Levers to optimize the IVT reaction for increased mRNA yield

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Abstract

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 scale. Individual constructs and demands require a tailored manufacturing process. It is therefore important to know the critical factors of the central *in vitro* transcription (IVT) reaction for the mRNA synthesis. Given the different constructs and applications, no universal protocol can be used for the IVT reaction, so optimizing is essential. Below we summarize some of the tools that can be utilized to set up and optimize the IVT reaction for enhanced mRNA yield. We demonstrate that factors such as magnesium 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.

Introduction

In research and early stages of development, off-the-shelf kits for in vitro transcription (IVT) of mRNA are often used. However, in the development of therapeutics, such kits may lack two important requirements to consistently produce high-quality RNA during the mRNA manufacturing process (general workflow described in Figure 1). First, the flexibility to optimize reagents for different mRNA molecules based on composition, length and template type. Second, the assurance that reagents used from research to production are of the same quality. 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.

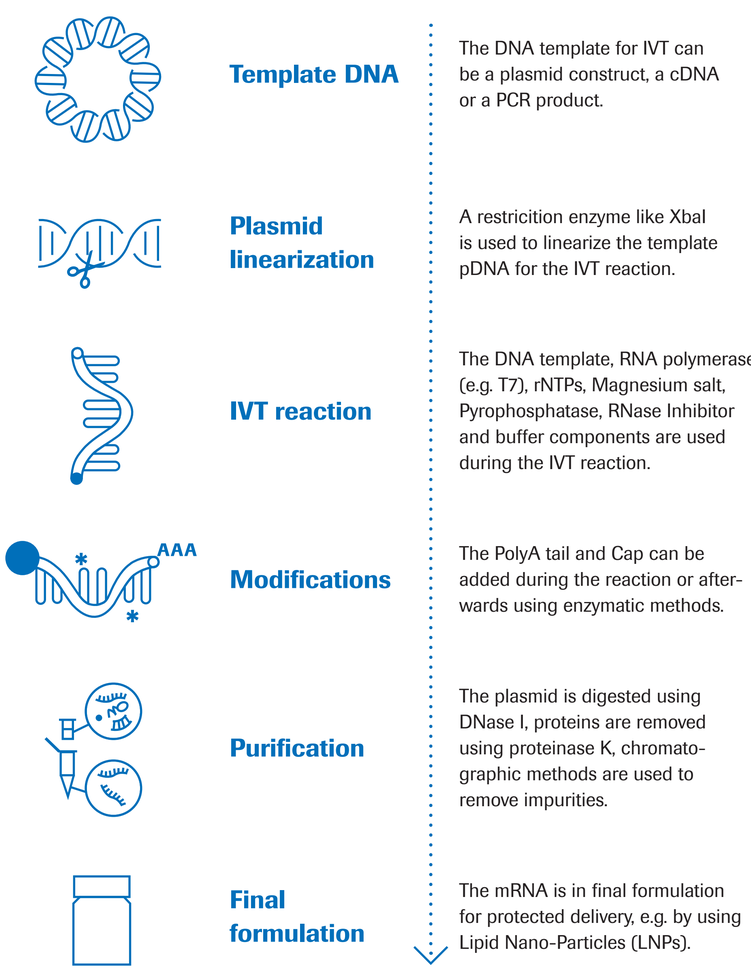

Figure 1: The general mRNA manufacturing workflow

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

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 (CustomBiotech Mat#08140669103), 1 µl Pyrophosphatase (CustomBiotech Mat#08140677103), 20 U RNase inhibitor (CustomBiotech Mat#09537643103), 2 µl reaction buffer, and RNase-free water to a final volume of 20 µl. 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 FFEPT 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 UV/Vis Spectrophotometer at 260 nm (NanoDropTM).

Experiment-specific adaptations are mentioned in the respective figure description.

Regulatory disclaimer

Roche CustomBiotech mRNA raw materials are for further processing only.

Results

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

pH maintenance during the IVT reaction is required, otherwise the pH would decrease with the incorporation of the ribonucleotides.[1)] To test if the choice of the respective buffering system is a critical factor for the overall performance of the IVT reaction, we performed the IVT reaction in HEPES and Tris buffer. Both Tris and HEPES buffers resulted in comparable, high mRNA yield, independent of template size ānd type.

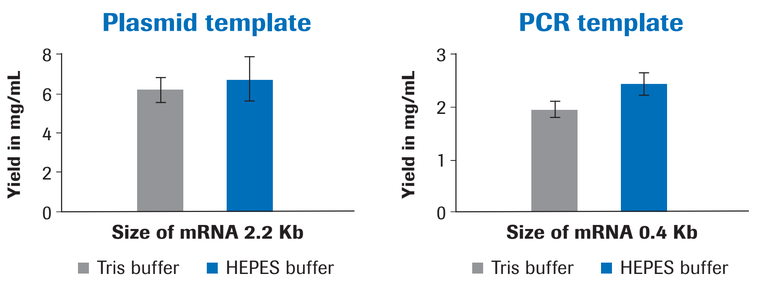

Figure 2: Testing the IVT reaction in HEPES and Tris buffer systems. Templates of 2.2 and 0.4 kB sizes and plasmid and PCR DNA templates were utilized for the mRNA synthesis. The following 10x buffer master mixes were used: 10x Tris: 400 mM Tris-HCl, 20 mM spermidine, 100 mM DTT, pH 7.9; 10x HEPES: 1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

Increasing the Mg^{2+} and ribonucleotide concentration can improve yields up to a certain concentration.

Mg^{2+} ions are important during the enzymatic catalysis of the IVT reaction.[2),3)] Therefore, the concentration of $MgCl_2$ is one of the critical parameters in the IVT reaction. We were testing different Mg^{2+} and ribonucleotide concentrations to see which concentrations might give the best overall yield in our set up.

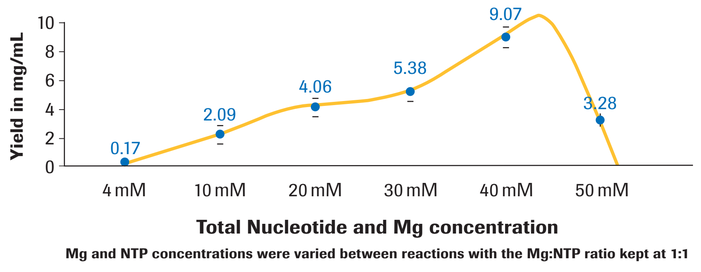

Figure 3: Titration of the Mg^{2+}/ribonucleotide concentration during the IVT reaction. Mg^{2+} and total rNTP concentrations varied between experiments but were kept at a constant ratio of 1:1 for a given experiment. Roche data on file.

Optimizing template concentration will help achieve the balance between yield and cost of production.

To identify to which extent the concentration of the used DNA template is influencing the overall yield of the IVT reaction, we titrated different amounts of DNA template used.

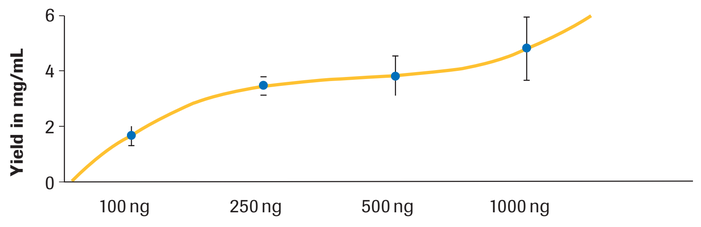

Figure 4: Titration of template amount during the IVT reaction. 100 ng - 1000 ng of a plasmid template for the 1.1 kb size mRNA construct was tested as template amount. Roche data on file.

Addition of pyrophosphatase (PPase) improves overall mRNA yield.

Pyrophosphate is produced during the IVT reaction as a byproduct and acts as an inhibitor of the IVT reaction through 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. To identify the impact of pyrophosphatase on the overall mRNA yield we performed comparison experiments, with and without the addition of pyrophosphatase.

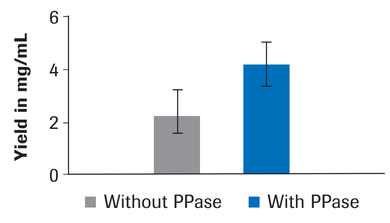

Figure 5: Impact of pyrophosphatase on the performance of the IVT reaction. IVT reactions were tested with and without the addition of PPase (1 µL (CustomBiotech Mat#08140677103) in total 20 µL reaction). Roche data on file.

The modified ribonucleotide N1-methyl-pseudo-UTP is efficiently incorporated by T7 RNA Polymerase, giving similar yield to the 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.

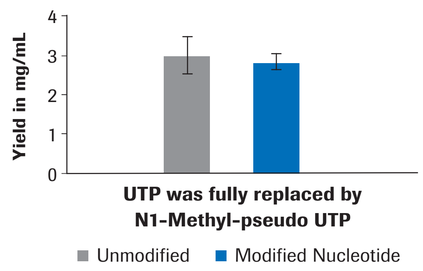

Figure 6: Incorporation of modified ribonucleotides during the IVT reaction. For the condition with modified nucleotide, the UTP was replaced by N1-Methyl-Pseudo-UTP (CustomBiotech Mat# 09522409103). Roche data on file.

Summary and discussion

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, 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 as 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. Pyrophosphate precipitates free Mg^{2+} ions and thereby reduces the free Mg^{2+} in the reaction, which inhibits the reaction.[4)]

RNase Inhibitor
RNases are ubiquitous in the environment and a significant risk factor during RNA production. The activity of RNases is blocked using RNase Inhibitor.[5)]

Template and T7 RNA polymerase concentration
T7 RNA Polymerase and template concentrations should be titrated and the overall reaction time optimized based on, for example, construct size.

References

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