Increasing yield of IVT reaction with at-line HPLC monitoring

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Abbreviations:

ARCA: Anti-reverse cap analog

DTT: dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

GFP: green fluorescent protein

GTP: guanidine triphosphate

HPLC: high pressure liquid chromatography

IVT: in vitro transcription

LNP: lipid nanoparticle

NTP: Nucleoside Triphosphate

mRNA: messenger ribonucleic acid

RNAP: RNA polymerase enzyme

Abstract

The COVID-19 pandemic triggered an unprecedented rate of development of mRNA vaccines, which are produced by in vitro transcription reactions. The latter has been the focus of intense development to increase productivity and decrease cost. Optimization of IVT depends on understanding of the impact of individual reagents on the kinetics of mRNA production and the consumption of building blocks, which

is hampered by slow, low-throughput, end-point analytics. We implemented a workflow based on rapid at-line HPLC monitoring of consumption of NTPs with concomitant production of mRNA, with a sub-3 min read-out, allowing for adjustment of IVT reaction parameters with minimal lag. IVT was converted to fed-batch resulting in doubling the reaction yield compared to batch IVT protocol, reaching 10 mg/mL for multiple constructs. When coupled with exonuclease digestion, HPLC analytics for quantification of mRNA was extended to monitoring capping efficiency of produced mRNA. When HPLC monitoring was applied to production of an ARCA-capped mRNA construct, which requires an approximate 4:1 ARCA:GTP ratio, the optimized fed-batch approach achieved productivity of 11.9 mg/mL with 79% capping.

The study provides a methodological platform for optimization of factors influencing IVT reactions, converting the reaction from batch to fed-batch mode, determining reaction kinetics, which are critical for optimization of continuous addition of reagents, thereby paving the way towards continuous manufacturing of mRNA.

Introduction

The COVID-19 pandemic triggered an unprecedented rate of development of mRNA vaccines, primarily due to the unprecedented clinical success of two vaccines based on this novel technology: BioNTech/Pfizer's Comirnaty and Moderna's Spikevax. Their impressive efficacy and rapid development exposed the strengths of mRNA technology as a promising modality for multiple disease areas, leading some to dub the era as the 'mRNA revolution'.

From a physico-chemical standpoint, mRNA is a challenging molecule to produce due to its large size and relative instability. The production process typically involves 10-15 steps including plasmid production, plasmid linearization, an *in vitro* transcription (IVT) reaction, mRNA purification, and lipid nanoparticle (LNP) production. Although manufacturing processes vary significantly, the common unit operation is the IVT reaction, which produces RNA from a DNA template.

For *in vivo* stability, mRNA must contain two crucial elements: 5' cap and 3' poly-adenylic acid tail. Without the former, endogenous exonucleases recognize the transcript as material of potentially viral origin and degrade it. Without the latter, *in vivo* stability is reduced with several hypotheses suggested, including preventing access to exonucleases, blocking enzyme association and steric inhibition. Therapeutic mRNA is therefore designed to include both features, which can be added either co- or post-transcriptionally. Co-transcriptional capping strategies underwent significant development from traditionally performed Anti-Reverse Cap Analog (ARCA) to highly efficient CleanCap technology. Post-transcriptional capping with, for example, vaccinia capping system is primarily used to reduce the cost of IVT reactions (*vide infra*) but introduces at least two additional unit operations to the production process.

The cost of mRNA production is dominated by the IVT reaction, with a single reagent, namely the capping reagent, driving the cost of the reaction. Optimization of mRNA yield in IVT is therefore crucial for lowering the cost of production. Computational studies have addressed the possibility of continuous manufacturing of mRNA , but to date there is no report of experimental verification of this approach.

The core IVT reaction mixture consists of a DNA template (typically linearized plasmid), RNA polymerase and nucleotide triphopshates (NTPs). To increase the rate of reaction and decrease degradation, various additional components are typically added: pyrophosphatase to remove poorly soluble pyrophosphate (P_iP_i) from the reaction, RNase inhibitor, spermidine, dithiothreitol and Mg^{2+} ions.

The central role in the IVT reaction is played by RNA polymerase enzyme (RNAP), typically T7 RNA Polymerase (T7 RNAP) due to its high enzymatic activity and ability to incorporate modified nucleotides. Kinetic studies of T7 RNAP report transcription rates varying between 40 and 90 nucleotides per second.

T7 RNAP is a magnesium-dependent enzyme, requiring Mg^{2+} ions for binding to DNA template. Mg^{2+} ion also forms complexes with NTPs prior to entering the active site, and catalyzes the incorporation of the

NTP into the RNA strand and elimination of two phosphates as a pyrophosphate. Between two to three Mg^{2+} ions are thought to be present in the active site.

A pyrophosphate ion is released for each NTP added to the nascent RNA strand. These ions can form insoluble complexes with Mg^{2+} ions and precipitate out of aqueous solution. To avoid precipitation, pyrophosphatase is used to convert one pyrophosphate ion to two phosphate ions, thus preventing unwanted precipitation, as well as removal of soluble Mg^{2+}ions, which would co-precipitate with pyrophosphate.

The literature mostly agrees on the fact that NTPs and Mg^{2+}, as well as their ratio and concentrations, have more influence on the reaction than other components. Some publications suggest exact Mg^{2+}:NTP ratios or exact concentrations needed for IVT. However, the reported ranges vary significantly. Akama *et al.* found that rate of synthesis did not increase when NTP concentrations were increased beyond 1.6 mM, while Li *et al.* observed that NTP concentration had to be 9 mM greater than Mg^{2+} to achieve efficient transcription. On the contrary, Kanwal *et al.* suggest optimal conditions that include a Mg^{2+} concentration of 45 mM and 8 mM individual NTP concentrations. Samnuan *et al.* identified NTP:Mg^{2+} ratio of 1:1.875 for optimal yield. This indicates that the reported ranges may be construct-specific, cannot be directly translated to other constructs / IVT designs, and need to be optimized. One of the main limitations of IVT optimization has been low throughput of analytics for quantification of mRNA and NTP precursors. RNA is typically quantified, with variable accuracy, by e.g. Ribogreen fluorescence, AGE or PAGE gels, while NTPs, and capping reagents are not frequently monitored.

Experimental Section

IVT reaction

All IVT reagents listed in Table 1 were preheated to 37°C, mixed in a 1.5 mL plastic tube in ThermomixerC (Eppendorf) and incubated at 37degC with shaking at 200 rpm unless otherwise stated. Scale-up was performed in 15 mL Falcon tube in ThermomixerC (Eppendorf) with a Smartblock^{TM} 15 mL adapter. For sampling, 2 µL aliquot were taken and quenched with addition of 2 µL of 100 mM EDTA for analytics at defined timepoints. mRNA yield and NTP consumption were monitored by CIMac PrimaS at each timepoint. Additions of NTPs and $MgCl_2$ were carried out based on CIMac PrimaS results as indicated in Results section.

Analytics

Qualitative analysis by anion exchange chromatography

Experiments were performed on a PATfix HPLC system composed of a quaternary pump, a multiwavelength UV-Vis detector (50 mm flow-cell path length), a conductivity, and a pH monitor (BIA Separations). ClarityChrom® software (Knauer) was used for instrument control and data acquisition. PATfix software (BIA Separations) was used for data analysis. CIMac PrimaS column with 0.1 mL bed volume and average channel size of 2 µm was used (BIA Separations). mRNA samples were equilibrated by a dilution with mobile phase A (MPA) (50 mM HEPES, pH 7.0). Injection volume was 25 µL. NTPs from the IVT reaction were separated in a linear gradient to 20% of mobile phase B (MPB) (50 mM HEPES, 200 mM Sodium pyrophosphate, pH 8.5). In the following isocratic elution, pDNA is eluted and finally mRNA is eluted in a step to 50 % MPB, followed by another step to 100% MPB and then CIP with 0.1M NaOH + 1M NaCl (MPC) to regenerate the column. Finally, the column is equilibrated with (MPD) 1.5 M HEPES pH 7 and MPA.

Agarose gel electrophoresis

Agarose gel electrophoresis (Advance, MupidOne) was used for visualization of mRNA and determination of mRNA quality produced with fed-batch approach. All samples were diluted 200-fold in ddH_2O. 18 µL of each diluted sample was mixed with 2 µL of TriTrack loading dye (ThermoScientific, R1161) and loaded on 1% agarose gel. RiboRuler HR (ThermoScientific, SM1831) was used as a marker. Agarose gel was made by dissolving 1 g of agarose (Sigma-Aldrich, A9539.) in 100 mL 1x TAE buffer (40 mM Tris-acetate, 1mM

EDTA pH 8.3). Electrophoresis was performed at 100 V for 60 min in 1x TAE as a running buffer. Gel was stained with SybrGold (Invitrogen, S11494) for 30 min in the dark and visualized with E-Gel Imager System (Invitrogen).

Dot-blot

Dot blot was used for detection of double-stranded RNA contaminant in mRNA samples. 1x TBS buffer (20 mM Tris-HCl, 150 mM NaCl pH 7.5) was prepared from 10x stock in advance as well as blocking buffer (5% milk in 1x TBS) and incubation buffer (1% milk in 1x TBS) where Skim Milk Powder (Milipore, 70166-500G) was used. Biodyne B Nylon membrane (Thermo Scientific, 77016) and Western blotting filter paper (Thermo Scientific, 88600) were prewetted in ddH_2O for 10 min and then put between sealing gasket and sample template of the Minifold 1, 96-well dot blot system apparatus (Whatman, 10447900). 1.13 – 1.39 μg of mRNA was loaded into each well and incubated for 60 min at room temperature. ddH_2O was used as a negative control and dsRNA Ladder (BioLabs, N0363S) in different concentrations (10 ng, 20 ng, 30 ng, 40 ng, 60 ng, 80 ng) was used as a positive control. After incubation, vacuum was applied to pull fluid through the membrane. Membrane was then transferred to clean incubation box with blocking buffer and incubated for 60 min while shaking. Membrane was later washed 3 times in 1x TBS buffer for 10 min. After washing, the membrane was transferred into fresh incubation box with incubation buffer where Monoclonal Antibody J2 (Scicons, 10010500) was diluted 5000-fold. After 1 hour, membrane was again washed 3 times in 1x TBS buffer for 10 min before 1 hour of incubation in incubation buffer with 5000-fold diluted HRP-conjugate Secondary Antibody to Mouse IgG (Jackson ImmunoResearch, 115-035-003). Membrane was washed 5 times in 1x TBS buffer and then soaked in 1-Step Ultra TMB-Blotting Solution (Thermo Scientific, 37574) until color was developed.

Capping efficiency

For determination of capping efficiency of IVT reactions, 2-step enzymatic degradation of uncapped mRNA was used as reported before. In brief, RNA 5'Polyphosphatase was used for the removal of phosphates from 5'-triphosphorilated RNA (uncapped mRNA). Mixture of 1x RNA 5' Polyphosphatase Reaction Buffer (Lucigen), 1 U/μL RNase Inhibitor (NEB, M0314S), 1 μg of mRNA, 10 U of RNA 5' Polyphosphatase (Epicentre, RP809H) and nuclease free water added up to 20 μL was incubated at 37°C for 60 min. After incubation, 5' monophosphorylated RNA was digested with Terminator 5'-Phosphate-Dependent Exonuclease. 1x Terminator Reaction Buffer A (Epicentre) 1 U/μL RNase Inhibitor (NEB, M0314S), 0,5 U of Terminator 5'-Phosphate-Dependent Exonuclease (Epicentre, TER510) and nuclease free water was added to reaction mixture from first step up to 40 μL reaction volume. Reaction was incubated at 30°C for 30 min and quenched with 5mM EDTA pH 8. mRNA concentration was determined with CIMac PrimaS analytical method as described above.

Results and discussion

We previously reported the development of a high-throughput HPLC assay for simultaneous quantification of NTPs, capping reagent, plasmid, and mRNA. Here, we applied this analytical method to follow the IVT reaction at-line with minimal analytical lag and efficiently improve the yield of the reaction (Figure 1).

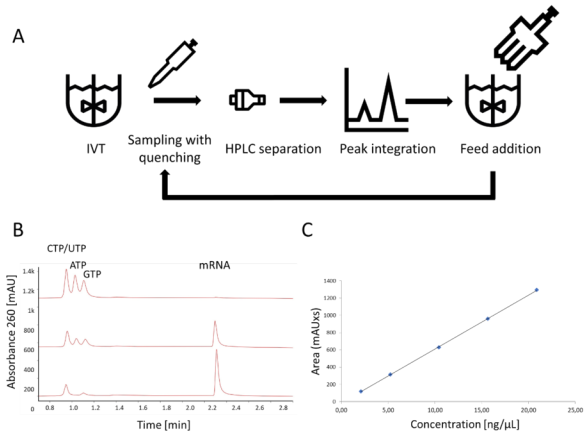

Figure 1: A) Schematic representation of IVT optimization workflow with CIMac PrimaS^{TM} quantification of NTPs and mRNA in an IVT reaction. B) Representative CIMac PrimaS chromatograms from t=0, mid-point, end-point of IVT reaction. B) CIMac PrimaS calibration curve for eGFP mRNA concentration.

Table 1: Summary of literature IVT protocols, and optimized standard conditions. 1: Protocol for Standard RNA Synthesis (NEB,); 2: Cold Assay (Roche;); 3: Conventional in vitro transcription (Thermo Scientific;); 4: T7 polymerase in vitro transcription (JenaBiosciences;); 5: Capped RNA Synthesis (NEB;); 6: Capped RNA Synthesis (TriLink); 7: 'Standard IVT process'.

	1	**2**	**3**	**4**	**5**	**6**	**7**
REAGENT	Final concentration	Final concentration	Final concentration	Final concentration	Final concentration	Final concentration	Final concentration
Tris-HCl	40 mM	40 mM	40 mM	40 mM	Not stated	40 mM	40 mM
MgCl2	6 mM	6 mM	8 mM	6 mM	Not stated	16.5 mM (MgOAc)	12 mM
DTT	1 mM + 5 mM	10 mM	0 mM	10 mM	Not stated	10 mM	1 mM
Spermidine	2 mM	2 mM	2 mM	2 mM	Not stated	2 mM	2 mM
NaCl	/	/	25 mM	10 mM	Not stated	/	/
pH	7.9@25°C	8.0 @20°C	8.0	7.9	Not stated	Not stated	7.9@25°C
RNAse inhibitor	1U/µL	1U/µL	1U/µL	1 U/µL	Not stated	1U/µL	1U/µL
ATP	0.5 mM	1 mM	2 mM	0.5 mM	2 mM	5 mM	4 mM
CTP	0.5 mM	1 mM	2 mM	0.5 mM	2 mM	5 mM	4 mM
UTP	0.5 mM	1 mM	2 mM	0.5 mM	2 mM	5 mM	4 mM
GTP	0.5 mM	1 mM	2 mM	0.5 mM	2 mM	5 mM	4 mM
Capping reagent	/	/	/	/	8 mM	4 mM	/
Lin. plasmid	0.5 µg – 2.5 µg	2.5 µg	1 µg	0.01–1 µg	1 µg	1 µg	1 µg
Pyrophosphatase	/	/	/	/	/	0.002 U/µL	0.001 U/µL

	1	**2**	**3**	**4**	**5**	**6**	**7**
RNA polymerase	200- 500 U/µg	40 U/µg	30 U/µg	100 – 10,000 U/µg	2 µL of T7 mix	400 U/µg	200 U/µg
Volume [uL]	50 µL	50 µL	50 µL	50 µL	20 µL	50 µL	50 µL

Numerous IVT reaction procedures are reported in the literature. Starting from a baseline IVT process designed based on comparison of published protocols (Table 1, column 7, 'Standard IVT process'), effect of Mg^{2+} concentration on the rate of IVT reaction was tested in the range of 6-50 mM (Figure 2A); 9-25 mM produced the same amount of mRNA (4 mg/mL) after 180 min, albeit at different rates. Initial rates increased from 9 (ng/µL)/min (6 mM Mg^{2+}), to 110 (ng/µL)/min (20 mM Mg^{2+}), then decreased again to 25 (ng/µL)/min (50 mM Mg^{2+}). Concentrations above 25 mM were inhibitory, contrary to previous reports suggesting that 50 mM is optimal, stressing the importance of optimization of IVT condition for each type of construct. Next, effects of NTP and plasmid concentration were investigated at a constant concentration of Mg^{2+}(Figure 2B): plasmid concentrations of 20, 40 and 100 ng/µL were tested in presence of 4 mM (standard NTP concentration) and 8 mM NTPs. 4 mM NTP reactions reached a plateau within approximately 90 minutes; analysis of NTP concentrations indicated that this correlated with the drop of NTP concentration to <20% of starting value (data not shown). Furthermore, results demonstrated that increasing the concentration of plasmid increased the rate of mRNA production, but did not result in a higher concentration of mRNA produced (Figure 2B). Starting concentration of 20 ng/µL pDNA resulted in a rate of approximately 46 (ng/µL)/min, which increased to 72 (ng/µL)/min if 100 ng/µL pDNA was used. Doubling the starting concentration of NTPs did not reach the same yield, but led to inhibition of transcription (Figure 2B). We reasoned that gradual addition (fed-batch), rather than high starting concentration of NTPs (batch), could therefore be beneficial for increasing the productivity of the IVT.

Fed-batch approach was tested in the presence and absence of additional Mg^{2+}. NTPs only were added to one reaction and NTP-Mg^{2+} complex (12 mM NTPs and 9 mM Mg^{2+}) was added to another. Two additions were performed in total, at minute 120 and minute 240, when at least one NTP decreasing to <20% of starting concentration. Reaction with added Mg^{2+} reached a significantly higher mRNA concentration (Figure 2Ci). At-line HPLC analytics suggested that addition of NTPs alone did not lead to increased mRNA production, but to accumulation of NTPs instead (Figure 2Cii). If NTP-Mg^{2+} was added, NTPs were consumed at a rate corresponding to the rate of mRNA production, suggesting that Mg^{2+}, rather than NTPs, was the limiting factor for progression of mRNA production. This was further tested by addition of equimolar concentration of EDTA, a strong Mg^{2+}chelator, at an early stage of IVT reaction (Figure 2D). Reaction was sampled before and after each addition to monitor the effects of addition on production of mRNA/consumption of NTPs. HPLC analytics indicated that mRNA production ceased immediately upon EDTA addition (Figure 2Di), as did NTP consumption (Figure 2Dii). After 60 minutes, an excess of Mg^{2+} (30 mM) was added to the reaction, and mRNA production, as well as NTP consumption, resumed. This experiment confirmed the central importance of Mg^{2+} to the transcription.

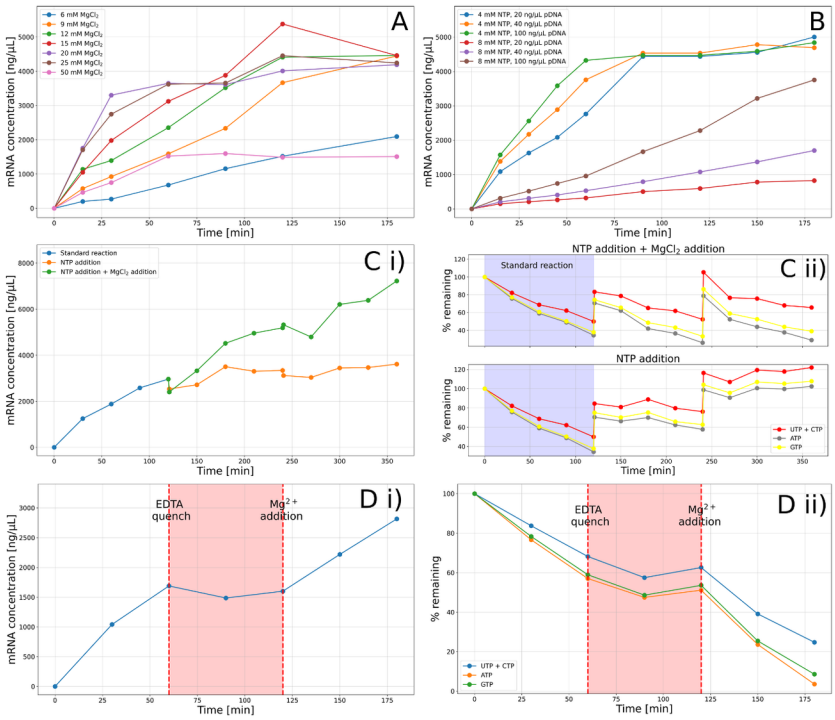

Figure 2: A) Impact of initial Mg^{2+} concentration on an IVT reaction. Very small (6 mM) and very large (50 mM) concentrations resulted in a low yield of mRNA production. Concentrations between 9 mM and 25 mM produced a comparable final yield after 175 min and a 12 mM Mg^{2+}concentration was chosen to be used in subsequent experiments. B) Effect of plasmid concentration (20, 40 and 100 ng/uL) was tested on two different NTP concentrations (4 and 8 mM). Higher plasmid concentrations increased the rate of mRNA production, but not the yield at plateau. Higher NTP concentration led to inhibition of transcription, suggesting gradual addition (fed-batch) could improve productivity. C) Mg^{2+} addition alongside NTPs is crucial for the production of mRNA: NTP-Mg^{2+} complex addition reached a significantly higher mRNA concentration (i); at-line HPLC analytics revealed that addition of NTPs alone led to accumulation of NTPs (ii). D) To confirm that $Mg^{2+}$was a limiting factor for progression of mRNA production, an IVT reaction was quenched with EDTA, a strong Mg^{2+} chelator, and resumed by addition of excess Mg^{2+} 60 minutes later. At-line HPLC analytics showed suspension of both mRNA production (i) and NTP consumption (ii) during the period without free Mg^{2+}.

IVT reactions are normally performed as batch processes. Considering their catalytic (enzymatic) basis, it should be possible to extend reaction times and yields by continuously adding consumed reagents to the reaction mixture, however fed-batch strategies have only been reported on short RNAs, e.g. 38mer RNA model systems. Application of this concept to therapeutically-relevant RNA lengths (several hundred nucleotides up to several thousand) has not yet been demonstrated in a scientific report. The fed-batch approach was therefore tested to increase the mRNA yield (Figure 3). A control reaction, including 20 ng/µL plasmid and 4 mM of each NTP was split into two samples after 180 minutes. One reaction was fed with bolus additions of NTP-Mg^{2+} (3 mM each NTP, 9 mM $Mg^{2+}$when at least one NTP fell >20%) while the control reaction was not fed. The latter did not show an increase in mRNA concentration, but the former did continue to produce mRNA. The rate of mRNA production was further increased by increasing the starting concentration of NTPs to 8 mM, and further still by increasing the plasmid concentration from 20 to 100 ng/uL, the latter reaching 9.3 mg/mL after approximately 5 hours (Figure 3A). Fed-batch strategy approach was tested on a larger mRNA construct, Cas9 (4495 nt); in absence of feeding, productivity of 4

mg/mL was reached, whereas with bolus additions of NTP-Mg^{2+}, productivity was increased to 9.5 mg/mL (Figure 3B).

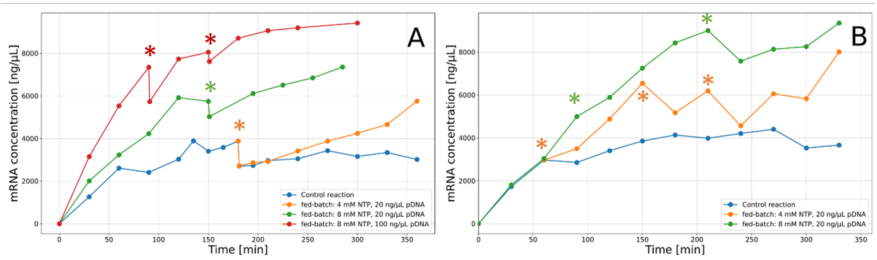

Figure 3: Optimisation of the fed-batch procedure on two plasmids. A) eGFP fed-batch: a control reaction, including 20 ng/uL plasmid and 4 mM of each NTP was split into two samples after 180 minutes. One reaction (orange) was fed with bolus additions of NTP-Mg^{2+} (3 mM each NTP, 9 mM Mg2+ when at least one NTP fell >20%) while the control reaction (blue) was not fed. Increasing starting NTPs to 8 mM at 20 ng/uL plasmid (green) led to a further increase in yield, and further still by starting NTPs to 8 mM at 100 ng/uL plasmid (red). B: Fed-batch production of Cas9 (4495 nt); in absence of feeding, productivity of 4 mg/mL was reached (blue). Bolus additions of NTP-Mg2+ increased yield to 8 mg/mL (orange).Increasing starting NTPs to 8 mM at 20 ug/uL plasmid further increased yield to 9.5 mg/mL. Bolus addition timepoints marked with asterisk.

In order to test reproducibility of fed-batch approach, two parallel reactions were performed on eGFP construct using the fed-batch approach (NTP-Mg^{2+} was added when at least one NTP fell to 20% starting value), both reactions reached 10 mg/mL productivity (Figure 4A). Figure 4B shows the consumption of NTPs averaged over both reactions. The rate of consumption is decreasing with time, which is consistent the decreasing rate of mRNA production. This is mostly likely due to the accumulation of mRNA, and concomitant pH decrease in the reaction vessel, which was not a controlled variable, and could be further explored in a bioreactor system. Quality assessment of mRNA produced over time showed no degradation of mRNA with time, and no increase in dsRNA content (Figure 4C/D).

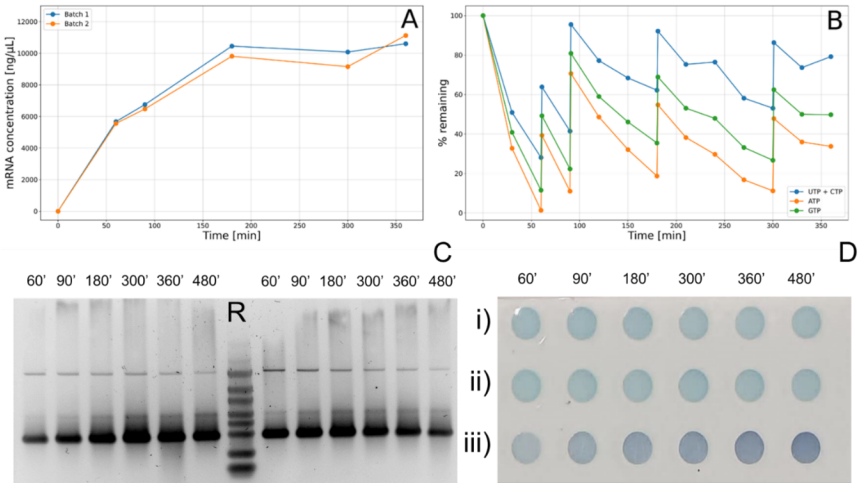

Figure 4: A: mRNA quantification with CIMac PrimaS of two parallel IVT reactions under optimized conditions. Sampling points before feeding are shown. B) Consumption of NTPs in Batch 1 reaction. Rate of NTP consumption decreased with each subsequent NTP addition, consistent with the reducing rate of

mRNA production. C) Agarose gel electrophoresis of fed-batch IVT with time showed no degradation of mRNA, left: Batch 1, right: Batch 2, R: Riboruler, timepoints 60, 90, 180, 300, 360, 480 min. D) dot-blot analysis of fed-batch IVT showed no increase in dsRNA content over time: i) Batch 1, ii) Batch 2, iii) serial dilution of dsRNA standard Magi2 (10 ng-80 ng).

IVT fed-batch approach of feeding NTP-Mg^{2+} boluses when at least one NTP fell to 20% starting value was then scaled-up 40-fold (to 6 mL reaction volume) to demonstrate scalability. Consistent with smaller reaction volumes, the reaction reached an mRNA concentration of over 10 mg/mL in less than 3 hours with rate of NTP consumption comparable to small-scale reaction. mRNA was purified with CIMmultus Oligo dT and resulting agarose gel demonstrated a single mRNA band at target molecular weight (Figure 5D).

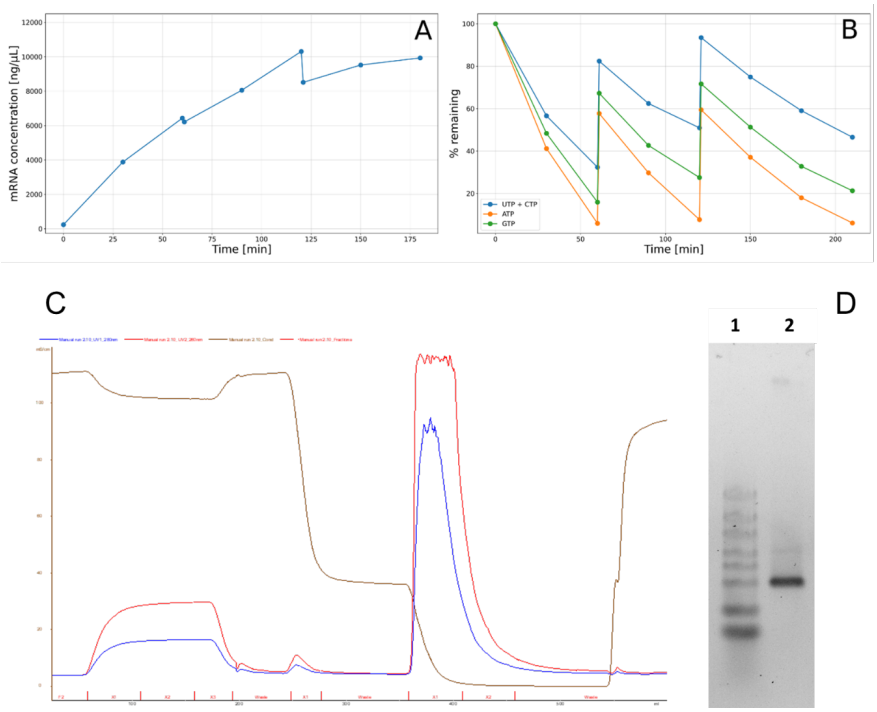

Figure 5: Scale-up and purification of mRNA produced by fed-batch approach. A: Monitoring of mRNA productivity with time (CIMac PrimaS method); B: monitoring of NTP concentration with time (CIMac PrimaS method); C: FPLC chromatogram of purification of 60 mg mRNA produced by fed-batch approach with CIMmultus Oligo dT (40 mL); D: Agarose gel electrophoresis of purified mRNA shows a single band at target molecular size (1000 nt). 1: Riboruler, 2: purified mRNA (eGFP).

Capping efficiency

mRNA could also be produced with co-transcriptional capping approach to produce 5' capped mRNA, necessary for in-vivo stability of mRNA. Correct ratio of NTPs:capping reagent is critical for high capping efficiency, in particular for capping analogue ARCA, which competes with GTP as initiation nucleotide; suggested ratio of ARCA:GTP for high capping efficiency was reported as 4:1.

Fed-batch approach based on HPLC monitoring was therefore applied to produce ARCA-capped mRNA. Concentration of 5' capped mRNA was determined by quantifying the amount of mRNA after sequential digestion with 5' polyphosphatase and terminator exonuclease as described previously. Concentration of undigested mRNA after enzymatic treatment was determined by CIMac PrimaS and compared with no-enzyme control, resulting in % capping ratio.

Four IVT reactions were performed to test the impact of feeding strategy on capping: fed-batch without controlling the GTP:ARCA ratio (Figure 6A), fed-batch with GTP:ARCA ratio ˜1:4 (Figure 6B); batch approach with GTP:ARCA ratio 1:4 (Figure 6C), a control without ARCA capping in batch approach (consumption of NTPs not shown). The control reaction without capping produced 3.7 mg/mL mRNA, polyphosphatase/exonuclease digestion resulted in completed digestion of mRNA, confirming that digestion protocol is specific for uncapped mRNA ('uncapped', Figure 6 D). Fed-batch without GTP:ARCA control produced 10.5 mg/mL mRNA, with only 37% capping. Controlling the GTP:ARCA ratio by supplementing the reaction with GTP concentration so as not to exceed 2 mM in reaction, based on at-line monitoring with CIMac PrimaS, significantly improved the capping efficiency (79%), while keeping the productivity high (11.7 mg/mL). ARCA was not supplemented, leading to gradual decrease from 8 mM to ˜6 mM. Performing same reaction in batch mode (no additions of NTPs and ARCA) resulted in comparable capping efficiency (79%) but poor yield (1.2 mg/mL). For production of ARCA-capped mRNA, fed-batch approach is thus favourable over the standard batch due to the low GTP content of the reaction; implementing at-line analytics to monitor the rate of GTP consumption prevents reaction from stopping, while also from over-feeding GTP.

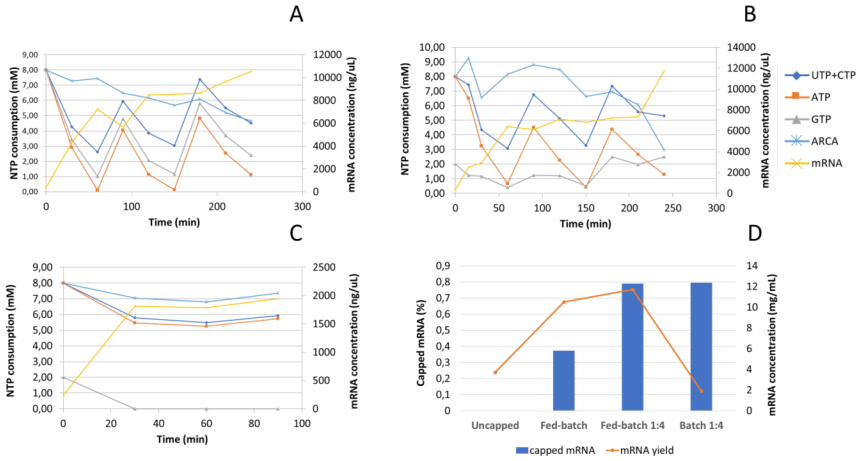

Figure 6: Increasing productivity and capping efficiency of fed-batch IVT reaction with ARCA capping. A: Monitoring of NTP, ARCA and mRNA concentration with CIMac PrimaS for fed-batch reaction with addition of equimolar NTP mixtures results in high productivity (10.5 mg/mL) and low capping efficiency (37%); B: Monitoring of NTP and mRNA concentration with CIMac PrimaS for fed-batch reaction without addition of 4:1 A/C/UTP: GTP mixtures results in high productivity (11.7 mg/mL) and high capping efficiency (79%); C: Monitoring of NTP and mRNA concentration with CIMac PrimaS for batch reaction (4:1 A/C/UTP: GTP mixture) results in low productivity (1.2 mg/mL) and high capping efficiency (79%); D comparative summary of yield and capping efficiency for four reactions A-C and uncapped control.

Concluding remarks

In order to optimize the yield of IVT reaction for production of mRNA, we developed a workflow based on at-line HPLC monitoring of consumption of NTPs and production of mRNA, with a sub-3 min read-out, to inform addition of reagents to IVT mixture. Application of at-line analytics enabled an insight into kinetics of mRNA production and NTP consumption, allowing optimization of individual IVT reaction components, such as concentration of Mg^{2+}, plasmid, NTP concentration, as well as adjusting the reaction parameters in near real-time. We demonstrate that optimal concentration of Mg^{2+} may be construct dependent, and high concentrations can be inhibitory; similarly, NTP concentration requires optimization in conjunction with Mg^{2+}. Plasmid concentration does not correlate with yield of mRNA produced, with important cost-of-goods implication – in context of limiting global capacity for production of plasmid, this provides a means for minimizing the concentration of plasmid to achieving target mRNA yield.

The workflow presented facilitates implementation of fed-batch approach resulting in doubling the reaction yield compared to standard IVT protocols, reaching 10 mg/mL for multiple constructs. Quantification of mRNA was also coupled with exonuclease digestion, leading to monitoring of capping efficiency of produced mRNA. When HPLC monitoring was applied to production of ARCA-capped mRNA construct, which requires an approximate 4:1 ARCA:GTP ratio, fed-batch approach achieved productivity of 11.9 mg/mL with 79% capping. The study provides a methodological platform for driving a better understanding of factors influencing IVT reaction, thereby increasing yield, and decreasing the cost of mRNA manufacturing. We foresee a coupling of this workflow with multiparallel automated bioreactor systems, e.g. Ambr®, allowing controlled, continuous addition of mRNA building blocks (e.g. NTPs, Mg^{2+}) based on read-out of consumption kinetics, with control of additional parameters, such as pH and temperature. This methodology also significantly reduces uncertainty in mRNA quantification in IVT mixtures prior to purification; it informs decisions on column sizing for downstream purification, which are otherwise hampered by interference of strongly UV absorbing components (NTPs, plasmid, proteins) or slow fluorescence-based analytics.

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Conflict of interest

Authors declare no conflicts of interest.

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