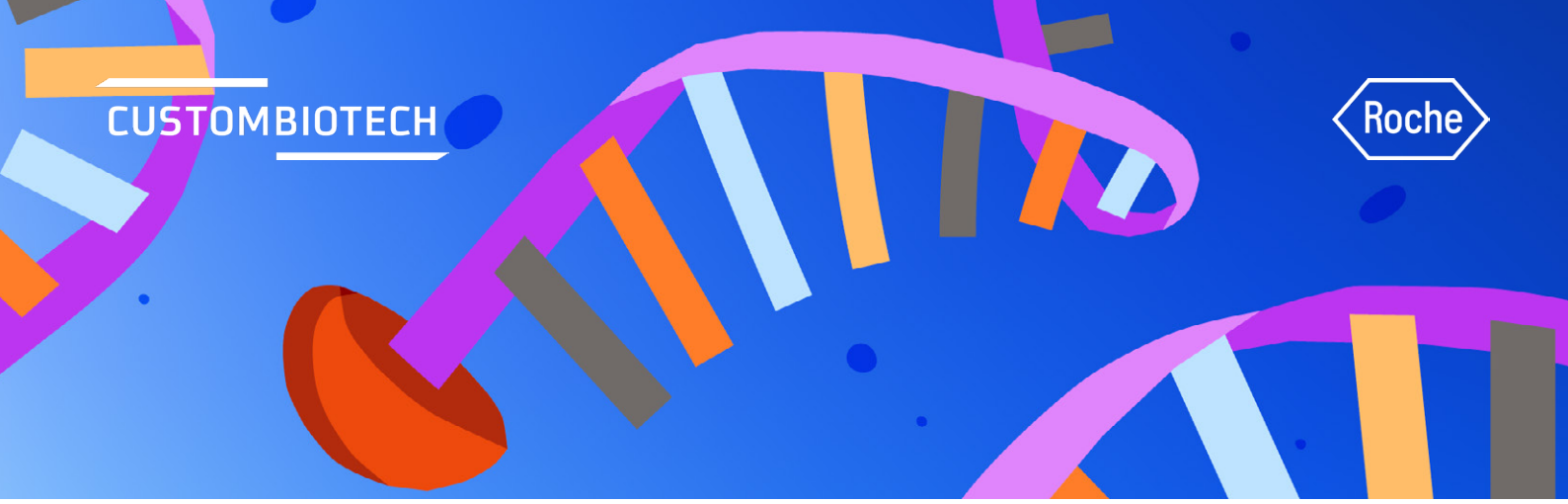

Levers to optimize the IVT reaction for increased mRNA yield

Technical Report

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Objective

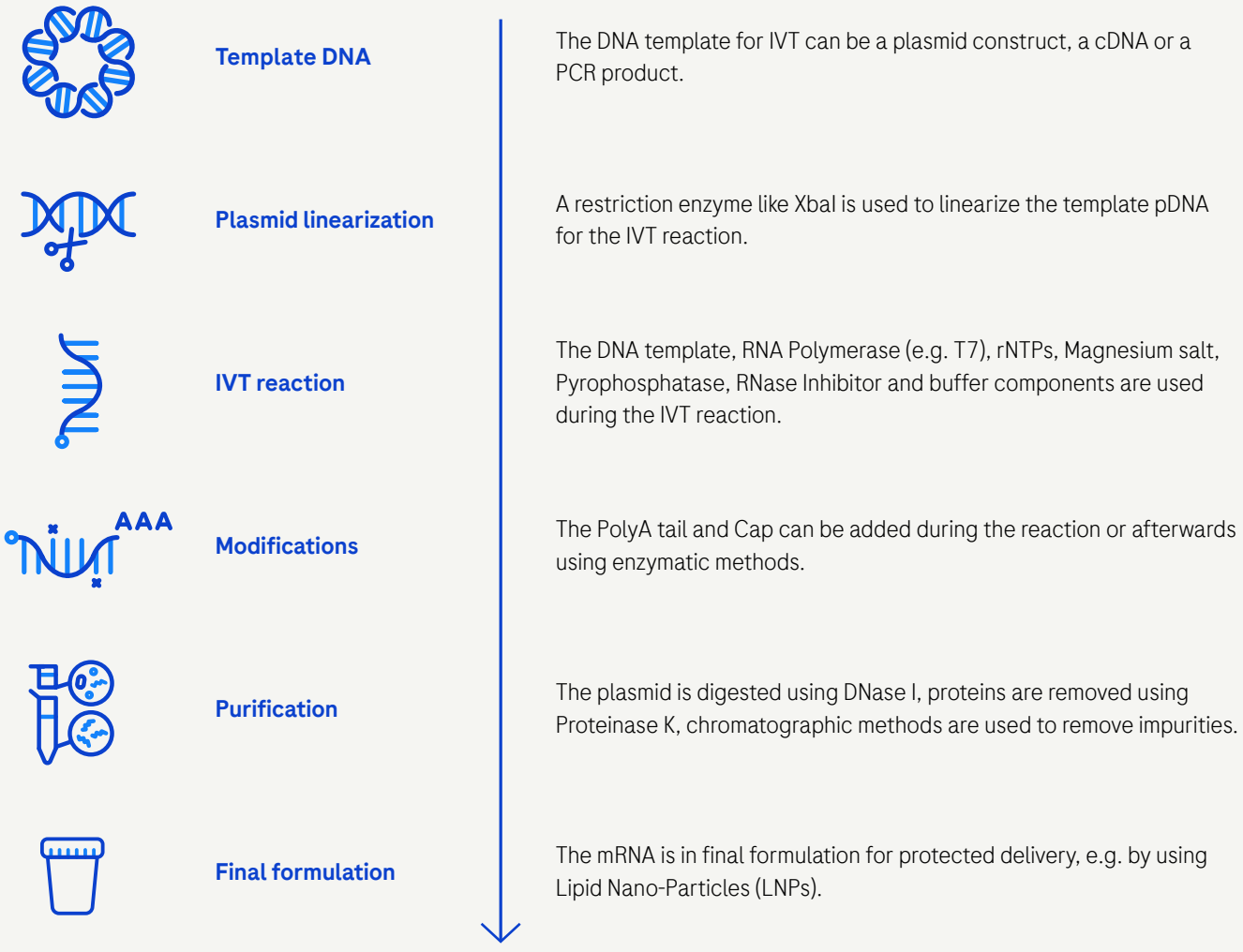
In vitro synthesis of single-stranded mRNA molecules is a widely used laboratory procedure that is central to RNA research and therapeutic development. Though already decades in development and use at smaller scales, the SARS-CoV-2 pandemic thrust mRNA therapeutics into the limelight with unprecedented demand in throughput. Individual constructs and intended uses require a tailored manufacturing process. It is therefore important to know the critical factors that influence the *in vitro* transcription (IVT) reaction.

Content Overview

We summarize some of the tools that can be used to set up and optimize the IVT reaction for enhanced mRNA yield. We demonstrate that factors such as magnesium ion and nucleotide concentrations impact the performance of the IVT reaction and the overall yield of mRNA significantly. Therefore, these critical parameters should be titrated and tested thoroughly to achieve reliable and efficient mRNA synthesis, a strategy to optimize its performance in your hands.

Introduction

Off-the-shelf kits for *in vitro* transcription (IVT) of mRNA are commonly used in research and early stages of development. However, for the specific requirements of therapeutics, such kits may lack two important features to consistently produce high-quality RNA during the mRNA manufacturing process (general workflow described in Fig. 1). First, flexibility to optimize reagents for different mRNA molecules based on composition, length and template type may be limited. Second, they may not operate with reagents of the same quality as needed for production. The aim of the first factor is to reduce production costs by achieving scale-appropriate yield under conditions of efficient reagent consumption. The aim of the latter is to ease the transfer from research to manufacturing scale.



01
The general mRNA manufacturing workflow

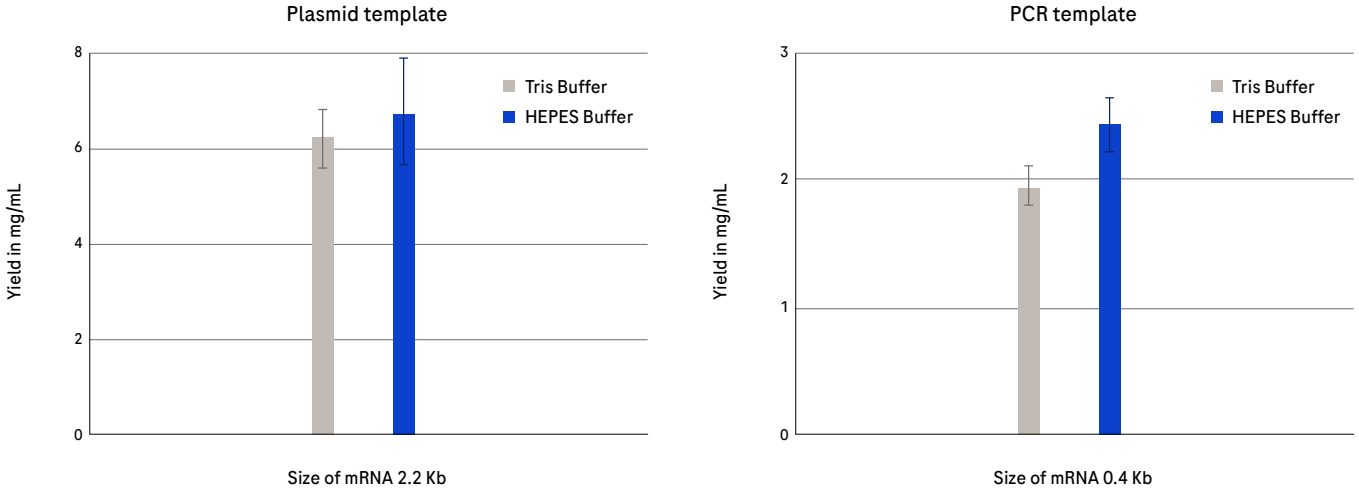
The main components of the IVT reaction are the DNA template, the RNA polymerase enzyme and the substrates. The latter include ribonucleoside triphosphates (rNTPs), and if required, modified ribonucleotides (e.g., N1-methyl-pseudo-UTP).

A transcription buffer solution containing Mg^{2+} ions from a magnesium salt (e.g. magnesium chloride) and other factors is also needed. Each of these components serves a specific function and can be optimized.

Results

Multiple buffer options give good overall yield with different template sizes and constructs

The pH during the IVT reaction must be maintained because it otherwise decreases with the incorporation of ribonucleotides.[1] To test if the choice of buffering system is a critical factor for the overall performance of the IVT reaction, we conducted an experiment with HEPES and Tris buffer. Both Tris and HEPES buffers resulted in comparably high mRNA yield, independent of template size and type.

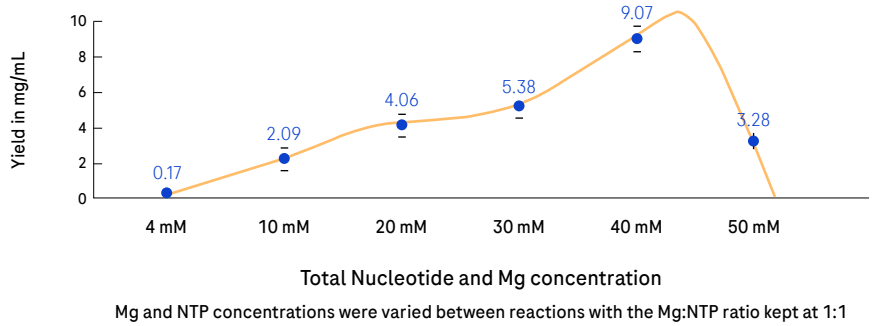


02

Testing the IVT reaction in HEPES and Tris buffer systems. Templates of 2.2 and 0.4 kB mRNA sizes (from plasmid and PCR DNA templates, respectively) were used for the mRNA synthesis. The following 10x buffer master mixes were used: 10x Tris: 400 mM Tris-HCl, 20 mM spermidine, 100 mM DTT, pH 7.9; 10x HEPES: 1 M HEPES-KOH, 20 mM spermidine, 400 mM DTT, pH 7.5. (Roche data on file, 2022, Latta A, application lab NAM)

Increasing the Mg^{2+} and ribonucleotide concentrations, up to a certain level, can improve yields

Mg^{2+} ions are important for the enzymatic catalysis of the IVT reaction.[2,3] Therefore, the concentration of $MgCl_2$ is a critical parameter in the IVT reaction. We tested a range of Mg^{2+} and ribonucleotide concentrations to determine the concentrations that generate the best overall yield in our setup.

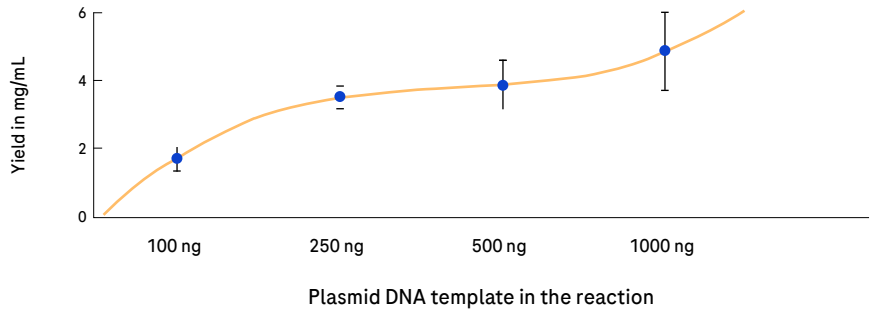


03

Titration of the Mg^{2+}/ribonucleotide concentration during the IVT reaction. Mg^{2+} and total rNTP concentrations varied between experiments but were kept at a constant ratio of 1:1 for a given experiment. (Roche data on file, 2022, Latta A, application lab NAM)

Optimizing template concentration helps achieve a balance between yield and cost of production

To examine the influence of DNA template concentration on the overall yield of the IVT reaction, we titrated different amounts of DNA template in a series of experiments.



04

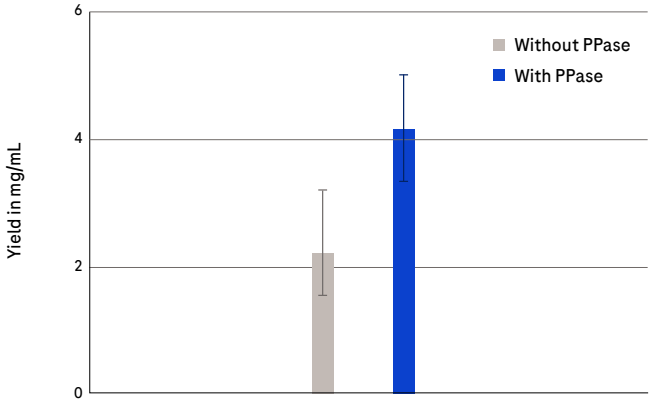
Titration of template amount during the IVT reaction. 100 ng – 1000 ng of a plasmid template for the 1.1 kb size mRNA construct was tested as template amount. (Roche data on file, 2022, Latta A, application lab NAM)

Addition of pyrophosphatase (PPase) improves overall mRNA yield

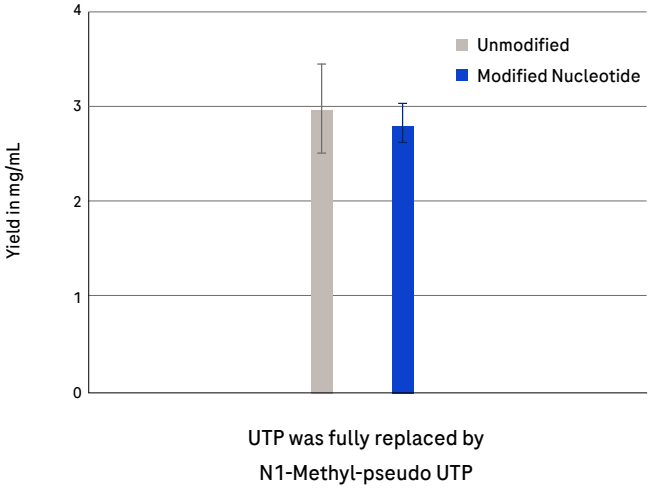
Pyrophosphate is a by-product of the IVT reaction and acts as an inhibitor of the reaction by causing the precipitation of Mg^{2+} ions.[4] The enzyme Pyrophosphatase can be added to catalyze the hydrolysis of pyrophosphates into inactive single orthophosphate ions that don't bind magnesium ions. Our comparison experiments with and without pyrophosphatase show that its addition increases overall mRNA yield.

The modified ribonucleotide N1-methyl-pseudo-UTP is efficiently incorporated by T7 RNA polymerase, giving similar yield to synthesis with unmodified ribonucleotides

To test if the incorporation of modified ribonucleotides has an impact on the overall performance and yield of the IVT reaction, we compared the condition of using unmodified ribonucleotides with the condition of replacing all UTP with N1-methyl-pseudo- UTP. The incorporation of the modified ribonucleotide does not change the yield in comparison to its unmodified counterpart. The usage of modified ribonucleotides is therefore not seen as a negative lever for the IVT reaction.



05
Impact of pyrophosphatase on the performance of the IVT reaction.
IVT reactions were tested with and without the addition of PPase (1 µL in total 20 µL reaction).
(Roche data on file, 2022, Latta A, application lab NAM)



06
Incorporation of modified ribonucleotides during the IVT reaction.
For the condition with modified ribonucleotide, the UTP was replaced by N1-methyl-pseudo-UTP.
(Roche data on file, 2022, Latta A, application lab NAM)

How CustomBiotech mRNA reagents help you to overcome mRNA synthesis challenges and achieve production goals

For over 30 years, Roche CustomBiotech has been a valued partner of therapeutic manufacturers, completing 150 projects to develop and produce enzymes, proteins and other raw materials for diagnostic and biopharmaceutical production. Always abreast with market developments, we are a key player in enabling a new generation of mRNA therapeutics. Roche CustomBiotech offers several key components needed for the IVT reaction and upstream and downstream manufacturing process, such as T7 RNA Polymerase, ribonucleotides (rNTPs), modified rNTPs (e.g., N1-methyl-pseudo-UTP or pseudo-UTP), RNase Inhibitor, pyrophosphatase and DNase I. All are supplied in a fit-for-purpose quality for the production of mRNA for therapeutics and vaccine manufacturing.

Experimental conditions

The reaction mix consisted of 500 ng DNA template of a linearized plasmid of 1.1 kb mRNA construct size, 1 µl T7 RNA Polymerase 1 µl Pyrophosphatase, 20 U RNase Inhibitor 40mM MgCl2, 10mM each of ATP, GTP, CTP and UTP. 2 µl reaction buffer, and RNase-free water to a final volume of 20 µl. For the Mg and NTP titration experiment the concentration is listed in Fig.03. For the Modified Nucleotide experiment, Fig.06, UTP was completely replaced by N1-Methyl-Pseudo-UTP. The reaction buffer was prepared from a 10X Tris transcription buffer master mix consisting of 400 mM Tris-HCl (pH 8.0), 20 mM spermidine and 100 mM DTT. The reaction was incubated for 1 h at 37°C. RNA was subsequently purified using the High Pure FFPET RNA Isolation Kit, sold by Roche Molecular, including Proteinase K digestion and DNase I treatment according to manufacturer's instructions. Experiments were performed in triplicates. Subsequently the RNA was analyzed using the Agilent® Bioanalyzer and quantified using a UV/Vis spectrophotometer at 260 nm (NanoDrop™). Experiment specific adaptations are mentioned in the respective figure descriptions.

Key takeaways

Our results show the advantage of spending some time optimizing the IVT reaction. It allows to balance costs and yields. We show in our results some of the most critical parameters to consider for optimization, like the right ratio and concentration of Mg^{2+} ions and ribonucleotides.

Buffer

The buffer system can be Tris or HEPES-based. The buffer used should have a good ionic strength for pH maintenance during the reaction as the pH of the IVT reaction can decrease when H^+ ions are released during rNTP incorporation.[1]

Mg^{2+} and nucleotide concentration

This parameter has the most significant impact on the RNA yield. An optimal balance of Mg^{2+} and rNTPs is needed for an effective IVT reaction.[2,3]

Pyrophosphatase

This enzyme removes pyrophosphate generated during the IVT reaction. Pyrophosphate can inhibit the IVT reaction as it precipitates free Mg^{2+} ions and thereby reduces the free Mg^{2+} ions in the reaction.[4]

RNase Inhibitor, template and T7 RNA Polymerase concentration

Additional parameters to assess include the addition of RNase inhibitor as well as the concentration of template and T7 RNA Polymerase concentration. RNases are ubiquitous in the environment and a significant risk factor during RNA production. The activity of RNases is blocked using RNase Inhibitor.[5] T7 RNA Polymerase and template concentrations should be titrated and the overall reaction time optimized based on, for example, construct size.

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