

INNOVATOR INSIGHT

Chromatographic purification with CIMmultus™ Oligo dT increases mRNA stability

Matevž Korenč, Nina Mencin, Jasmina Puc, Janja Skok, Kristina Šprinzar Nemec, Anže Martinčič Celjar, Pete Gagnon, Aleš Štrancar and Rok Sekirnik

One of the major challenges of mRNA based vaccines has been their requirement for distribution and storage at extremely low temperatures, indicating that exposure of mRNA to suboptimal physico-chemical conditions can result in degradation and loss of potency; it is unclear whether this is due to instability of mRNA drug substance, or LNP-encapsulated mRNA, or both. In this study we compare the stability of model mRNA drug substance (eGFP, 995 nt) prepared by affinity chromatography with the stability of mRNA purified by precipitation. We show that both purification methods lead to highly pure mRNA drug substance, however, mRNA purified by chromatography remains stable for 28 days at 37°C, whereas mRNA purified by precipitation is subject to significant degradation under the same storage conditions. We conclude that chromatography eliminates elements and/or conditions with adverse impact on the quality of mRNA to a greater extent than precipitation method and that choosing appropriate purification strategy is crucial not only to achieve target purity but also to obtain a stable product with retained integrity.

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Strengths of mRNA technology were recently demonstrated by the extraordinarily rapid development and clinical success of two vaccines against SARS-CoV-2 and variants [1–3]. However, the challenges of global

roll-out exposed limitations associated with (ultra)cold-storage requirements [4]. Moderna's Spikevax vaccine requires long-term storage between -15 and -25°C and BioNTech/Pfizer's Comirnaty vaccine between -60 and

-90°C [5]. Both mRNA vaccines use lipid nanoparticle (LNP) formulation of mRNA drug substance and it is unclear what factors lead to the different temperature requirements for long-term storage of the two vaccines - whether these arise from instability of mRNA drug substance, LNP-encapsulated mRNA ('drug product'), or a combination thereof [5]. A recent publication evaluated stability of LNP-encapsulated mRNA and demonstrated its stability at 25°C for 7 days, suggesting that encapsulation itself has a stabilizing effect on mRNA [6]; instability of mRNA vaccines could thus potentially stem from instability of mRNA drug substance itself.

With the proviso that mRNA stability can be highly sequence-dependent [7,8], it is possible that for a given sequence, impurity profile and/or purification approach, which could induce physico-chemical or mechanical stress, lead to instability of mRNA drug substance. Ribonucleic acids (RNA) are relatively unstable biomolecules, especially when compared to deoxyribonucleic acids (DNA). Degradation rates are very heterogeneous in solution because of the variety of local RNA conformations, which determine hydrolysis rates [9]. Hydrolytic cleavage of the phosphodiester bond is catalyzed by some metallic complexes, including with Mg²⁺, one of the critical cofactors required during production of mRNA [9], some reports even proposed that full protection from atmosphere is needed to prevent hydrolysis for long term storage [10]. mRNA stability is therefore highly dependent on the chemical environment to which it is exposed during production process.

An IVT reaction mixture contains a number of components, including enzymes, residual NTPs and DNA template, as well as small molecule additives used to improve mRNA yield (spermidine, Triton, etc.), that need to be removed during the manufacturing process. Traditional lab-scale purification methods are based on DNA removal by DNase digestion followed by lithium chloride (LiCl) or ethanol precipitation, but these methods are chemically harsh,

introduce toxic chemicals into the final product and are challenging to scale-up [11].

Chromatography offers a highly selective as well as scalable approach to purification, which when fully optimized delivers high purity of therapeutic agents. Due to its large size (1000 nt mRNA is approximately 400 kDa, nearly 3-times larger than an IgG), mRNA diffusion coefficient is low, rendering traditional chromatographic approaches, which depend on diffusive mass transport, less suitable as a purification tool for this therapeutic class [12]. Convective-flow purification media, such as monoliths, are more suitable for purification of such large biomolecules, providing higher binding capacity, faster purification cycles and lower shear [11,13-16]. For mRNA constructs containing a polyadenylic acid (PolyA) tail, the mRNA can be isolated from the IVT mixture in a pseudo-affinity mode, using affinity of chromatography support-immobilized poly-deoxythymidine for mRNA containing PolyA tail. For constructs that do not contain a polyA tail, charge or hydrogen bonding interactions can be employed [12].

In this study we evaluated the contribution of purification methodology to quality of mRNA drug substance, using stability as an index. Two purification approaches were evaluated, both expected to yield highly pure mRNA: RNA extraction kit was chosen as a standard RNA purification tool used in many laboratories working with RNA, and monolithic chromatography media was selected for their ability to support rapid high-resolution separation of very large molecules in a low-shear environment [11,13-16]. Stability of mRNA at 37°C (optimal temperature for activity of RNAses), 4°C (typical refrigeration temperature), -20°C and -80°C was assessed by a selection of standard physicochemical analytical techniques used for mRNA drug substance quality attribute assessment (UV spectroscopy (A₂₆₀ nm) for content determination, agarose gel electrophoresis for molecular mass and RNA integrity assessment, chip capillary electrophoresis for quantification

of fragmentation) [4-6, 17], and two HPLC methods which are reported for the first time: quantification of poly-adenylated mRNA using CIMac™ Oligo dT and purity assessment of mRNA by CIMac PrimaS™. Freeze-thaw stability was also assessed after each purification approach using the same set of analytical methods. For the full materials and methods, please refer to the [supplementary data](#) for this article.

RESULTS & DISCUSSION

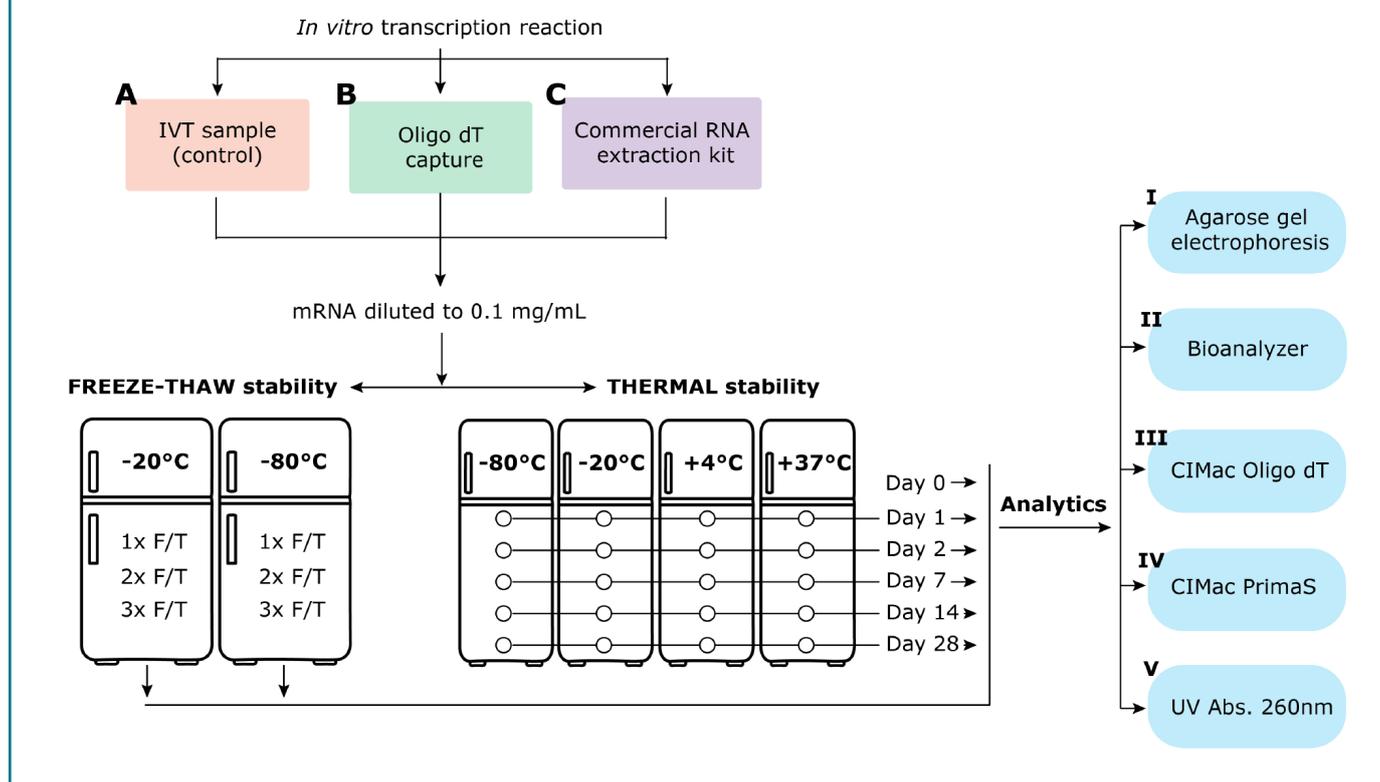
After IVT production of mRNA from a plasmid encoding for eGFP (950 nucleotides) and poly-adenosine tail (45 nucleotides) according to a standard IVT protocol (Figure 1), the reaction mixture was either diluted in ddH₂O, purified using affinity chromatography (CIMmultus™ Oligo dT), or purified using a standard RNA extraction kit. Purified mRNA was incubated at defined storage conditions until stability time-points. Note that

capital letters label purification approaches and roman numerals label analytical methods in all figures presented.

mRNA at expected molecular size (995 nt) was observed by AGE and BioAnalyzer for all samples. Oligo dT eluate showed a minor band at 2000 nt by both AGE and BioAnalyzer, corresponding to a dimeric form of mRNA, which disappeared with denaturing the sample by heating it at 70°C for 5 min (Supplementary Figure 1). For diluted IVT, analytical affinity chromatography (CIMac™ Oligo dT) revealed the expected flow-through peak, corresponding to UV-absorbing IVT reaction components (nucleotides, enzymes), and the elution peak, corresponding to polyadenylated RNA. Peak area corresponded to expected mRNA concentration (0.1 mg/mL). mRNA purified by affinity chromatography and extraction kit only showed elution peak at expected concentration and no flow-through peak. Similarly, CIMac PrimaS™ chromatogram demonstrated the presence of nucleotide-like reaction components

► **FIGURE 1**

mRNA production, purification and stability testing scheme.



(capping reagent and residual NTPs) and RNA in the IVT sample, but only RNA in samples purified by affinity chromatography and extraction kit (Figure 2), suggesting that both purification methods yield highly pure, poly-adenylated RNA.

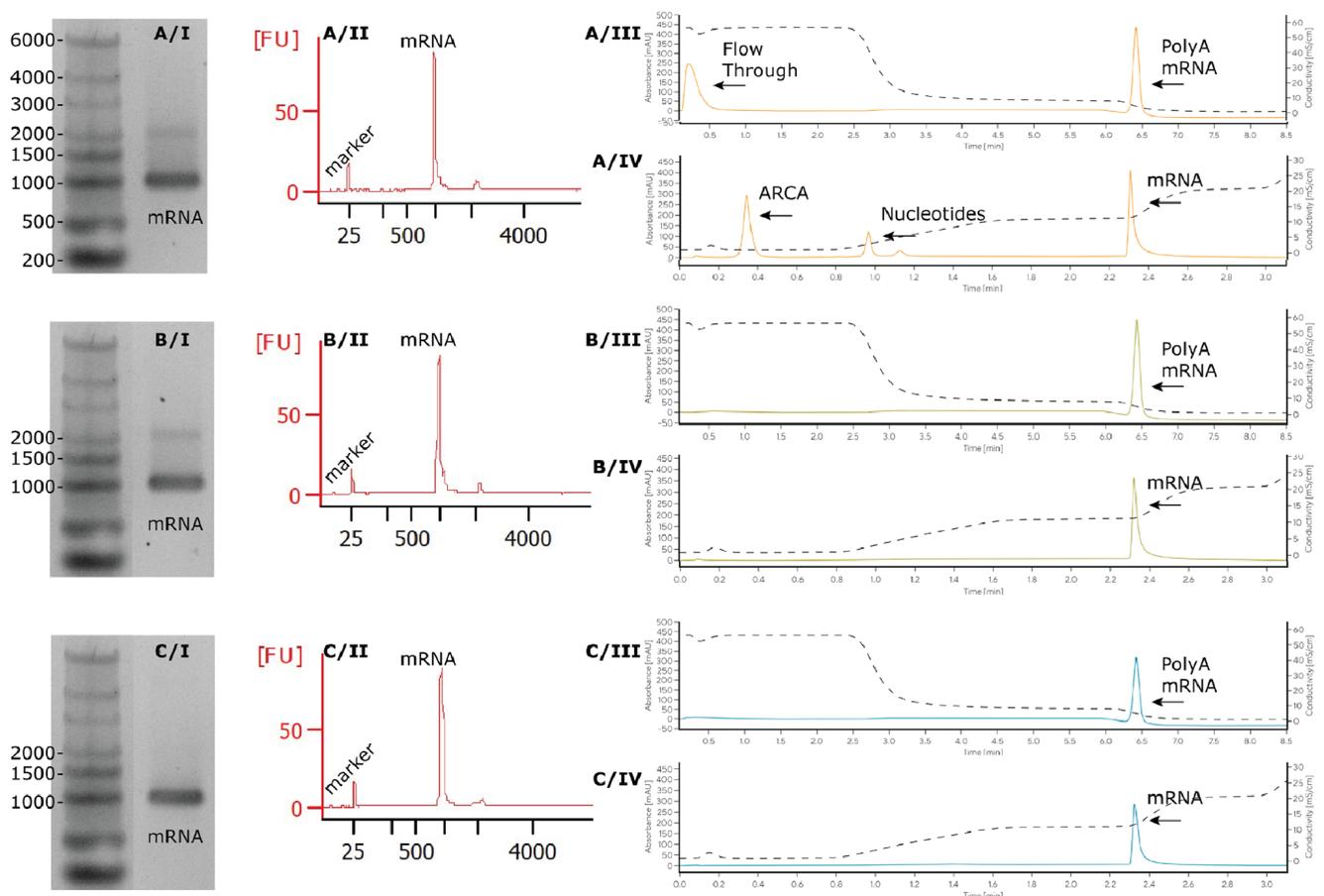
Stability of mRNA prepared according to the three methods was then evaluated at different temperatures over a period of 28 days, and freeze-thaw stability at -20°C/-80 was evaluated for up to three freeze-thaw cycles (Figure 1).

All three preparation methods resulted in mRNA that was stable at -80, -20 and 4°C for up to 28 days, and up to three freeze-thaw cycles at -20°C and -80°C (Figure 3) as assessed by electrophoretic methods (AGE

and BioAnalyzer, Figure 4), and chromatography (Figure 5). Incubating mRNA at 37°C, however, resulted in significant degradation of mRNA produced by extraction kit or dilution, but not when purified chromatographically. Purity of extraction kit- purified material decreased in a time-dependent fashion; it was less than 20% by day 14 and 6% by day 28 (as determined by BioAnalyzer, Figure 4 (C/II), Supplementary Figure S2). Similarly, smearing of RNA band at 1000 nt was observed by agarose gel electrophoresis which resulted in no observable band at 1000 nt by day 28 (Figure 4 (C/I)). In comparison, when mRNA purified by CIMmultus™ Oligo dT was incubated at 37°C, it retained 80% integrity (as determined by BioAnalyzer) by day 14 and

► **FIGURE 2**

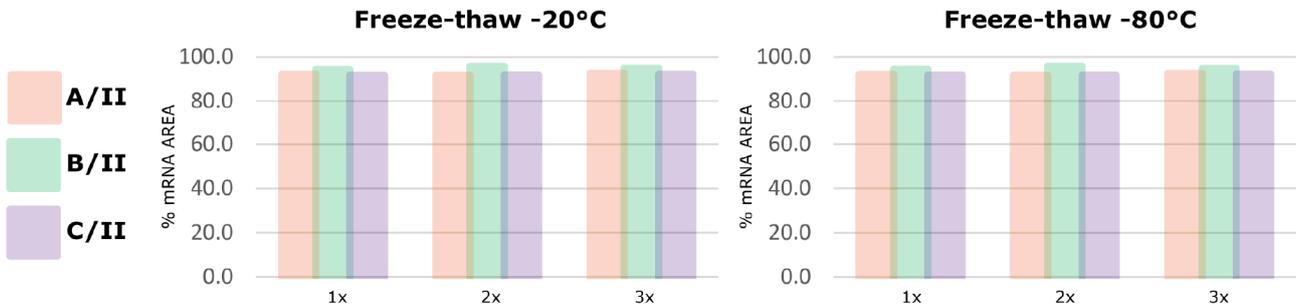
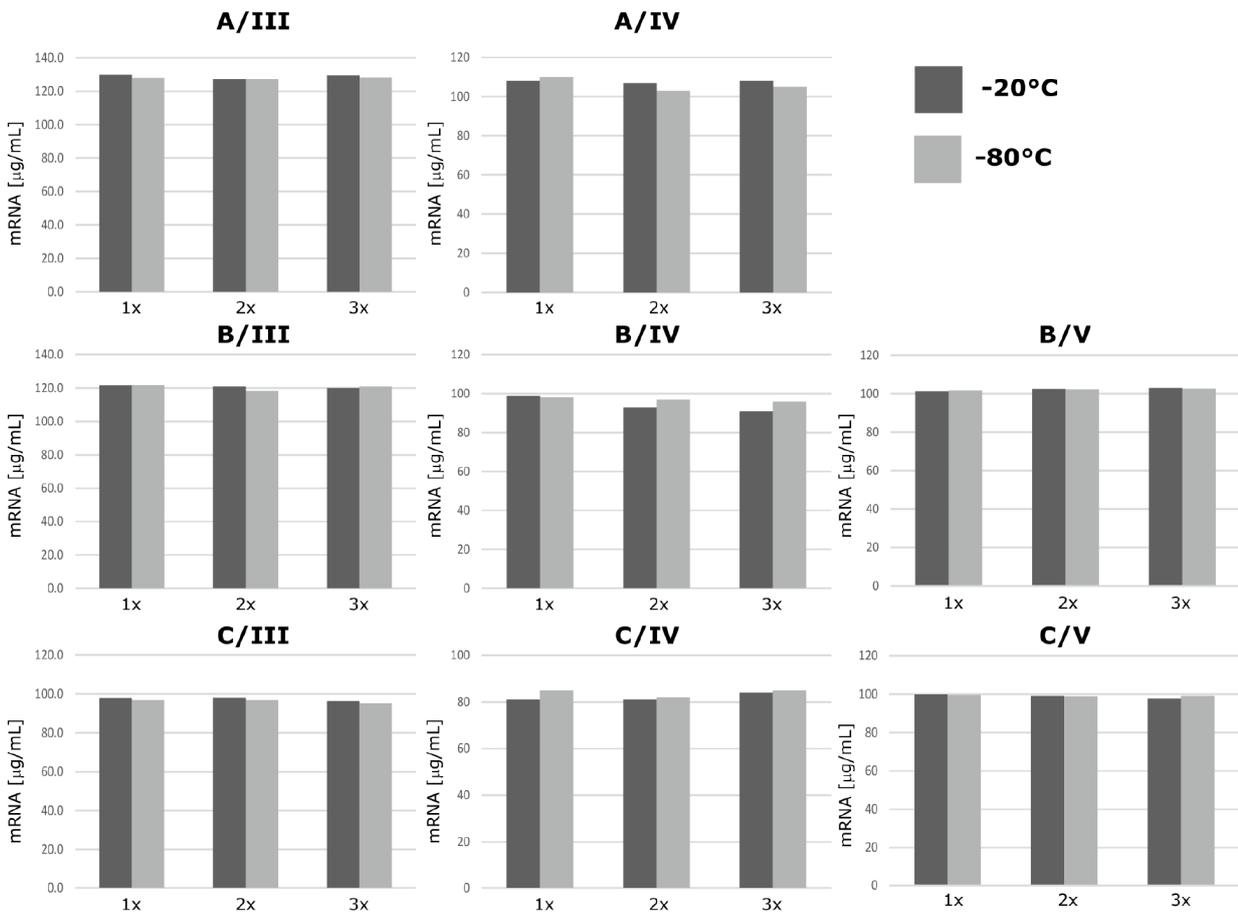
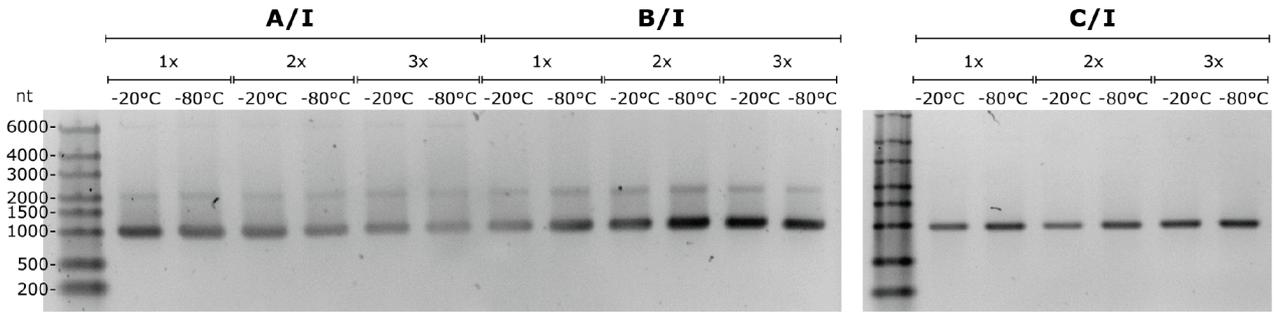
Electrophoretic and chromatographic properties of mRNA produced by A) IVT, and purified by B) Oligo dT and C) extraction kit. I) Agarose gel electrophoresis, II) BioAnalyzer, III) CIMac Oligo dT, IV) CIMac PrimaS.



FT: flow-through, ARCA: anti-reverse cap analog,

▶ **FIGURE 3**

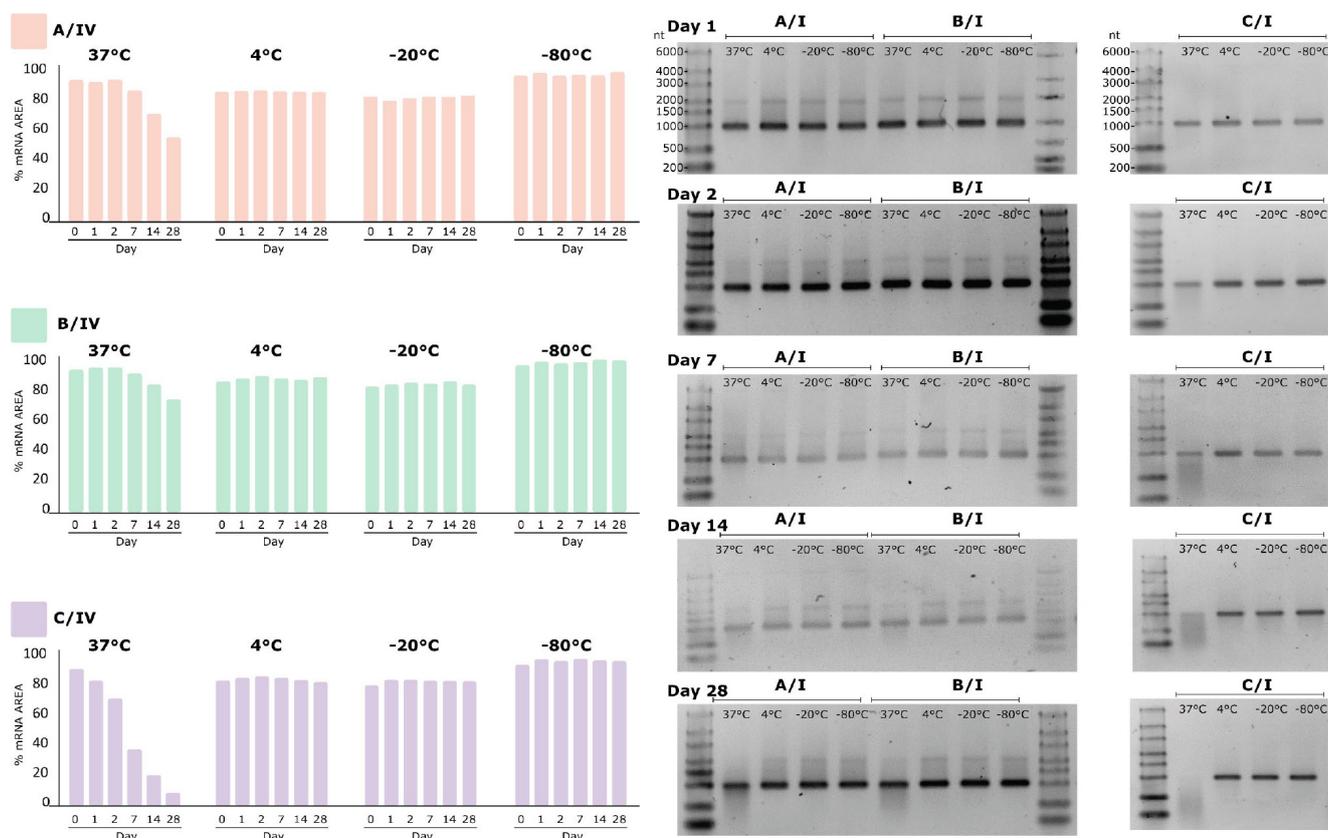
Freeze-thaw stability of mRNA.



I) Agarose gel electrophoresis, II) BioAnalyzer, III) CIMac Oligo dT, IV) CIMac PrimaS and V) UV (260 nm, right).

► **FIGURE 4**

Thermal stability of mRNA at -80/-20/4/37° for up to 28 days.



Left: BioAnalyzer; and right: Agarose gel electrophoresis of mRNA prepared by A) IVT (diluted), B) Oligo dT-purified and C) extraction-kit purified.

72% by day 28 (Figure 4 B/II, Supplementary Figure S2); only barely detectable smearing of agarose gel band was observed by day 28.

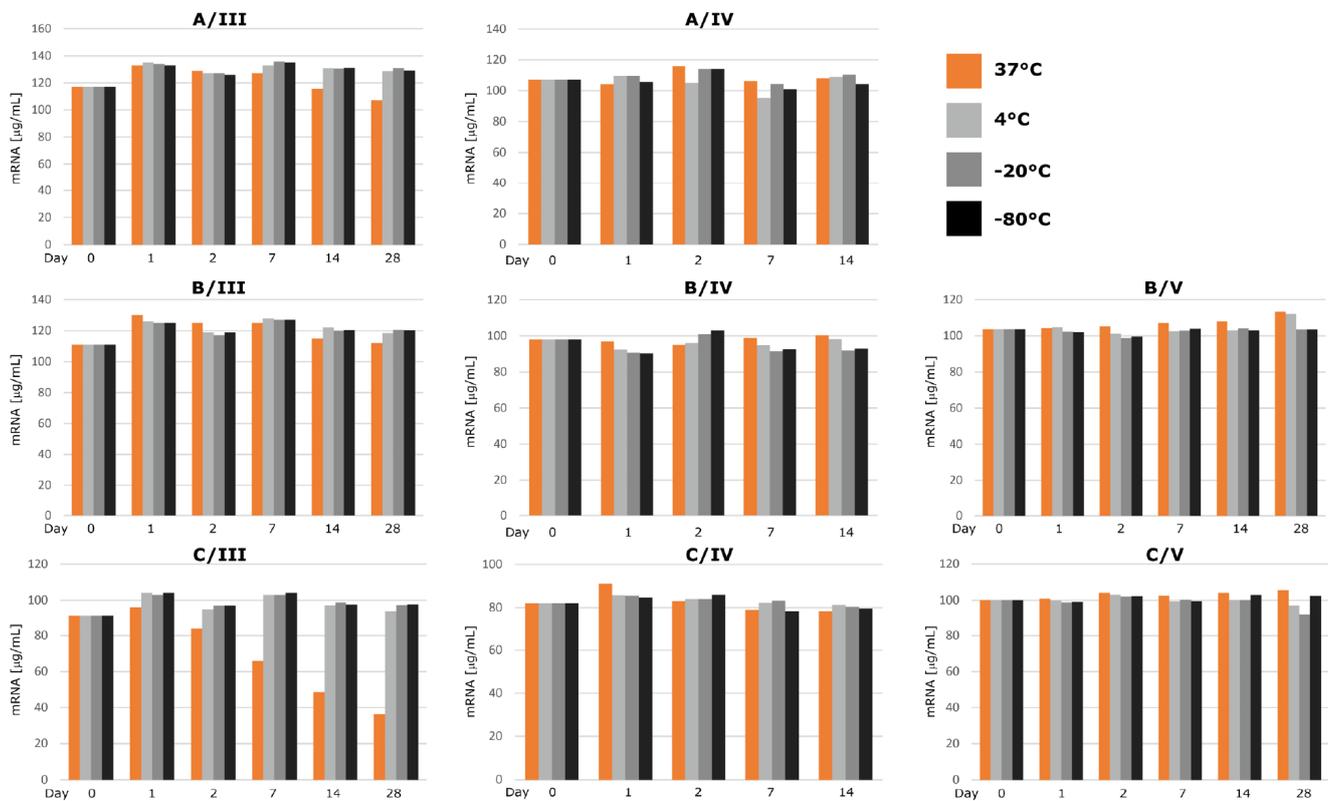
Chromatographic analysis by CIMac™ Oligo dT (labeled with roman number III in all figures) was in close agreement with electrophoretic results. The content of mRNA prepared by all three methods did not differ significantly for incubation temperatures of -80/-20/4°C, but a significant decrease in content (from starting 90 µg/ml to 38 to µg/ml by day 28) was observed for mRNA purified by extraction kit, but not other methods, when incubated at 37°C for up to 28 days (Figure 5 C/III). CIMac PrimaS™ analysis, which resolves mRNA from NTPs, capping reagent and plasmid, resulted in mRNA peak intensity which did not change with incubation time or temperature (Figure

5C/IV). Interestingly, analysis of peak corresponding to residual ARCA capping reagent (retention time of 0.4 min, Figure S3) in diluted IVT sample indicated a shift to 0.8 min observed at 37°C from day 1 onwards, suggesting possible degradation of the reagent. Although IVT reactions are seldomly carried out beyond 3-6 hours, stability of reactants under IVT conditions (e.g. 37°C) should nonetheless be evaluated to avoid potential safety issues due to incorporation of degradation products into nascent mRNA.

As expected, UV absorbance of stability samples resulted in no apparent difference in content as measured by absorbance at 260 nm even when other analytical methods showed severe degradation (Figure 5 (B/V and C/V)). Content by UV is one of the critical quality attributes assessed for release

► **FIGURE 5**

HPLC and UV analytics of mRNA stability.



III) CIMac Oligo dT, IV) CIMac PrimaS, V) UV absorbance at 260 nm (Nanodrop). mRNA was prepared by A) dilution of IVT, B) CIMmultus Oligo dT and C) RNA extraction kit.

of mRNA vaccines [5]; our results point to the need to evaluate the UV results in conjunction with other purity methods, as it is not indicative of content of intact target molecule.

CONCLUSIONS

In this study, we compared stability of mRNA drug substance (995 nt) prepared by chromatographic method using CIMmultus Oligo dT column versus a precipitation technique using commercial RNA extraction kit. mRNA purified by chromatography was

shown to remain stable for 28 days at 37°C, whereas purification by precipitation led to significant degradation of mRNA at 37°C, detectable by electrophoretic (AGE and Bio-Analyzer), chromatographic (CIMac™ Oligo dT), but not UV-spectrophotometric method. Although follow-up studies on a wider range of mRNA sizes and modalities are warranted in the future, our results demonstrate the need for careful selection of purification strategy during development of mRNA therapeutics, which should be based on consideration of long-term stability impact, as well as scalability and compatibility with GMP standards.

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AFFILIATIONS

Matevž Korenč

BIA Separations d.o.o., a Sartorius company, Mirce 21, 5270 Ajdovščina, Slovenia

Nina Mencin

BIA Separations d.o.o., a Sartorius company, Mirce 21, 5270 Ajdovščina, Slovenia

Jasmina Puc

BIA Separations d.o.o., a Sartorius company, Mirce 21, 5270 Ajdovščina, Slovenia

Janja Skok

BIA Separations d.o.o., a Sartorius company, Mirce 21, 5270 Ajdovščina, Slovenia

Kristina Šprincar Nemeč

BIA Separations d.o.o., a Sartorius company, Mirce 21, 5270 Ajdovščina, Slovenia

Anže Martinčič Celjar

BIA Separations d.o.o., a Sartorius company, Mirce 21, 5270 Ajdovščina, Slovenia

Pete Gagnon

BIA Separations d.o.o., a Sartorius company, Mirce 21, 5270 Ajdovščina, Slovenia

Aleš Štrancar

BIA Separations d.o.o., a Sartorius company, Mirce 21, 5270 Ajdovščina, Slovenia

Rok Sekirnik

Author for Correspondence:
Rok Sekirnik, BIA Separations d.o.o.,
Mirce 21, 5270 Ajdovščina, Slovenia
Tel.: +386 59 699 500;
Fax: +386 59 699 599
E-mail: rok.sekirnik@biaseparations.com



AUTHORSHIP & CONFLICT OF INTEREST

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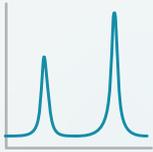
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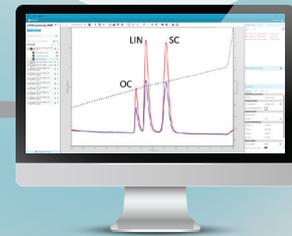
pDNA standard included



pDNA isoform separation



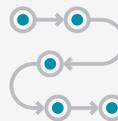
Sensitive isoform concentration readouts



Remote work enabled



User-friendly data processing interface



Built-in methods & SOP



Step-by-step user guide



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