

Technical Report

Levers to optimize the IVT reaction for increased mRNA yield



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.

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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 Figure 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.

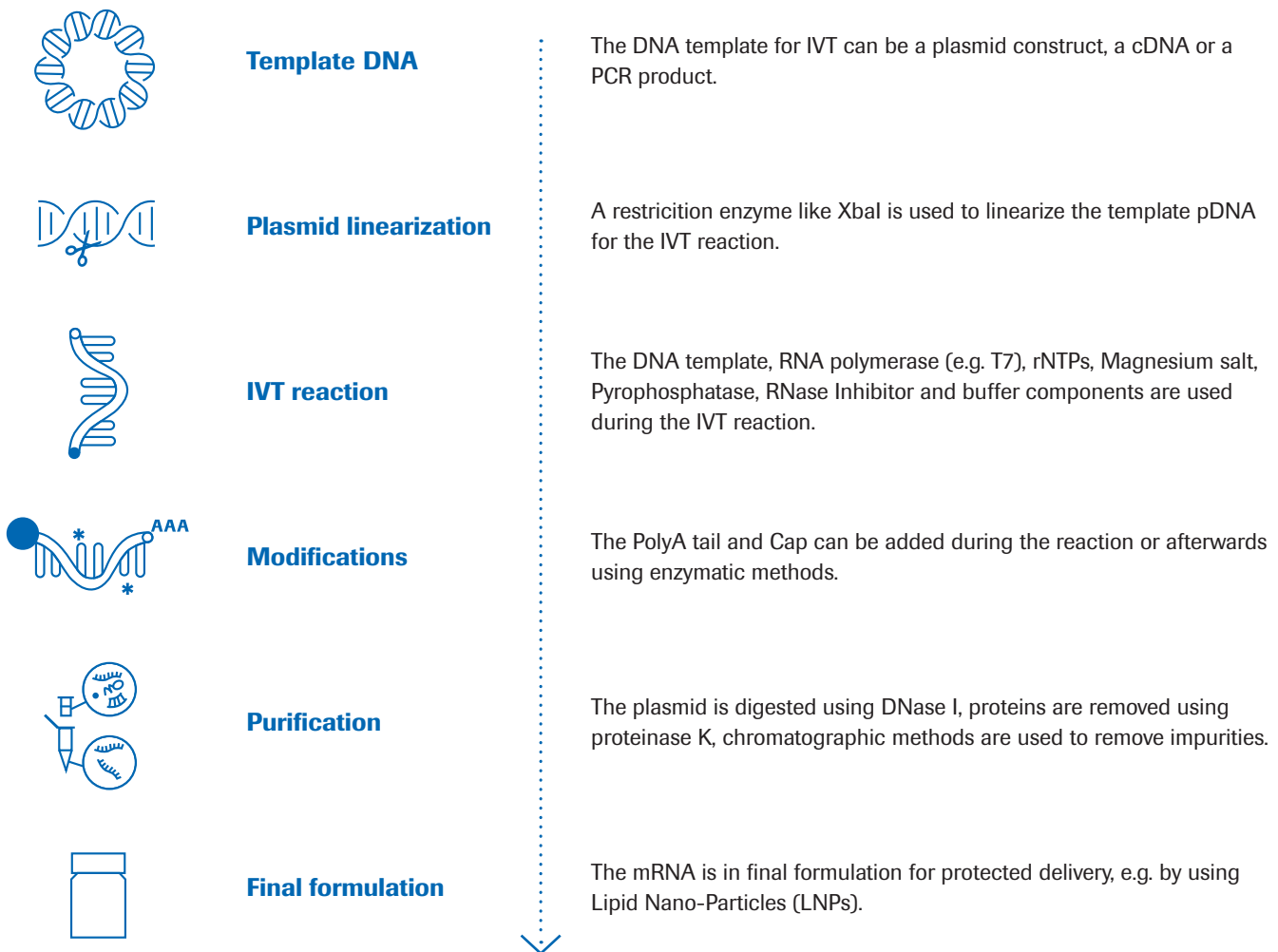


Figure 1: The general mRNA manufacturing workflow

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.

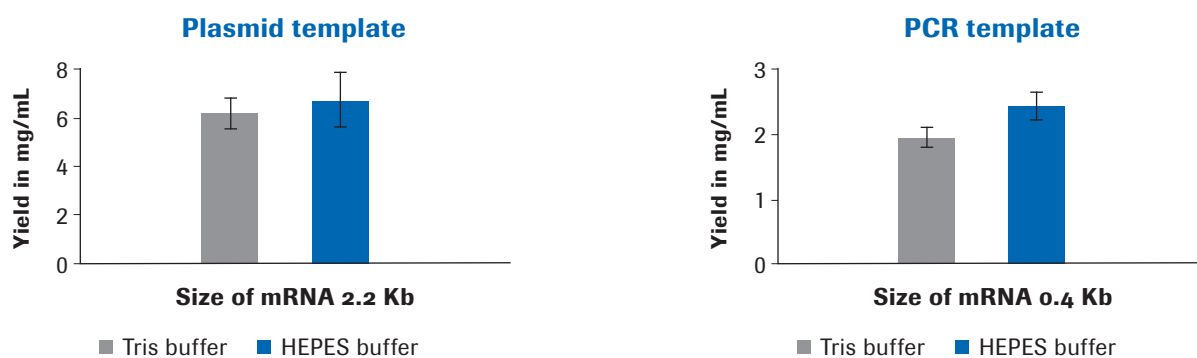


Figure 2: Testing the IVT reaction in HEPES and Tris buffer systems. Templates of 2.2 and 0.4 kB mRNA sizes (from plasmid and PCR DNA templates, respectively) were used for the mRNA synthesis. The following 10x buffer master mixes were used: 10x Tris: 400 mM Tris-HCl, 20 mM spermidine, 100 mM DTT, pH 7.9; 10x HEPES: 1 M HEPES-KOH, 20 mM spermidine, 400 mM DTT, pH 7.5. Roche data on file.

Increasing the Mg²⁺ and ribonucleotide concentrations, up to a certain level, can improve yields

Mg²⁺ ions are important for the enzymatic catalysis of the IVT reaction.^{2),3)} Therefore, the concentration of MgCl₂ is a critical parameter in the IVT reaction. We tested a range of Mg²⁺ and ribonucleotide concentrations to determine the concentrations that generate the best overall yield in our setup.

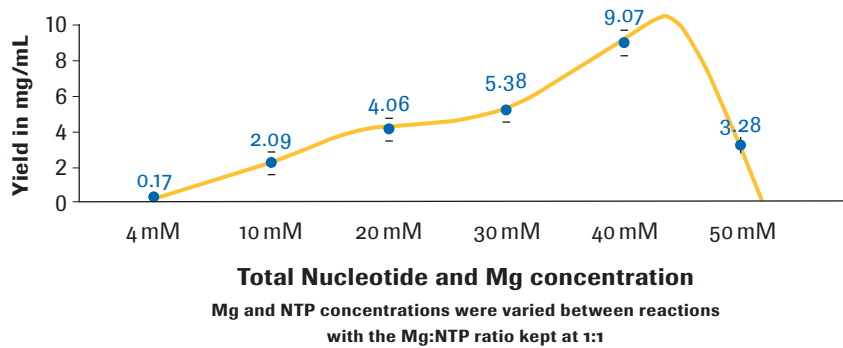


Figure 3: Titration of the Mg²⁺/ribonucleotide concentration during the IVT reaction. Mg²⁺ and total rNTP concentrations varied between experiments but were kept at a constant ratio of 1:1 for a given experiment. Roche data on file.

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.

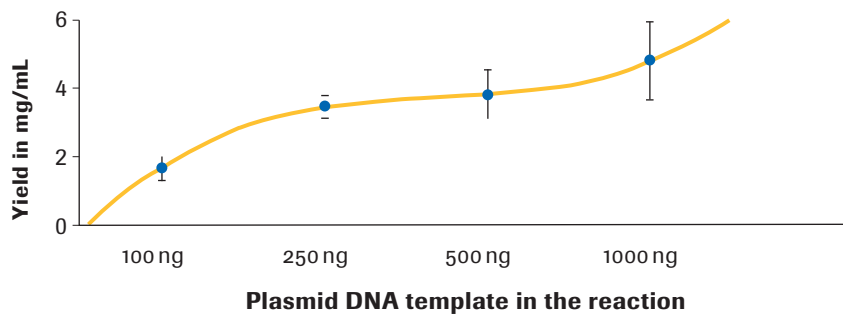


Figure 4: Titration of template amount during the IVT reaction.

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.

Addition of pyrophosphatase (PPase) improves overall mRNA yield

Pyrophosphate is a by-product of the IVT reaction 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.

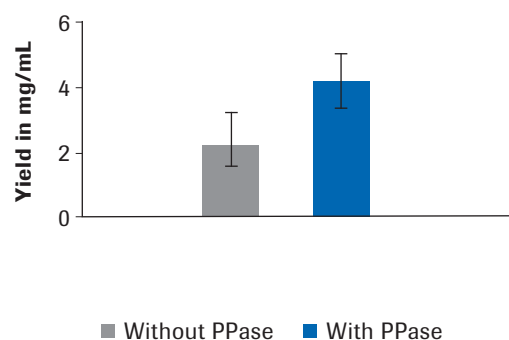


Figure 5: Impact of pyrophosphatase on the performance of the IVT reaction. IVT reactions were tested with and without the addition of PPase (1 μ L (CustomBiotech Mat#08140677103) in total 20 μ L reaction). Roche data on file.

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.

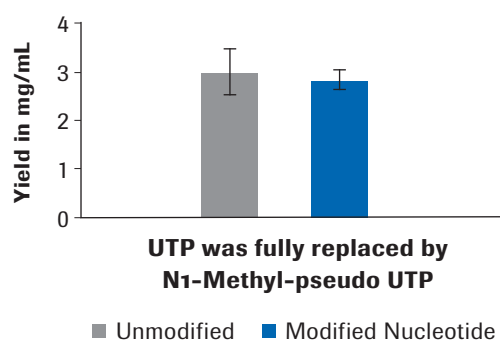


Figure 6: Incorporation of modified ribonucleotides during the IVT reaction. For the condition with modified nucleotide, the UTP was replaced by N1-methyl-pseudo-UTP (CustomBiotech Mat# 09522409103). Roche data on file.

Product portfolio of fit-for-purpose mRNA reagents

Our fit-for-purpose requirements for raw materials set and control property tolerances based on the sensitivity of their intended use to those specific attributes. Beyond meeting standards of consistent performance and material purity, our reagents for mRNA synthesis are produced under stringent conditions and undergo additional scrutiny.

| Product | Catalog number | Pack size | GMP Grade | Animal-origin-free ¹ (AOF) | Antibiotic-free | Tested for RNase/DNase activity | Extended impurity testing ⁴ |
|------------------------------------|----------------------------------|--------------------------------|---------------------------|---------------------------------------|--------------------------|---------------------------------|--|
| Proteins | | | | | | | |
| T7 RNA Polymerase, rec., GMP Grade | 08 140 669 103 | 10 ml (ca. 10 mg) | Yes | Yes | Yes | Yes | Yes |
| Pyrophosphatase, rec., GMP Grade | 08 140 677 103 | 20 ml (ca. 40 mg) | Yes | Yes | Yes | Yes | Yes |
| RNase Inhibitor, rec., GMP Grade | 09 537 643 103 09 537 589 103 | 100 kU 2 MU | Yes | Yes | Yes | Yes | Yes |
| DNase I, rec., RNase-free | Inquire 03 539 121 103 | 20 kU 200 kU | Yes | No ² | Yes | Yes | Yes |
| Proteinase K, rec. | 03 654 672 103 | 850 mL | Yes (except for Hb assay) | No ² | Yes | Yes | Partially |
| SP6 RNA Polymerase, rec. | 09 788 131 103 | 25 mL | Planned | Yes | Yes | Yes | Yes |
| Xba I rec. | 09 520 848 101 09 520 848 103 | 5 ml (50 kU) 25 ml (250 kU) | No | Yes | Yes | No | Partially |
| Product | Catalog number | Pack size | GMP Grade | Animal-origin-free ¹ (AOF) | β-Lactam-antibiotic-free | Tested for RNase/DNase activity | Extended impurity testing ⁴ |
| Ribo-Nucleotides | | | | | | | |
| ATP | 04 980 824 103 | 100 mL | Yes | No ^{2,3} | Yes | Yes | No |
| CTP | 04 980 875 103 | 100 mL | Yes | No ^{2,3} | Yes | Yes | No |
| GTP | 04 980 859 103 | 100 mL | Yes | No ^{2,3} | Yes | Yes | No |
| UTP | 04 979 818 103 | 100 mL | Yes | No ^{2,3} | Yes | Yes | No |
| N1-Methyl-Pseudo-UTP, GMP Grade | 09 744 878 103 09 744 762 103 | 100 mL 1.0 mL | Yes | Yes | Yes | Yes | Yes |
| Pseudo-UTP | 09 754 341 103 09 538 798 103 | 100 mL 1.0 mL | No | Yes | Yes | Yes | Yes |

¹ For details see Certificates of Origin.

² TSE/BSE certificate available.

³ Orthogonal virus depletion steps included in manufacturing process (e.g. virus retentive filter). Further information on virus depletion study is available.

⁴ Includes e.g. testing for bioburden, endotoxin, heavy metals, host-cell DNA, host-cell protein, if applicable = subject to change in on-going project.

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^+ 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+} 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.

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 μ l T7 RNA polymerase (CustomBiotech Mat#08140669103), 1 μ l pyrophosphatase (CustomBiotech Mat#08140677103), 20 U RNase inhibitor (CustomBiotech Mat#09537643103), 2 μ l reaction buffer, and RNase-free water to a final volume of 20 μ l. 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 FFPE RNA Isolation Kit (Mat # 06650775001, sold by Roche Molecular Systems; www.lifescience.roche.com), 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.

References

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Regulatory disclaimer

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