Determination of the optimal buffer conditions and nucleotide concentrations to maximize mRNA yield using *in vitro* transcription

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Abstract

In this study we determined optimal buffer conditions and nucleotide concentrations for maximizing the yield and quality of mRNA produced by *in vitro* transcription (IVT). By using design of experiments (DoE) principles and analytics, a variety of IVT reaction conditions were assessed for their impact on the IVT reactions. DoE experiments using full factorial design were employed to discover which factor(s) had the biggest influence on mRNA transcript yield and to identify potential interactions between the factors tested.

In these experiments, IVT reaction factors such as type of buffer, pH, Mg^{2+} concentration, reaction accessories, and nucleotide concentration and ratios were examined. Results of the DoE experiments indicate that magnesium anion, a cofactor for T7 RNA polymerase [1], had the most significant impact on mRNA yield. The Mg^{2+}:NTP ratio was further identified to be a significant factor in the yield of the IVT reaction. It was also observed that the type of buffer and counter ion used when producing mRNA affected the overall IVT reaction.

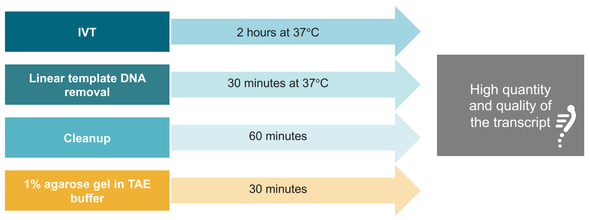
Introduction

The growing use of RNA-based vaccines and therapies has focused much attention and effort on developing conditions for maximizing the yield and efficiency of the IVT reactions used to produce mRNA. IVT procedures involve the biosynthesis of RNA from a linear DNA template containing an appropriate transcription promoter. mRNA is typically enzymatically synthesized by T7 RNA polymerase using nucleotide triphosphate (NTP) building blocks. The IVT reaction mixture will also contain a number of IVT reaction accessories such as ribonuclease inhibitor (RI) and inorganic pyrophosphatase (IPP) that help increase the efficiency and yield of the IVT reaction. A variety of buffer systems and additives can also be used when performing IVT.

In this study, we examined the impact of different buffer conditions and nucleotide concentrations on the IVT reaction. A design of experiments (DoE) approach was utilized to determine the optimal reaction buffer and nucleotide concentration for achieving the highest mRNA yield and quality for an IVT reaction. A full two-level factorial DoE was initially employed to test and optimize IVT buffer conditions. Some of the IVT buffer variables examined included type of buffer, Mg^{2+} concentration and salt form, and spermidine. Once an optimal IVT buffer composition had been identified, further DoE follow-up experiments were conducted with nucleotide concentrations and ratios to maximize mRNA yield and quality.

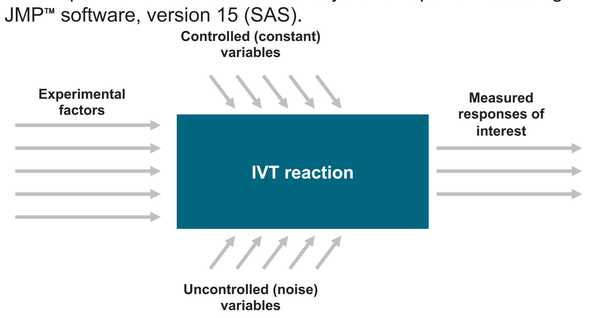
Materials and methods

Materials



IVT reactions were performed using a linearized plasmid vector (pTri-Xef) with a T7 RNA polymerase promoter and a 1.8 kb insert for mRNA synthesis. All enzymes, nucleotides, IVT reaction accessories, and buffer chemicals were obtained from Thermo Fisher Scientific and were Thermo Scientific™ TheraPure™ GMP* products whenever possible. Invitrogen™ MEGAclear™ Transcription Clean-Up Kit (Cat. No. AM1908) and Invitrogen™ Dynabeads™ magnetic beads (Cat. No. 61006), were used for mRNA purification. mRNA yield and quality were measured by A_{260} measurements on a Thermo Scientific™ NanoDrop™ spectrophotometer and electrophoresis on a 1% agarose gel with TAE buffer.

DoE experimental and statistical analysis were performed using JMP™ software, version 15 (SAS).



We performed a full factorial design. The aim was to determine the components of the IVT buffer that have the highest statistical significance on our response of interest, which was the RNA yield. A simple linear regression model was used for the analysis of the data, a fit model of standard least squares.

Methods

IVT protocol

Component	Amount
IVT reaction buffer	2 µL
100 mM nucleotides	2 µL each
IVT enzymes	2 µL
T7 RNA polymerase (200 U/µL)	
RNase inhibitor (40 U/µL)	
Pyrophosphatase (1 U/µL)	
1 µg linear template	X µL
Water, nuclease free	To 20 µL

The IVT reaction was allowed to proceed for 2 hours at 37°C. Then, 1 µL of DNase I (1 U/µL) was added to the IVT mixture and incubated for a further 30 minutes at 37°C. The resulting mRNA was purified using Dynabeads magnetic beads following the manufacturer's instructions [2]. Synthesized mRNA was quantitated by A_{260} measurement and purity/quality was assessed by electrophoresis (1% agarose gel). For the analysis, 300 ng of product was loaded into each well.

Table 1. DoE two-level factorial experimental design.

Name	Units	Type	Domain/range
Buffer	Buffer choice	Categorical	HEPES-NaOH, Tris-HCl
Buffer concentration	mM	Real number	[20, 60]
Cofactor (Mg acetate)	mM	Real number	[20, 60]
Spermidine	mM	Real number	[1, 3]
NTPs	mM	Real number	[5, 15]
Evaluation	Yield		

Name	Units	Type	Domain/range
Buffer	Buffer choice	Categorical	HEPES-NaOH, Tris-HCl
Buffer concentration	mM	Real number	[20, 60]
Cofactor (Mg chloride)	mM	Real number	[10, 45]
Spermidine	mM	Real number	[1, 3]
NTPs	mM	Real number	[5, 15]
Evaluation	Yield		

For the DoE studies, a full two-level factorial design, testing six factors (buffers, cofactors, spermidine, and NTP concentrations), at two levels (low and high concentrations) was employed. The DoE was designed to explore the differences in mRNA yield of IVT between HEPES-NaOH and Tris-HCl buffer, the effect of magnesium, and the difference between chloride ions and acetate ions. Full factorial analysis included the effect of relationships between each factor tested in the IVT buffer.

Results

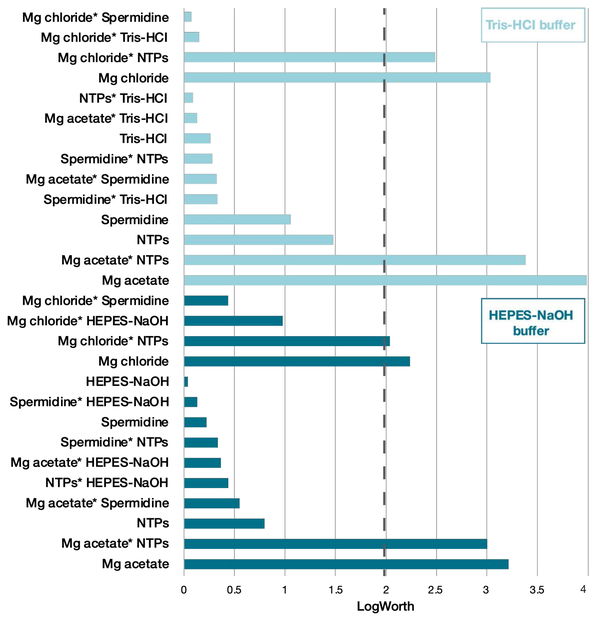


Figure 1. Determining the most significant factors impacting IVT.

After performing the DoE guided experiments detailed above, JMP software generated a fit model of standard least squares, according to the measured RNA yield for each set of IVT reaction conditions. Figure 1 shows the indicated values for LogWorth of each factor tested and their secondary interactions. In the context of this analysis, LogWorth values larger than 2 are significant. Results indicate that magnesium had the highest significance in terms of impact on mRNA yield.

Source	LogWorth	p-value	
Mg acetate	3.987	0.00061	HEPES-NaOH [20, 60]
Mg acetate:NTPs	3.000	0.00101	
Mg chloride	2.240	0.00575	
Mg chloride:NTPs	2.040	0.00911	

Source	LogWorth	p-value	
Mg acetate	3.987	0.00010	Tris-HCl [20, 60]
Mg acetate:NTPs	3.384	0.00041	
Mg chloride	3.039	0.00091	
Mg chloride:NTPs	2.489	0.00325	

Figure 2. Magnesium counter-ion effect on mRNA yield.

Magnesium was shown to have the greatest significance in terms of mRNA transcript yield in the initial DoE studies. When we further examined the effect of magnesium on IVT, we found that the type of magnesium counter ion (acetate or chloride) also had a significant effect on mRNA yield. Acetate ions with a LogWorth of 3.213 and p-value of 0.00061 stood out as most significant. The same tendency was seen in the examination of secondary interactions between magnesium and NTPs. Results are in accordance with recently published data, where Mg^{2+} is the most significant component for IVT and where acetate ions result in higher yields than chloride ions [3].

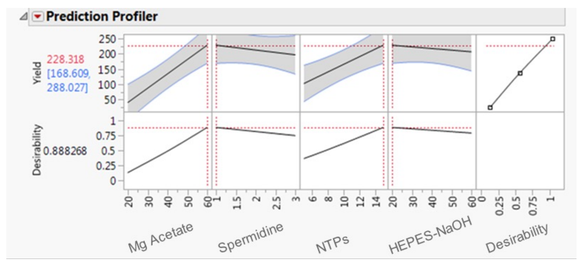


Figure 3. Determining IVT buffer conditions to optimize mRNA yield.

The results of the DoE studies described above (Figures 1 and 2) were utilized to determine the optimal combination of IVT buffer factors that would result in the maximum mRNA yield.

The prediction profiler indicated that to maximize RNA yield, the best IVT buffer conditions would be to utilize HEPES-NaOH as the IVT solution buffer with magnesium acetate as the source of the magnesium ions required for IVT (corresponding to the final, optimized composition for IVT buffer published [3], with Mg-acetate and HEPES-NaOH in the reaction mixture).

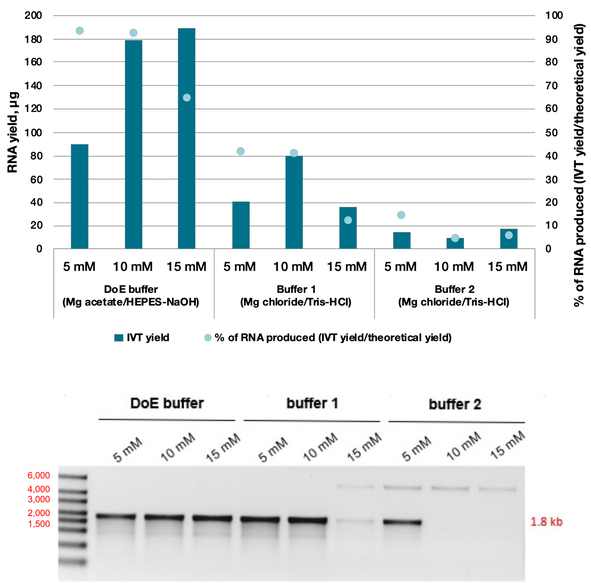


Figure 4. Confirming buffer and nucleotide concentration effects on mRNA yield and purity.

Once the optimal buffer salt and magnesium source had been determined, we performed several follow-up experiments to further optimize the IVT buffer conditions. A comparison of the mRNA yield and mRNA transcript quality from the chosen optimal IVT buffer conditions (HEPES-NaOH and magnesium acetate) was performed against the mRNA yield and mRNA transcript quality obtained from other IVT buffers using different concentrations of Tris-HCl and magnesium chloride (Buffers 1 and 2).

We also tested the efficiency of the IVT reaction with different NTP concentrations (5,10, and 15 mM) in both buffer conditions. We compared the percentage of produced RNA with theoretical yield, while using different NTP concentrations.

Results from these experiments, shown in Figure 4, indicate that IVT reactions performed using the optimal IVT buffer (HEPES-NaOH and magnesium acetate), as determined by DoE, gave the highest mRNA yields with maximized yield at 10 mM NTP concentrations. Analysis of the mRNA transcripts also indicated that the optimal IVT buffer also produced the expected mRNA at high quality. Gel electrophoresis results shown in Figure 4 indicate the correct transcript size (1.8 kb) was produced using the optimal IVT buffer and with significantly less aberrant mRNA transcripts than were observed in the other IVT buffer conditions.

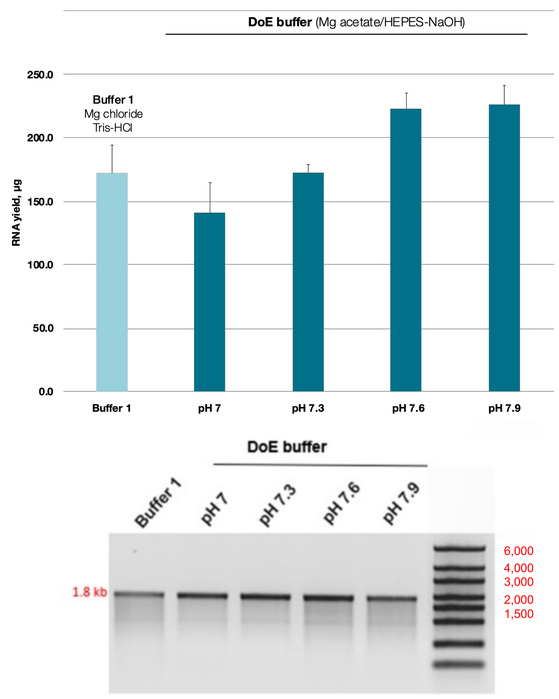


Figure 5. Effect of pH on mRNA yield.

The effect of a range of pH values on mRNA yields from IVT produced using the optimal IVT buffer described previously was also examined. IVT was performed in the optimal buffer at a range of pH values from pH 7 to 7.9. Higher pH values increased the reaction efficiency and mRNA yield up to pH 7.6 with no obvious reduction in the quality of mRNA transcript produced.

Conclusions

Using a DoE approach to optimize IVT reaction buffer conditions, we were able to produce 27% higher yields of mRNA transcript than were obtained using non-optimized IVT buffers. These results demonstrate the impact that each component of an IVT buffer can have on mRNA transcription efficiency and overall mRNA yield in the IVT reaction.

These results indicate that of the factors examined, magnesium ions have the most significant impact on mRNA yield. Magnesium counter-ions also have an impact on mRNA yield with acetate being preferred over chloride. The type of buffer system utilized can also impact mRNA yield, with the use of HEPES-NaOH leading to higher mRNA yields than Tris-based buffer. Finally, the nucleotide concentration and pH also impact mRNA yields. It was found that when using an optimized IVT buffer, mRNA yields were maximized at 10 mM and 15 mM nucleotide concentration, but important to note—IVT was not optimal at 15 mM, producing only 65% of expected mRNA amount. Results also strongly indicate that the quality of the mRNA produced using the optimized IVT buffer conditions is excellent.

References

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