Simplifying Progress

Advancements in mRNA Synthesis: Real-Time Quantification and Characterization of In Vitro Transcription Reactions Using Modified Nucleotides

Anže Martinčič Celjar[1]*, Raymond Feng[2], Janja Skok[1], Andreja Gramc Livk[1], Aleš Štrancar[1]

[1] Sartorius BIA Separations, Mirce 21, SI-5270 Ajdovščina, Slovenia
[2] Sartorius Stedim North America, Inc., United States of America
* Corresponding author: anze.m.celjar@biaseparations.com

Introduction

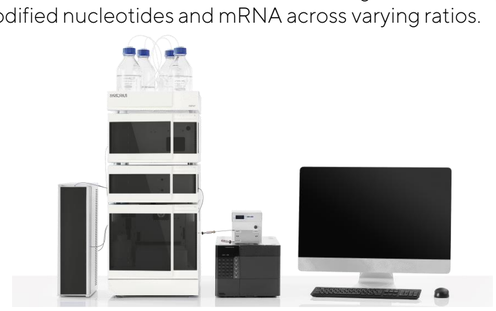
The recent push to make a viable mRNA-based vaccine against COVID-19 highlighted the significance of modified nucleosides, as one of the candidate vaccines failed precisely because only regular NTPs were used to produce the mRNA (1). The groundbreaking discovery made by Karikó and Weissman in 2005, demonstrating that mRNA synthesized with ΨTP instead of UTP exhibits reduced immunogenicity, was recognized with the Nobel Prize in Chemistry in 2023.
Modified nucleosides can change the structure, stability and even affect the rate of translation of the mRNA. As more and more research is done in this field, we focused on developing a method enabling at-line IVT reaction monitoring using two naturally occurring modified nucleotides; 1-methylpseudouridin triphosphate (N¹meΨTP) and 5-methylcytidine triphosphate (m5CTP), on a multimodal CIMac PrimaS® column using PATfix® analytical system. The developed method facilitates the monitoring of in vitro transcription (IVT) reactions by accommodating the quantification of modified nucleotides (N¹meΨTP and/or m5CTP), unmodified nucleotides and mRNA across varying ratios.

1. IVT reactions

In IVT reactions, a 4000-nucleotide mRNA (mFix4) was produced* using different nucleotide mixtures:

- IVT1: 4 mM CTP, ATP, GTP and N¹meΨTP,
- IVT2: 4 mM CTP, ATP and GTP, 2 mM UTP and N¹meΨTP,
- IVT3: 4 mM 5mCTP, ATP, GTP and UTP,
- IVT4: 4 mM ATP and GTP, 2 mM CTP, 5mCTP, UTP and N¹meΨTP.

*IVT reactions were prepared by the IVT procedure described by Pregeljc et al.(2).



PATfix chromatographic system

CIMac PrimaS analytical column

2. At line IVT monitoring using CIMac PrimaS

The novel method enables chromatographic separation of CTP, N¹meΨTP, UTP, ATP, GTP, linearized pDNA template and mRNA.

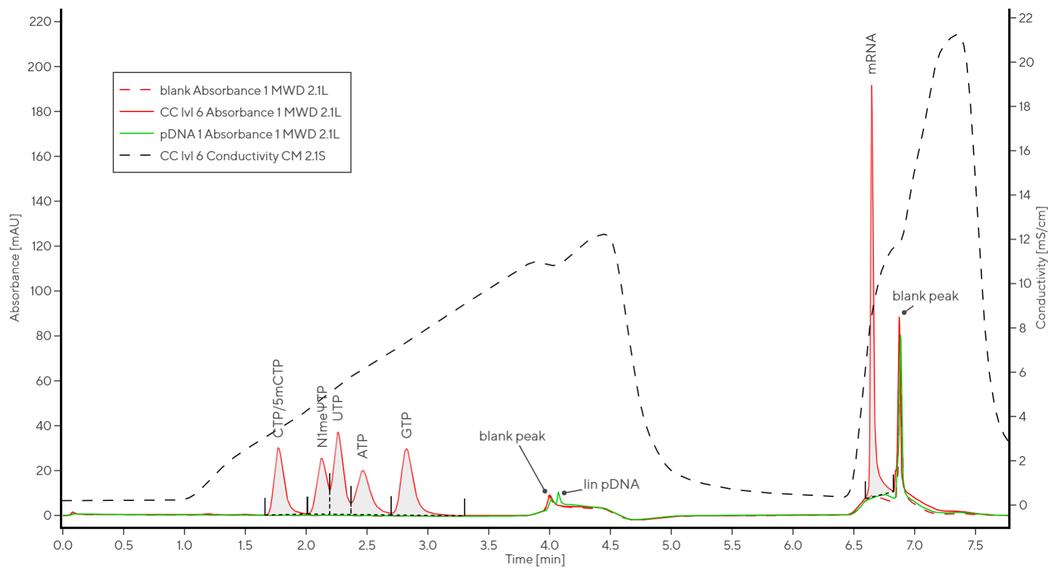
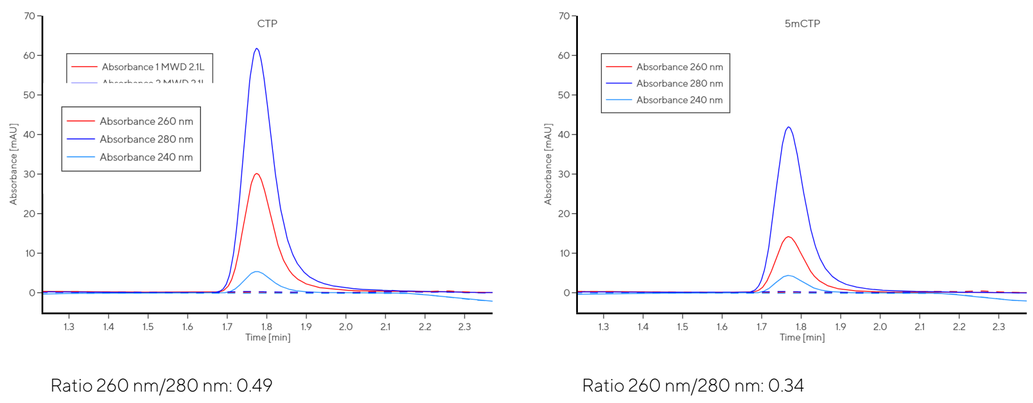


Figure 1: Example chromatogram of calibration curve standard and lin pDNA samples, analyzed with the developed method.

Under shown chromatographic conditions, CTP and 5mCTP cannot be chromatographically separated. It is however still possible to deconvolute them and determine their concentration in the IVT samples from the difference in their UV absorbance ratios at 260 nm, 280 nm and 240 nm.



Ratio 260 nm/280 nm: 0.49
Ratio 260 nm/240 nm: 5.63

Ratio 260 nm/280 nm: 0.34
Ratio 260 nm/240 nm: 3.25

Figure 2: CTP and 5mCTP absorbance ratios.

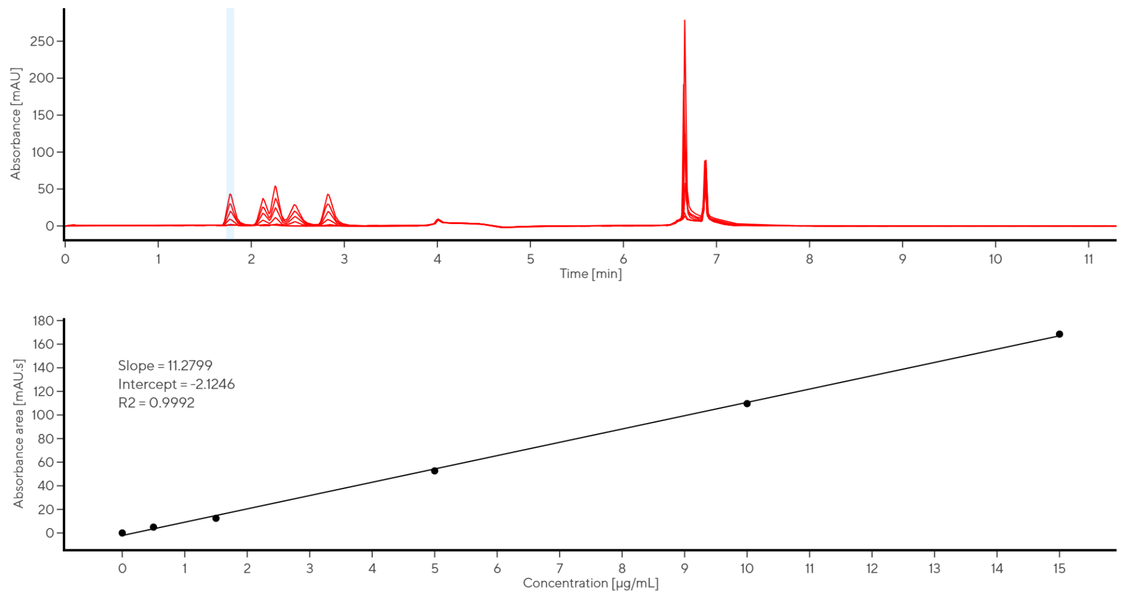
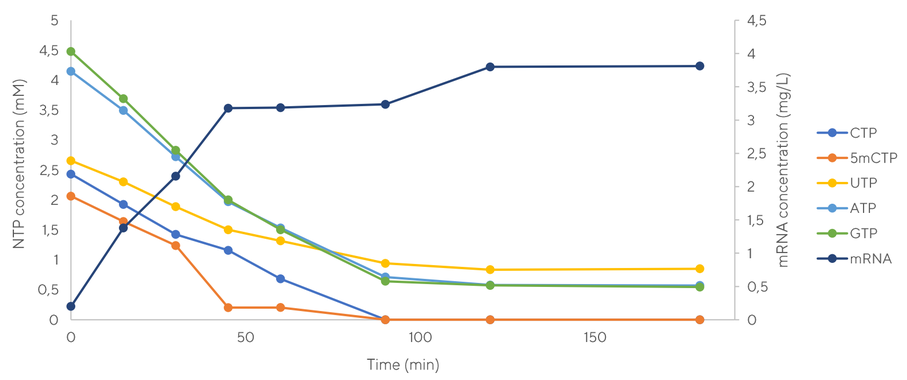


Figure 3: Calibration curve of CTP/5mCTP as calculated in the PATfix softwere.

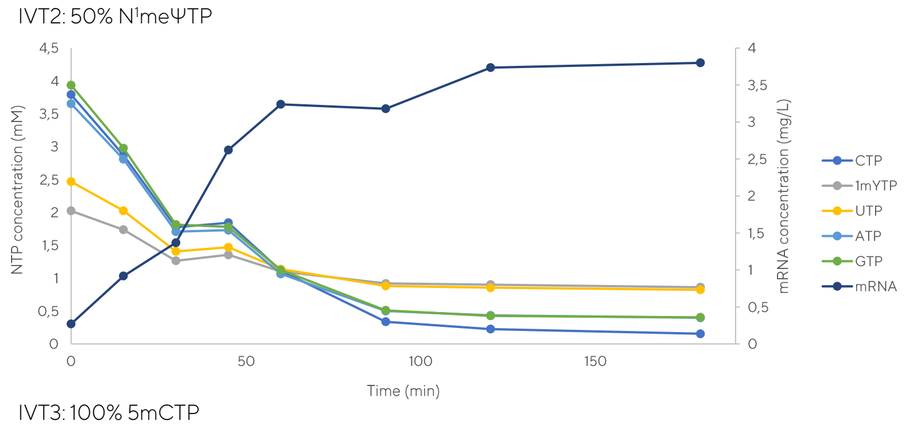
3. Results – real-time IVT monitoring

Diverse IVT reactions can be monitored efficiently with the developed method, as shown below.

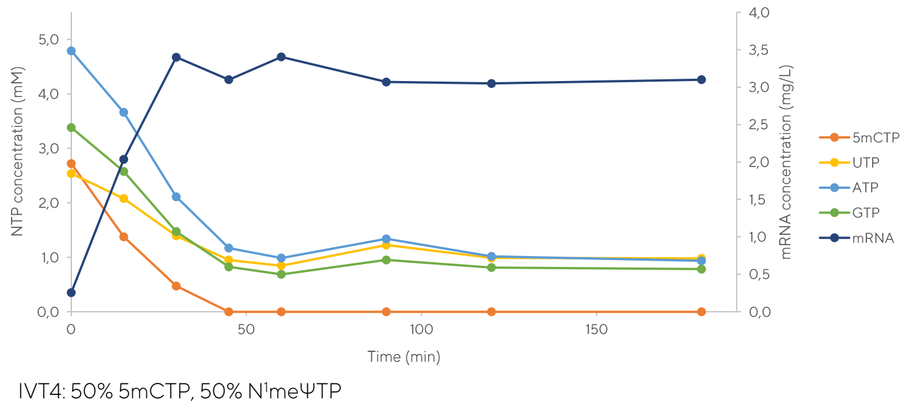
IVT1: 100% N¹meΨTP



IVT2: 50% N¹meΨTP



IVT3: 100% 5mCTP



IVT4: 50% 5mCTP, 50% N¹meΨTP

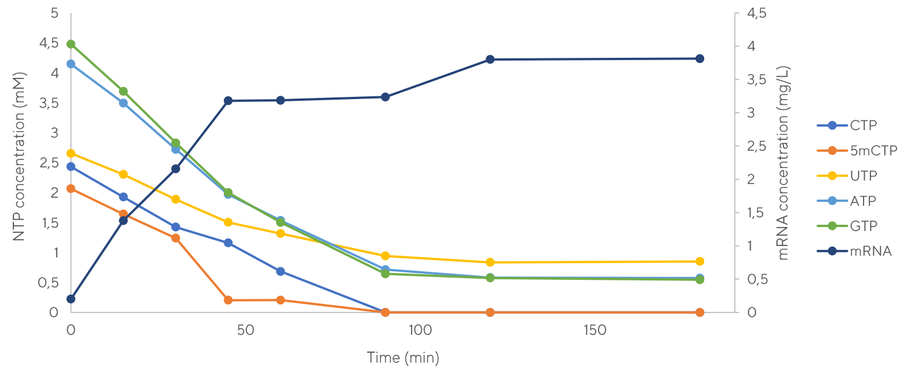


Figure 4: Time-course plots for all four IVT reactions.

4. Summary

Herein, a novel analytical chromatographic method was developed on the CIMac PrimaS column, that enables separation and quantification of six different nucleotides and mRNA in less than 7 minutes.

Using this method as at-line monitoring of IVT reactions, even in the presence of complex mixtures of modified nucleotides, such as 5mCTP and N¹meΨTP, is an efficient way to optimize and control IVT reactions.

References:
1) Morais P, Adachi H and Yu Y-T (2021) The Critical Contribution of Pseudouridine to mRNA COVID-19 Vaccines. Front. Cell Dev. Biol. .doi: 10.3389/fcell.2021.789427
2) Pregeljc D, et al. (2023) Increasing yield of in vitro transcription reaction with at-line high pressure liquid chromatography monitoring. Biotechnol. Bioeng. doi: 10.1002/bit.28299