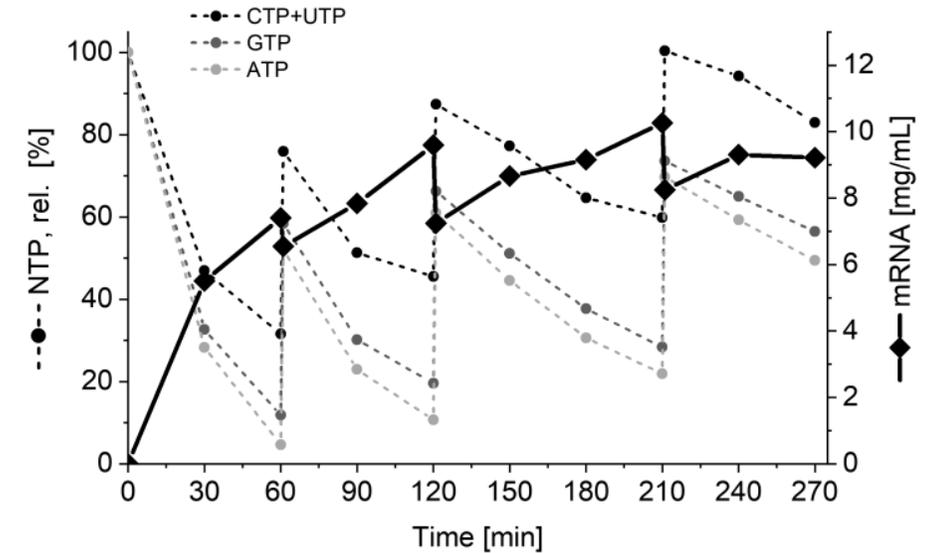
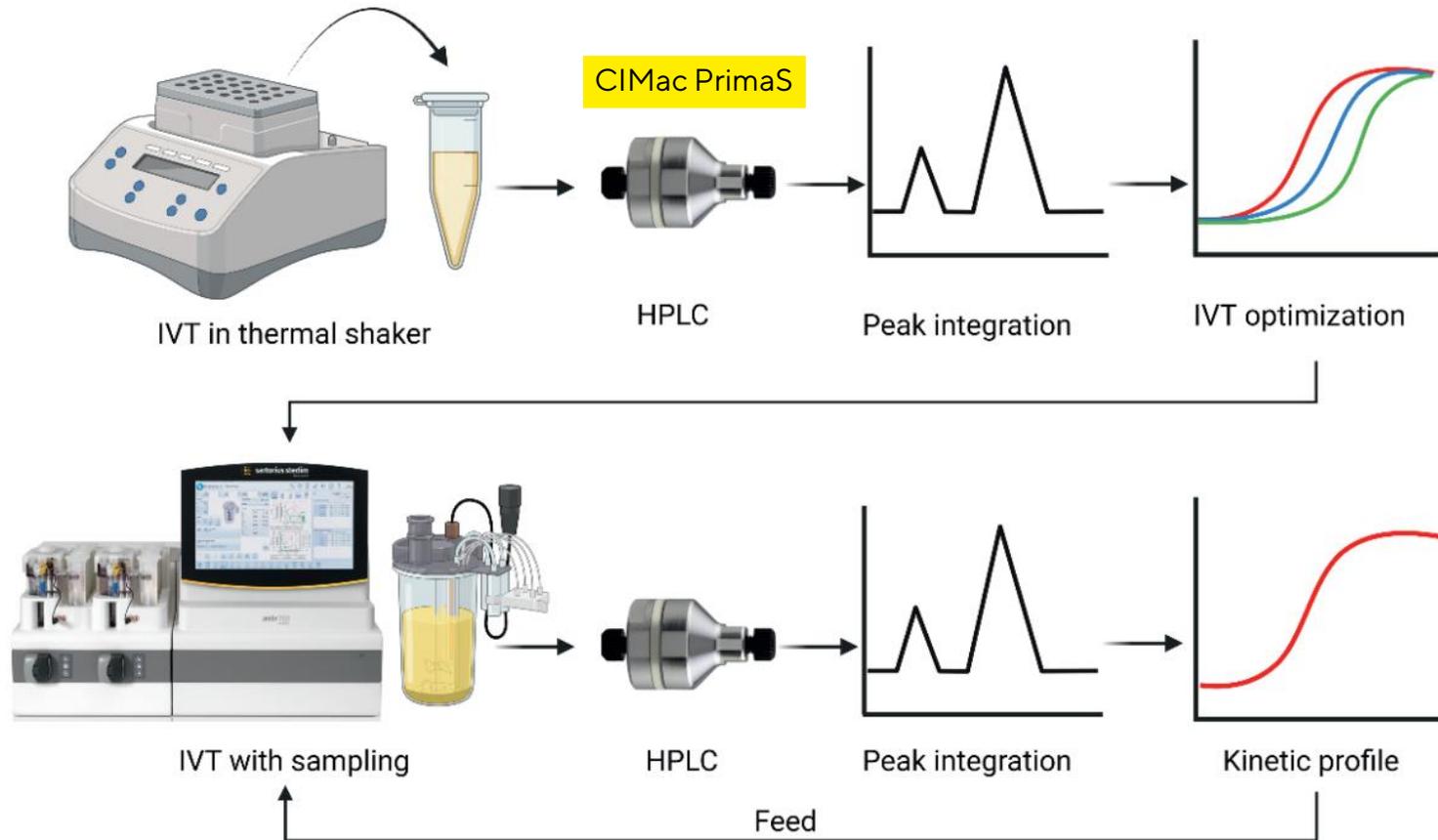
- 2.5 min for results, 8 min method
- SST-validated
- Includes additional methods for orthogonal insight



- 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



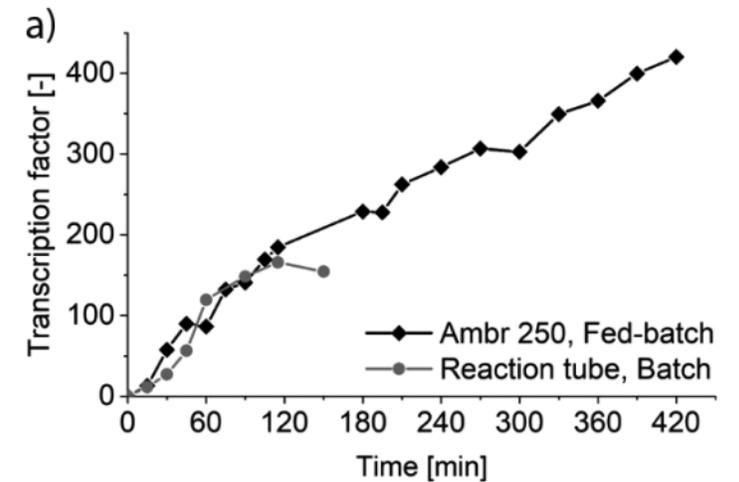
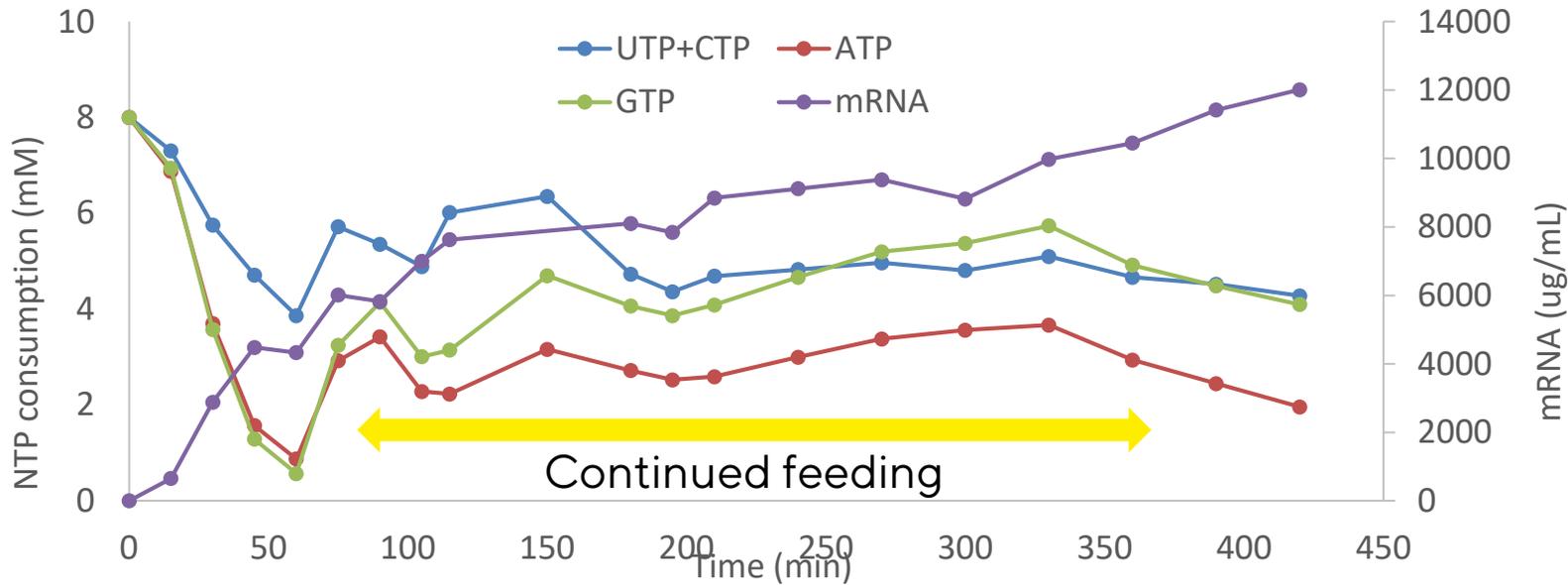
CIMac PrimaS Provides Tight Control Over IVT Reaction



- 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, Janja, et al. "Gram-Scale mRNA Production Using a 250-mL Single-Use Bioreactor." *Chemie Ingenieur Technik* 94.12 (2022): 1928-1935.

A Step Towards Continuous Manufacturing: Fed-Batch



- Real-time monitoring of NTP in reaction container.
- Reaction kinetics in scale-up comparable to thermal shaker (linear increase in transcription factor)

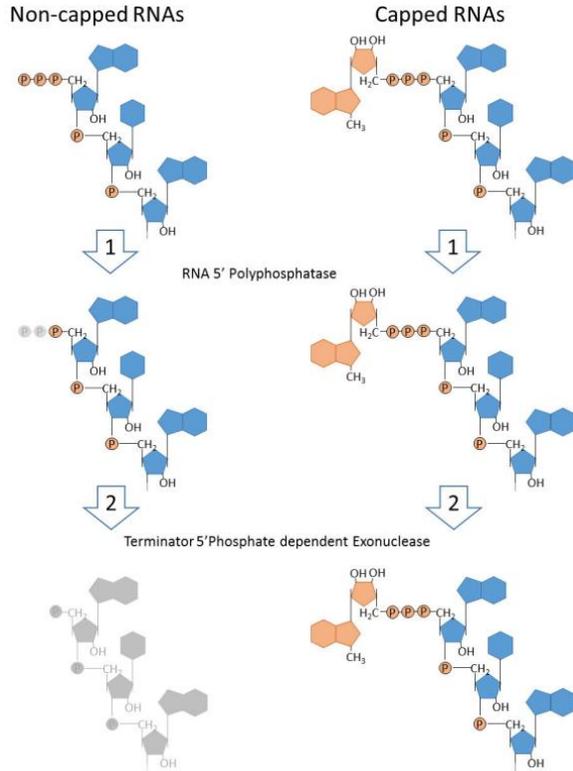
Skok, Janja, et al. "Gram-Scale mRNA Production Using a 250-mL Single-Use Bioreactor." *Chemie Ingenieur Technik* 94.12 (2022): 1928-1935.

CIMac PrimaS: Capping Efficiency (with Enzymatic Digestion)

IVT reaction



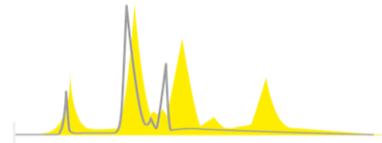
5' polyphosphatase + 5' terminator exonuclease



Enzymatic treatment selectively degrades uncapped mRNA



CIMac PrimaS



IVT with capping reagent, e.g. ARCA or CleanCap

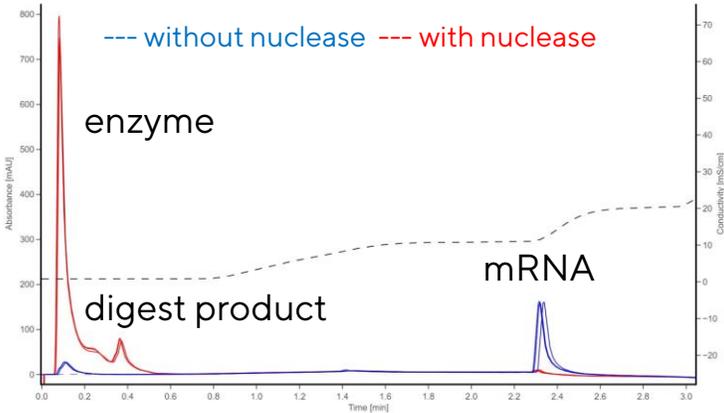
Systematic study of variables, e.g. GTP:ARCA ratio, addition of NTPs

Quantification of undigested mRNA by PATfix mRNA analytics
 Calculation of %undigested mRNA by comparison to no-enzyme control

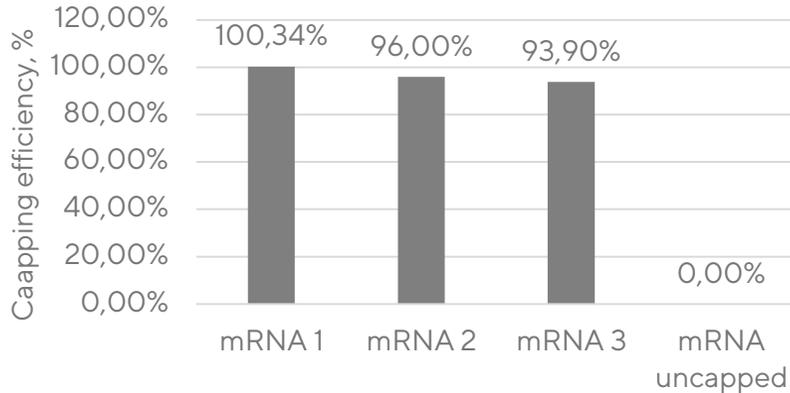
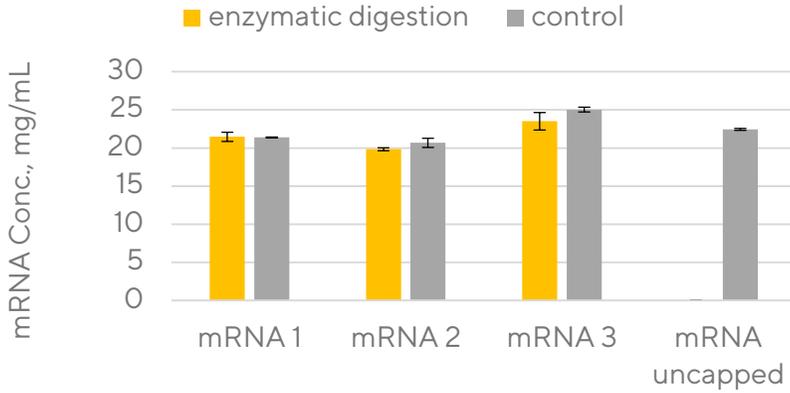
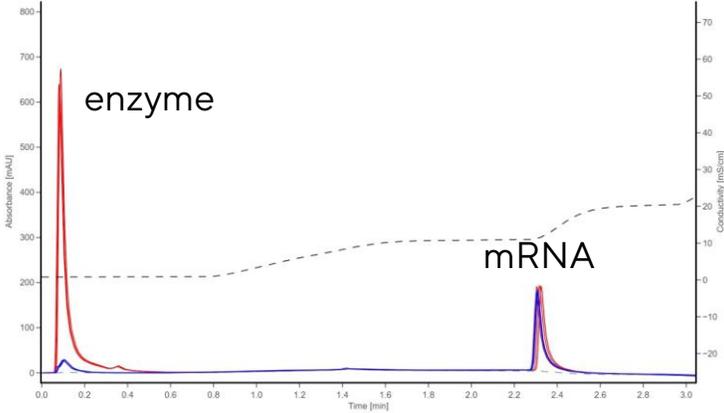
Chiron, Stéphane, and Philippe H. Jais. "Non-radioactive monitoring assay for capping of messenger RNA." *Transl Genet Genom* 1 (2017): 46-49.

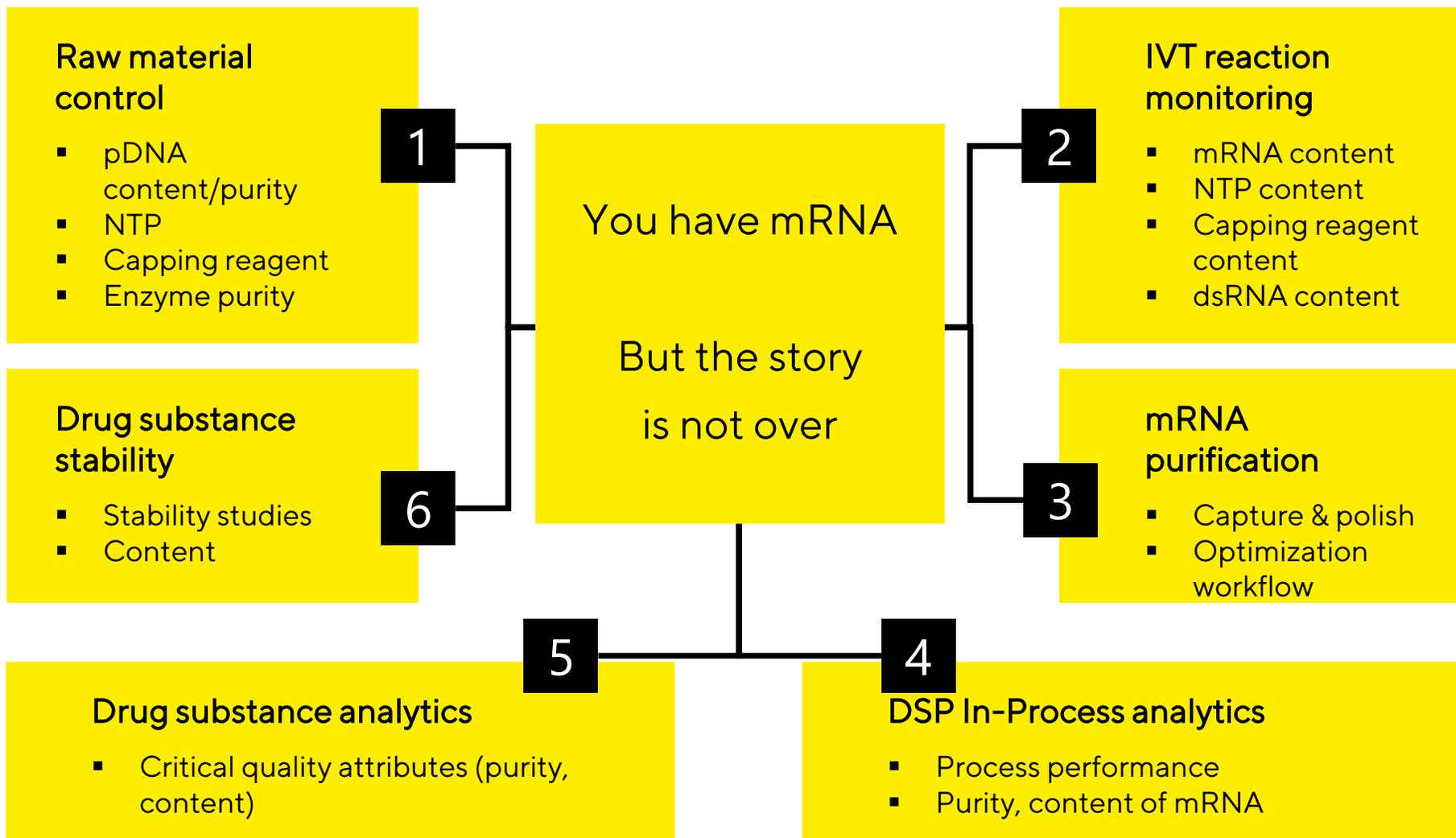
Capping Analysis of mRNA

Uncapped mRNA



Capped mRNA

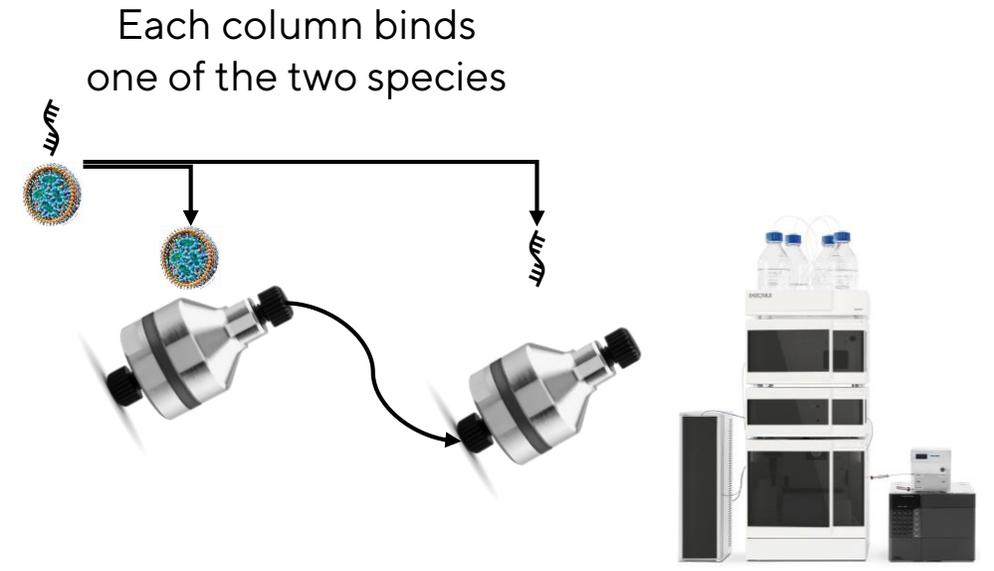
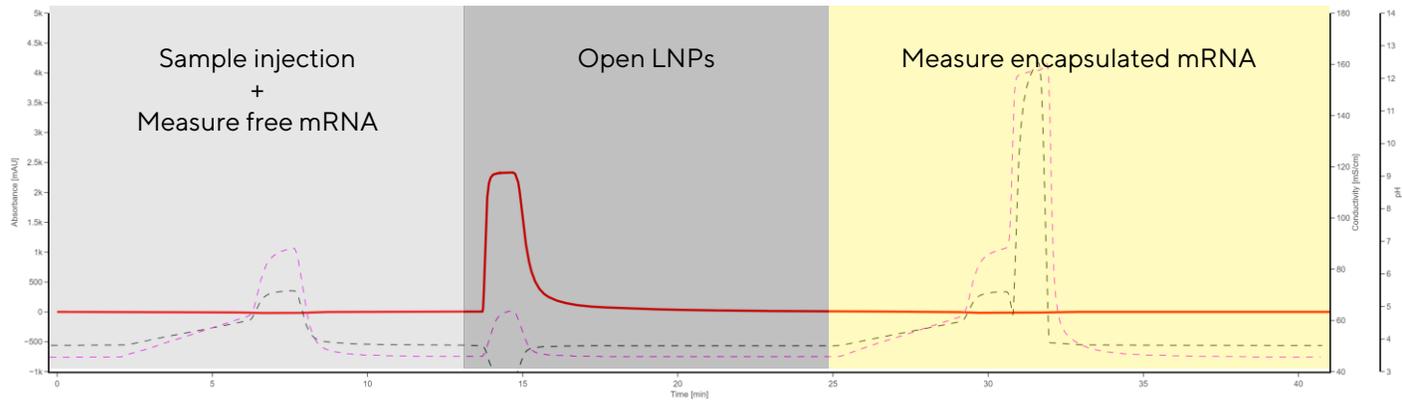




Sekimik, Kostelec, Chromatography in mRNA Production Workflows, BPI December 2021, [Chromatography in mRNA Production Workflows - BIA Separations](#)

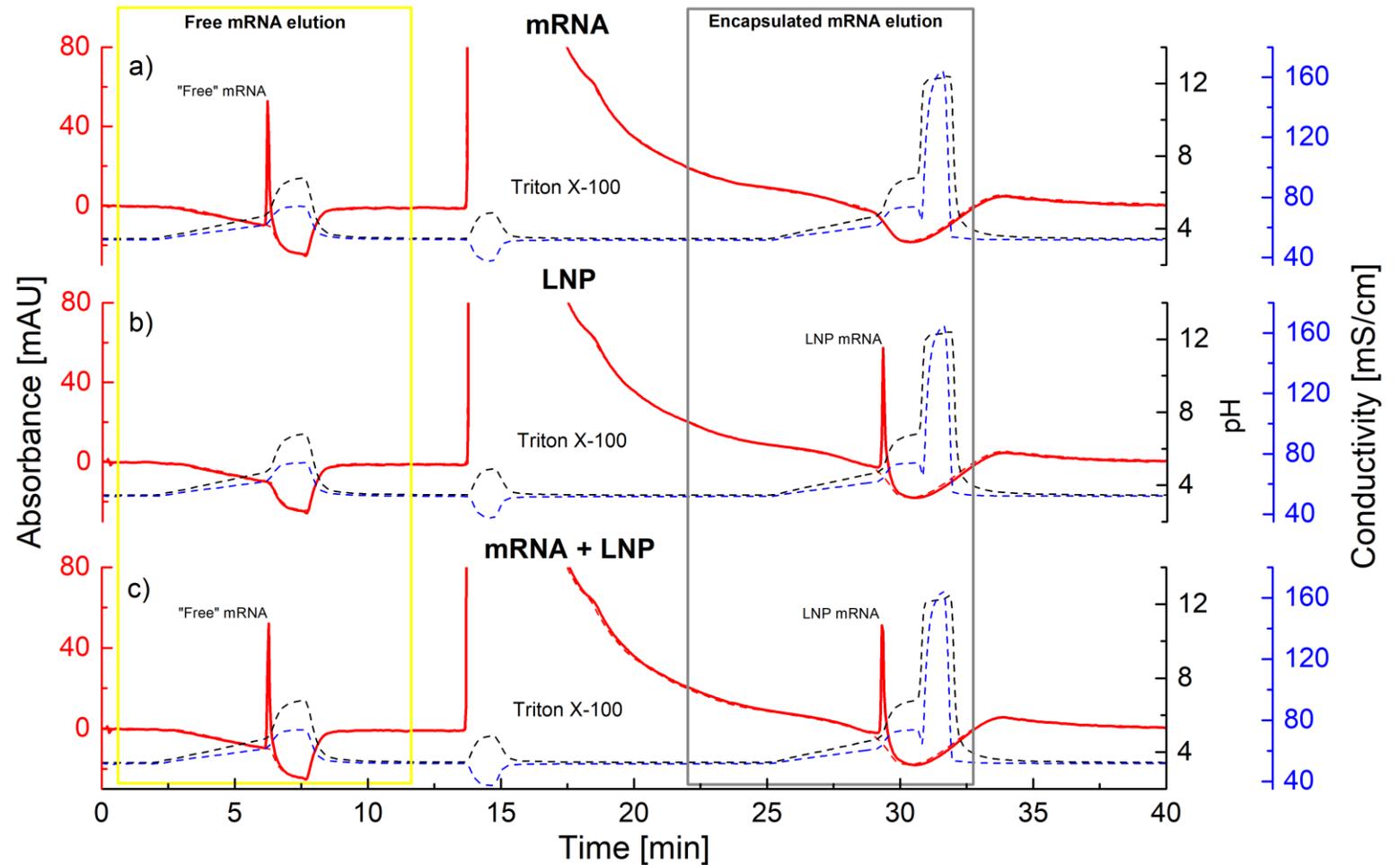
Monitoring Encapsulation Efficiency

- No dyes required
- Double column setup
- At-line quantification with immediate feedback
- Method steps overview on a blank sample:



Monitoring Encapsulation Efficiency

- Comparison chromatograms of free vs encapsulated mRNA
 - mRNA sample is mFix4
 - LNP sample is encapsulated mFix4
 - 50/50 mixture of the two
- Triton X-100 is used for opening the LNP particles
- mRNA from LNPs is then captured on the second column
- A gradient is used for elution of mRNA from the second column



Take home messages

General

- pDNA has trackable isoforms
- mRNA has a dynamic structure
- **Separation before quantification**
- Absorbance is matrix dependent
- Dyes can have off-target effects

pDNA a critical raw material

- pDNA purification
 - Monitor pDNA purification
 - Track sc/oc content and ratio
- pDNA linearization
 - Monitor sc/oc/lin
 - Determine & optimize kinetics

mRNA process development

- mRNA synthesis
 - Track all key components
 - Determine & optimize synthesis kinetics - **double yields**
- mRNA purification
- Capping analytics
- mRNA encapsulation analytics
 - What would you want to see?

Stop by our poster

PC4 team: Andreja Gramc Livk, Nejc Pavlin, Ana Ferjančič Budihna, Anže Martinčič Celjar

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Chromatographic platform for characterization of mRNA samples

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Introduction

mRNA has been at the forefront of both scientific and general public interests from the start of the COVID-19 pandemic. However, there are still limited options available for rapid characterization of mRNA containing samples. For precise characterization of an mRNA sample, first the presence and concentration of mRNA molecules in the sample needs to be identified. In the second step, any contaminants in the sample coming from the IVT reaction need to be identified and quantified. All major components of the IVT reaction: nucleotides, capping reagent, and DNA template may be present in the mRNA sample. In addition, impurities such as short, incomplete RNA fragments, and in particular, dsRNA may also be present. Contaminants may also come from the mRNA *in vivo* instability, caused by spontaneous hydrolysis of the mRNA backbone. These issues can be mitigated using appropriate analytical tools throughout the mRNA production and purification steps.

1. PATfix™ mRNA analytical platform

PATfix™ analytical system with embedded, thoroughly validated methods provides a platform for robust mRNA characterization of complex samples using three complementary analytics:

- Oligo dT affinity chromatography with CIMac™ Oligo dT column,
- PrimaS™ bimodal analysis and hydrogen bonding with CIMac™ PrimaS™ column,
- SDBV reverse phase with CIMac™ SDBV column.

Oligo dT affinity chromatography with CIMac™ Oligo dT column

The most important part of sample characterization is the mRNA quantification, which can be carried out with the Oligo dT analytics in a wide range of complex samples. This affinity column only binds the mRNAs that have a polyadenylated tail (polyA). They elute in the elution step. The species without a polyA tail elute within the first minute under non-binding conditions in the flow-through, as shown in Figure 1.

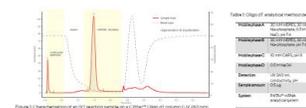


Figure 1: Observation of mRNA species in a complex sample using CIMac™ Oligo dT column (UV260 nm).

PrimaS™ bimodal analysis with CIMac™ PrimaS™ column

Using CIMac™ PrimaS™ column, full-line analysis of the IVT reaction components can be achieved with a method able to quantify throughout the IVT reaction the depletion of individual nucleotides, capping reagent, and generation of mRNA, as shown in Figure 2.



Figure 2: Bimodal analysis of a complex sample using CIMac™ PrimaS™ column (UV260 nm). Full-line analysis of the IVT reaction components is shown in the area of interest.

SDBV reverse phase with CIMac™ SDBV column

SDBV analytics provides data on mRNA integrity and size, and the amount of dsRNA in the sample.

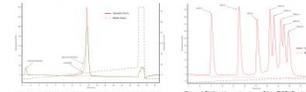


Figure 3: Reverse phase analysis of a complex mRNA sample using CIMac™ SDBV column (UV260 nm).

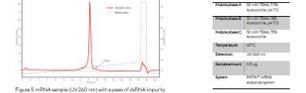


Figure 4: Reverse phase analysis of a complex mRNA sample using CIMac™ SDBV column (UV260 nm).

Conclusion

In this work, PATfix mRNA analytical platform is presented. Platform combines three different chromatographic methods specially developed and validated for characterization of mRNA samples during upstream and downstream process. The versatility of the mRNA platform was demonstrated on case study example, where three different mRNA samples were evaluated.

2. Case study: Characterization of unknown mRNA samples using PATfix™ mRNA analytical platform

Determination of impurities in the final product, introduced during IVT reaction (such as DNA template, unreacted nucleotides, mRNA fragments, double-stranded RNAs) or purification process is a critical step in any development and manufacturing of drug substance. The described selective analytical methods, which utilize three different column chemistries, deliver an analytical platform for mRNA quantification and characterization.

Oligo dT analytics

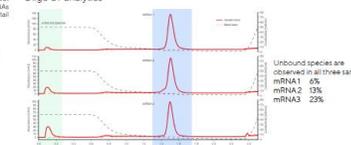


Figure 5: Characterization of three unknown mRNA samples using CIMac™ Oligo dT column (UV260 nm). Unbound species are observed in all three samples: mRNA 1: 65%, mRNA 2: 13%, mRNA 3: 22%.

PrimaS analytics

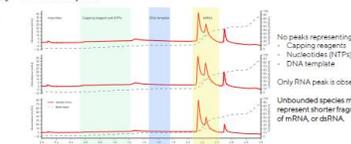


Figure 6: Characterization of three unknown mRNA samples using CIMac™ PrimaS™ column. No peaks at the retention time of impurities from the IVT reaction were observed.

SDBV analytics

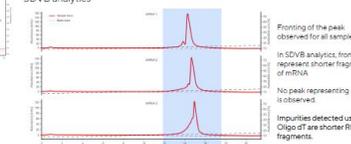


Figure 7: Characterization of three unknown mRNA samples using CIMac™ SDBV column (UV260 nm). Fronting of the main mRNA peak is observed in all three samples.