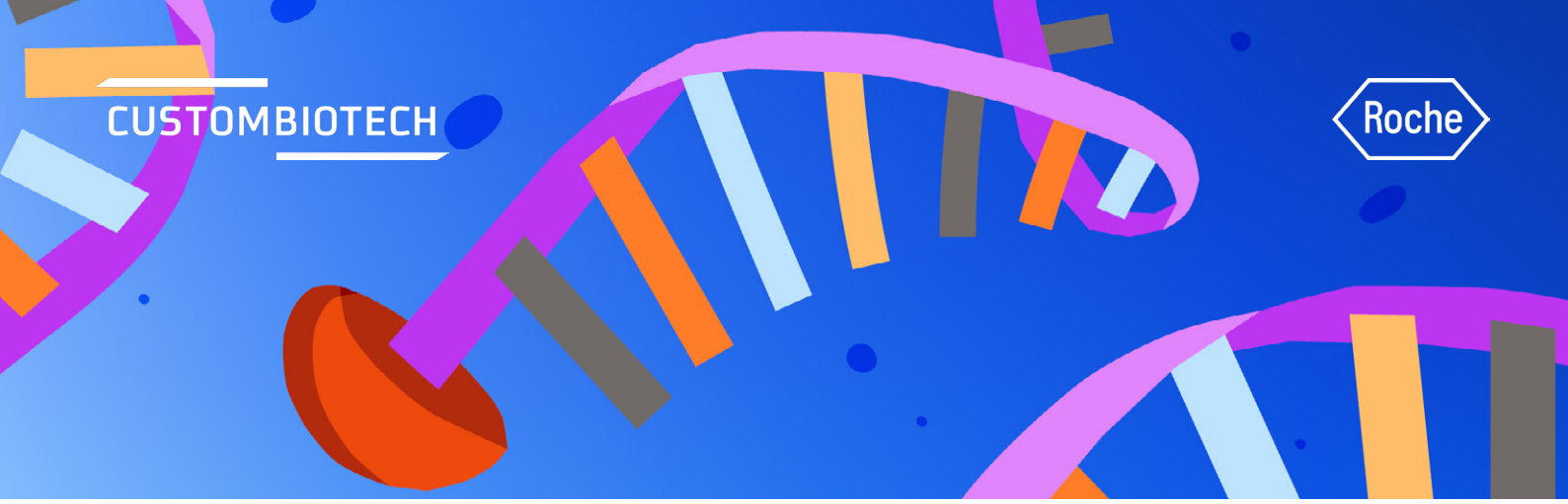


DoE of multiple inputs of IVT to develop a performance model optimized for yield and quality of the mRNA product

Technical Report

2023
CPI Innovation Services Limited (CPI), United Kingdom for CustomBiotech, Roche Diagnostics GmbH

Scope

Therapeutic mRNAs and vaccines are being developed for a broad range of human diseases, including COVID-19. However, optimizing mRNA production is complicated by interdependencies among factors of the *in vitro* transcription (IVT) reaction that impact yield and quality. Here, we employ the design of experiment (DoE) approach to analyze the influence and interdependence of factors (T7 RNA polymerase concentration, DNA template amount, and use of modified versus unmodified ribonucleotides) to develop an IVT performance model for assessing process performance using yield, integrity, and purity data of the mRNA product.

Aim & Objectives

The aim of this study was to identify an optimum set of conditions for the IVT reaction that produces high-quality mRNA in high yield using a design of experiment (DoE) approach. Our individual objectives were to:

- Complete a screen with different IVT reagents and use analytics to assess the yield and integrity of mRNA while quantifying dsRNA impurity, one of the biggest challenges in process development.
- Perform a statistical analysis to identify the reagents and any interactions among them that have a significant impact on IVT.
- Compare the performance of IVT with and without the use of a modified nucleotide (N1-methyl-pseudo-UTP).
- Recommend potential IVT setups that, based on the statistical analysis, favor the generation of mRNA in high yield and of high quality.

Introduction

Previous optimization data generated by Roche CustomBiotech demonstrated that factors such as magnesium and nucleotide concentrations significantly impact the yield performance of IVT reactions.[1] Other studies also show that the interaction between optimal amounts of these components is essential to produce high yields, especially with special construct designs like self-amplifying RNA (saRNA).[2] Table 1 lists the components selected for this DoE study.

IVT component	Units	Condition 1	Condition 2
T7 RNA polymerase	Activity units per µL of IVT	100	200
Magnesium:nucleotide ratio (1:1)	mM	30	40
DNA template linearized plasmid	µg per µL of IVT	0.025	0.05
Modified nucleotide (N1-methyl-pseudo-UTP) as a substitute for UTP	n/a	With	Without

T01
Reaction components tested in DoE. All critical raw materials used (enzymes, nucleotides) were from the Roche CustomBiotech mRNA portfolio, except for CleanCap AG (Trilink Biotechnologies) and the template GFP plasmid (CPI Innovation Services Limited). (Roche data on file DOE 2023)

Results

Yield of mRNA

The IVT reaction was set up with co-transcriptional capping using CleanCap AG. mRNA yield was quantified using the Nanodrop™ spectrophotometer and outcomes are listed in Table 2. In summary, good mRNA yields were observed across all conditions tested. Differences in the average yield (6–15 g/L) depended on the IVT reaction condition screened.

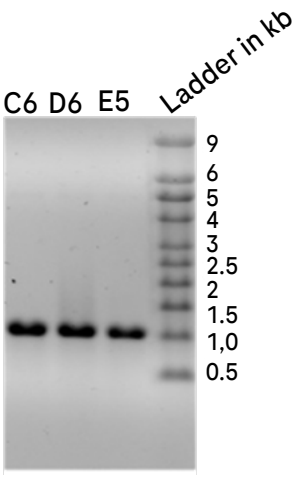
			CAP								
DNA in µg/µL			0.025 µg				0.05 µg				Nucleotide used
T7 Pol.	Mg/NTP conc.	Row	1	2	3	Avg.	4	5	6	Avg.	
100 U/µl	30 mM	A	7383.1	7706.4	15268.9	**7544.8**	14347.7	14623.8	14215.4	**14395.6**	modified
		B	11032.1	16148.7	16525.2	**16337.0**	12498.3	11675	10267.4	**11480.2**	unmodified
	40 mM	C	9171.4	8550.2	9153.9	**8958.5**	12776.7	9783.7	11096.2	**11218.9**	modified
		D	8529	8725.5	9196.7	**8817.1**	12227.5	9348	11332.7	**10969.4**	unmodified
200 U/µl	30 mM	E	13172	10176.3	12261.2	**11869.8**	17756.7	9214.8	6503.5	**7859.2**	modified
		F	7520.2	4829.6	6094.7	**6148.2**	7183.2	13619.2	12707.8	**13163.5**	unmodified
	40 mM	G	9353.9	1556.2	1621.8	**9353.9**	10058.3	8916.7	10909.8	**9961.6**	modified
		H	9115.7	10164.1	11780.4	**10353.4**	13690.3	10525.6	15822.7	**13346.2**	unmodified

T02
IVT yields in the DoE screen. The mRNA yields in the table are in µg/mL. The replicates in gray were considered technical outliners and were excluded from the statistical analysis. (Roche data on file DOE 2023)

mRNA Integrity

Agarose gel electrophoresis

After LiCl precipitation and resuspension, mRNA samples were separated by agarose gel electrophoresis to check integrity in a qualitative manner. Figure 1 shows the single band of the GFP mRNA product at the expected size of 1.1 kb. No prematurely terminated or longer 3′-extended mRNA products were observed. This level of integrity was consistent across all conditions.

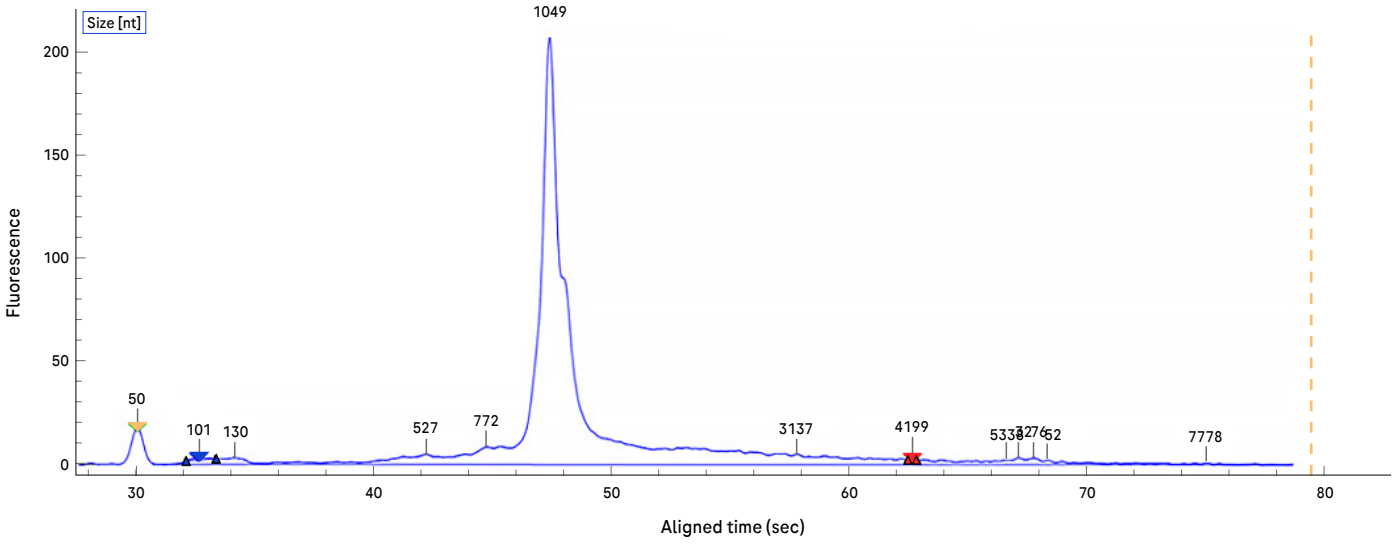


01

A representative image of purified mRNA from an IVT reaction separated on 1% w/v agarose gel. Approximately 500 ng per sample were loaded. The data shown is for one replicate per condition (C6, D6, E5). The sizes of the RNA ladder are listed in kilobases. (Roche data on file DOE 2023)

LabChip analysis

The mRNA integrity was additionally analyzed using LabChip® (Fig. 2). A main peak was detected close to the expected product size (1.0–1.2 kb). However, a secondary shoulder peak and other small peaks were detected in some samples.

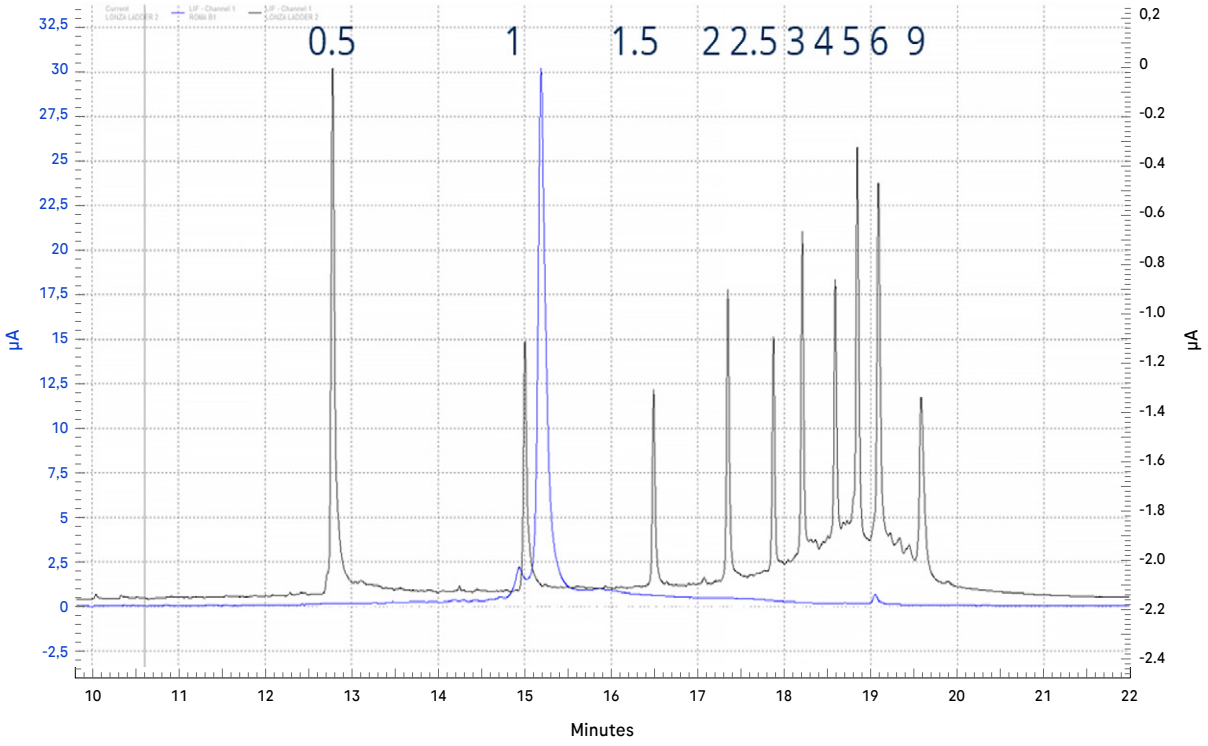


02

mRNA integrity analysis using the LabChip instrument. The example shown here was for the IVT reaction using 100 U/µL T7 RNA polymerase, 0.025 µg template DNA, 30 mM Mg:NTP ratio, the non-modified nucleotide, and CleanCap reagent (B2 in Tab. 3). All detected peaks are shown and the approximate molecular weight (bp) is displayed above each peak.

Capillary electrophoresis

To ascertain if the additional peaks in the Labchip analysis were artifacts, samples were evaluated at higher resolution using capillary electrophoresis. The analysis detected one main peak at the expected product size of 1.1 kb, as shown in Figure 3. Similar results were observed for other samples tested.



03

Capillary electrophoretic analysis of the sample in Figure 2. The 0.5–9 kb standard is shown in black and sizes (kb) are displayed above each peak. Peaks detectable in the mRNA sample are shown in blue.(Roche data on file DOE 2023)

Summary of mRNA integrity

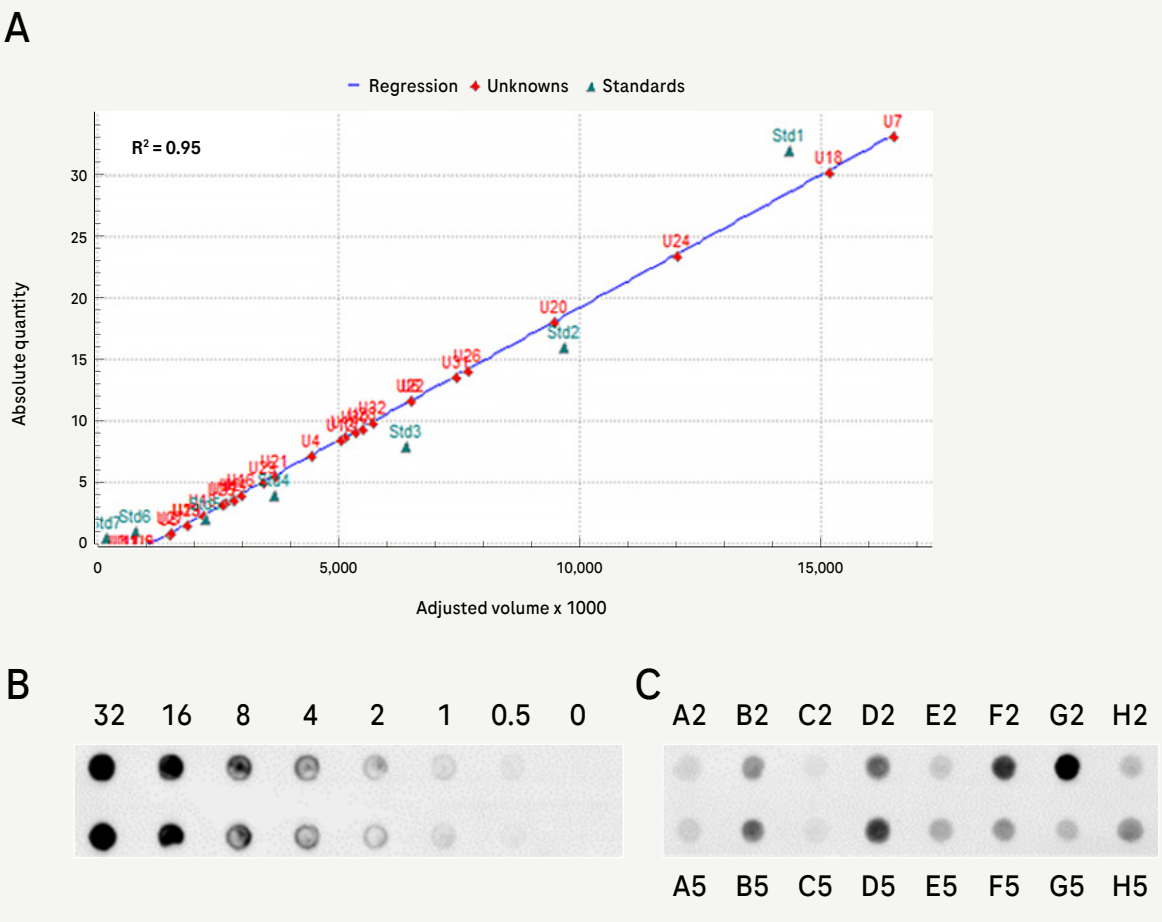
Overall, the IVT conditions chosen produced high-integrity RNA. The samples tested contained the mRNA product of interest. No major species beyond the expected mRNA product were detected.

mRNA purity

Dot-blot analysis for dsRNA

Double-stranded RNA (dsRNA) is a known by-product during the IVT reaction resulting from the "self-priming" activity of RNA polymerase.[3] These dsRNA byproducts trigger a cellular immune response that detrimentally impacts the safety and efficacy of an mRNA therapeutic or vaccine. Therefore, an optimization goal for IVT reactions is to keep dsRNA levels low and any production is removed by extensive downstream purification using, for example, chromatographic columns.[4,5] A low dsRNA amount is an important quality parameter in mRNA production process design.

The amount of dsRNA in each sample of the DoE screen was evaluated with a quantitative dot blot assay. A standard curve for dsRNA was generated by serial dilution (Fig. 4A), spanning a concentration range from 32 to 0.5 µg/mL. Purified mRNA test samples (5 µL) at a concentration of 200 µg/mL and standards (5 µL) were loaded on the blots (Fig. 4B). The quantified amount of dsRNA in the samples is summarized in Table 3.



04

dsRNA measured by quantitative dot blot (A). The standard curve for quantification with the standards marked in green and samples marked in red (B). Dot blots of the standards (in duplicate) with concentrations indicated in µg/mL (C). Dot blot of representative samples (one replicate for each condition). G2 was considered an outlier based on yield data and thus, its dsRNA measurement was excluded from the overall analysis.(Roche data on file DOE 2023)

DNA in µg/µL			0.025 µg						0.05 µg						
T7 Pol.	Mg/ NTP conc.	Sample	1		2		3		4		5		6		Nuc. used
			µg/mL	% of yield	µg/mL	% of yield	µg/mL	% of yield	µg/mL	% of yield	µg/mL	% of yield	µg/mL	% of yield	
100 U/µl	30 mM	A	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	modified
		B	535.1	4.9	379.5	2.4	57.8	0.4	462.4	3.7	496.2	4.3	421	4.1	unmodified
	40 mM	C	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	modified
		D	285.7	3.4	357.7	4.1	349.5	3.8	403.5	3.3	514.1	5.5	170	1.5	unmodified
200 U/µl	30 mM	E	0	0.0	30.5	0.3	30.7	0.3	0	0.0	142.8	1.6	152.8	2.4	modified
		F	319.6	4.3	253.6	5.3	280.4	4.6	237	3.3	306.4	2.3	235.1	1.9	unmodified
	40 mM	G	0	0.0	263	16.9	120	7.4	65.4	0.7	84.7	1.0	0	0.0	modified
		H	54.7	0.6	76.2	0.8	17.7	0.2	0	0.0	263.1	2.5	142.4	0.9	unmodified

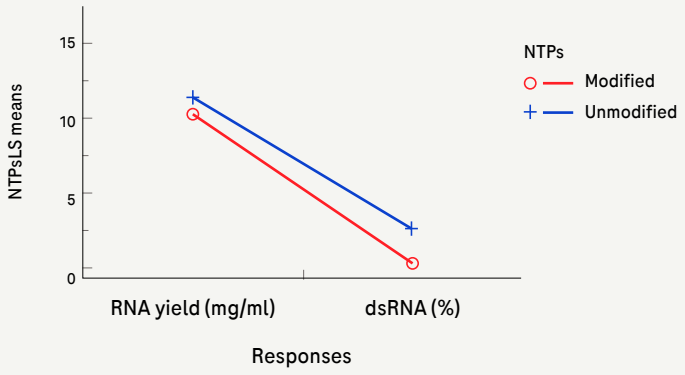
T03

The dsRNA concentrations in the table are presented in µg/mL and as a percentage of the mRNA yield. The replicates in gray were considered technical outliers based on the earlier results for yield and were excluded from the statistical analysis. Samples with dsRNA levels below the limit of detection were assigned a value of 0. (Roche data on file DOE 2023)

Overall, dsRNA levels for most of the conditions in the DoE were less than 5% of mRNA yield. Results were comparable across all three replicates for each condition. In general, a higher template concentration generated more dsRNA than lower template amounts. Furthermore, dsRNA impurities were lower in samples using the modified nucleotide, averaging 0.33% versus 2.76% of the mRNA yield.

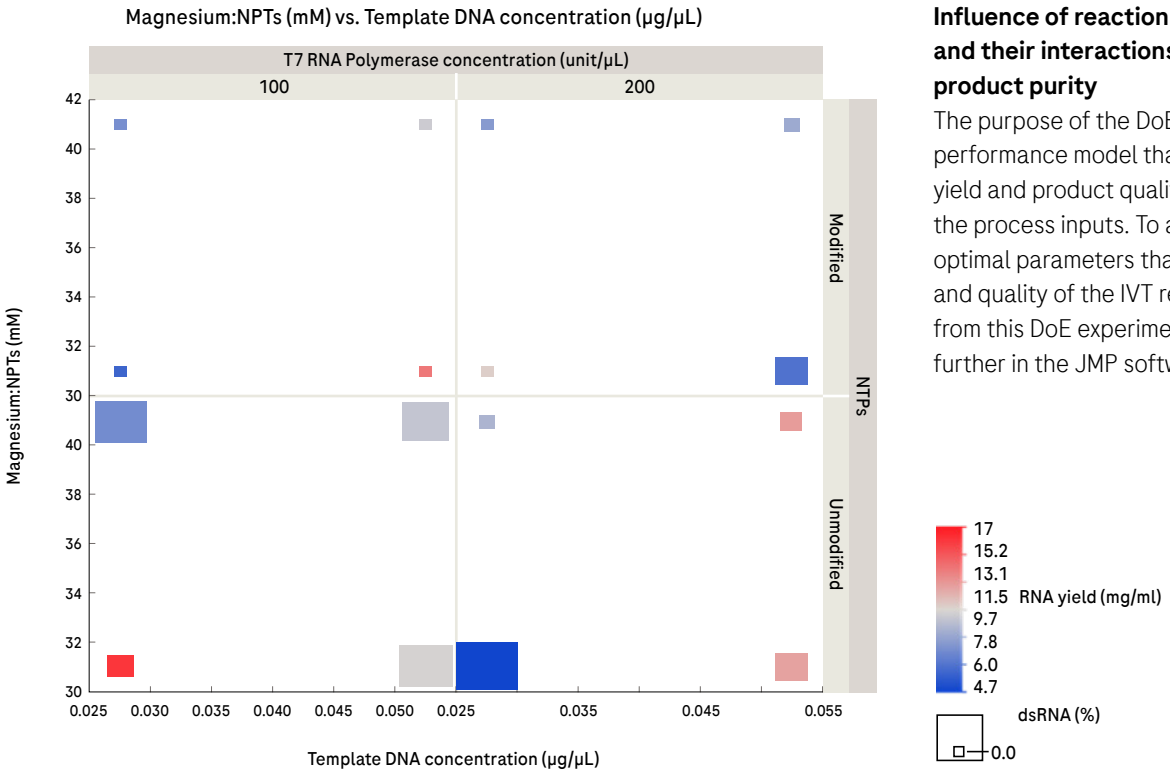
Use of modified nucleotide N1-methyl-pseudo-UTP leads to lower amounts of dsRNA generation

The observed decrease in dsRNA generated in IVT reactions using a modified nucleotide was corroborated by a MANOVA analysis. The difference in dsRNA between IVT conditions including modified versus unmodified nucleotides was statistically significant (P<0.05), while mRNA yields were similar (Fig. 5).



05

Results from a MANOVA analysis comparing the impact of using modified (red) and unmodified (blue) nucleotides on mRNA yield (mg/mL) and dsRNA content relative to mRNA yield (%). There is a statistically significant difference in dsRNA (%) between modified and unmodified nucleotides ($p < 0.001$) but no difference in mRNA yield ($p = 0.56$). (Roche data on file DOE 2023)



Influence of reaction components and their interactions on IVT yield and product purity

The purpose of the DoE was to generate a performance model that predicts process yield and product quality depending on the process inputs. To aid with identifying optimal parameters that maximize yield and quality of the IVT reaction, the results from this DoE experiment were analyzed further in the JMP software.

06

The figure axes summarize the different IVT parameters tested in the DoE screen. mRNA yield (color scale) and the dsRNA content relative to mRNA yield (symbol size) measured in all sample conditions are represented as a function of the inputs. The optimal result would be a small (low dsRNA) and red (high yield) rectangle. (Roche data on file DOE 2023)

Evident in Figure 6 is the importance of interactions among the input variables of the DoE in determining the outputs, both mRNA yield and purity. In identifying optimal conditions for the IVT reaction, trade-offs between mRNA yield and purity must be considered and multiple conditions can produce mRNA in good yield with low dsRNA.

The lowest dsRNA levels with high mRNA yield as determined in this DoE screen was achieved using 0.05 µg template DNA, 100 U/µL T7 RNA polymerase, 30 mM magnesium:nucleotide ratio, and the modified nucleotide N1-methyl-pseudo-UTP (lower right corner of the upper left quadrant in Fig. 6).

The highest mRNA yield with low levels of dsRNA was achieved using 0.025 µg template DNA, 100 U/µL T7 RNA polymerase, 30 mM magnesium:nucleotide ratio, and an unmodified nucleotide (lower left corner of the lower left quadrant in Fig. 6).

It is noteworthy that keeping all inputs the same for the above two conditions and only increasing the T7 RNA polymerase concentration from 100 to 200 U/µL changes the results drastically to a low-yielding and high-impurity reaction.

Components and their interactions should be carefully assessed to select the optimal conditions for a specific therapeutic. These conditions must balance target yield and purity with the cost of raw materials for mRNA generation and purification.

A model accounting for the different inputs and their interactions is represented by cross terms:

$$RNA\ yield = a * NTP + b * T7 + c * DNA + \ldots$$
$$+ f * NTP * T7 + g * NTP * DNA + h * T7 * DNA + \ldots$$
$$+ m * NTP * T7 * DNA + \ldots$$

mRNA titre and purity data generated were analyzed further via statistical analysis/modeling in JMP. A fit model of standard least squares for effect screening was used to analyze the mRNA yield and purity data of the DoE. Least squares is a form of mathematical regression analysis used to determine the line of best fit for a set of data, demonstrating the relationship between the data points.[6]

Table 4 lists cross terms from the model carrying the highest impact in the fitted model in predicting the outcome. Factors with p-values less than 0.05 had a statistically significant impact. Logworth values show that the highest impact came from the interactions of T7 RNA polymerase, template, and the use of modified nucleotides.

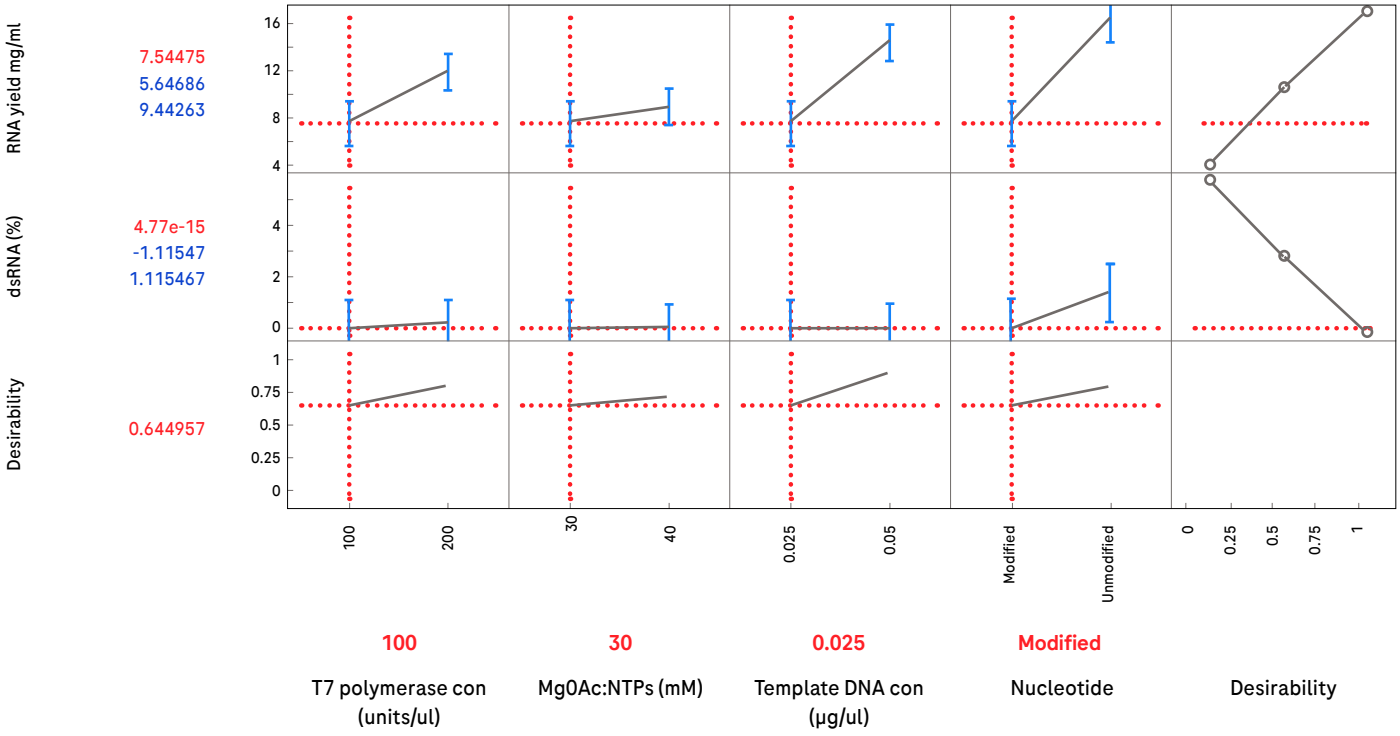
Source	Logworth	p-value
T7 polymerase conc. (U/µL)*template DNA conc. (µg/µL)*NTPs	**7.218**	**0**
Template DNA conc. (µg/µL)*NTPs	**6.87**	**0**
NTPs	6.089	0
Magnesium:NTPs (mM)*template DNA conc. (µg/µL)*NTPs	**5.784**	**0**
T7 polymerase conc. (U/µL)*magnesium:NTPs (mM)*template DNA conc. (µg/µL)*NTPs	**5.613**	**0**
Magnesium:NTPs (mM)(30 mM, 40 mM)	3.973	0.00011
T7 polymerase conc. (U/µL)*magnesium:NTPs (mM)	**3.679**	**0.00021**
T7 polymerase conc. (U/µL)*NTPs	**2.714**	**0.00193**
Magnesium:NTPs (mM)*NTPs	**2.608**	**0.00246**
T7 polymerase conc. (U/µL)*magnesium:NTPs (mM)*NTPs	**2.533**	**0.00293**
Magnesium:NTPs (mM)*template DNA conc. (µg/µL)	**1.857**	**0.01389**
T7 polymerase conc. (U/µL)*magnesium:NTPs (mM)*template DNA conc. (µg/µL)	**1.759**	**0.0174**
T7 polymerase conc (U/µL)(2U/µL,4U/µL)	1.502	0.03148

T04

Summary effects on IVT based on a least squares fit analysis of reaction components and their interactions. Unbold text are single-input impacts whereas multi-input interactions are in bold. NTPs here represent modified or unmodified UTP. (Roche data on file DOE 2023)

Prediction of process performance based on the yield and dsRNA data from the DOE

Different inputs, as shown in Figure 7, and their interactions, as shown in Figure 6, affect the final output of the IVT reaction in terms of yield and purity. DoE followed by statistical analysis in the JMP software identified a set of starting input conditions that will generate high mRNA yield and lower amounts of process impurities like dsRNA. Those conditions are 100U/µL T7 RNA polymerase, 30 mM magnesium:NTPs, 0.025µg/µL template DNA, and the use of modified NTPs.

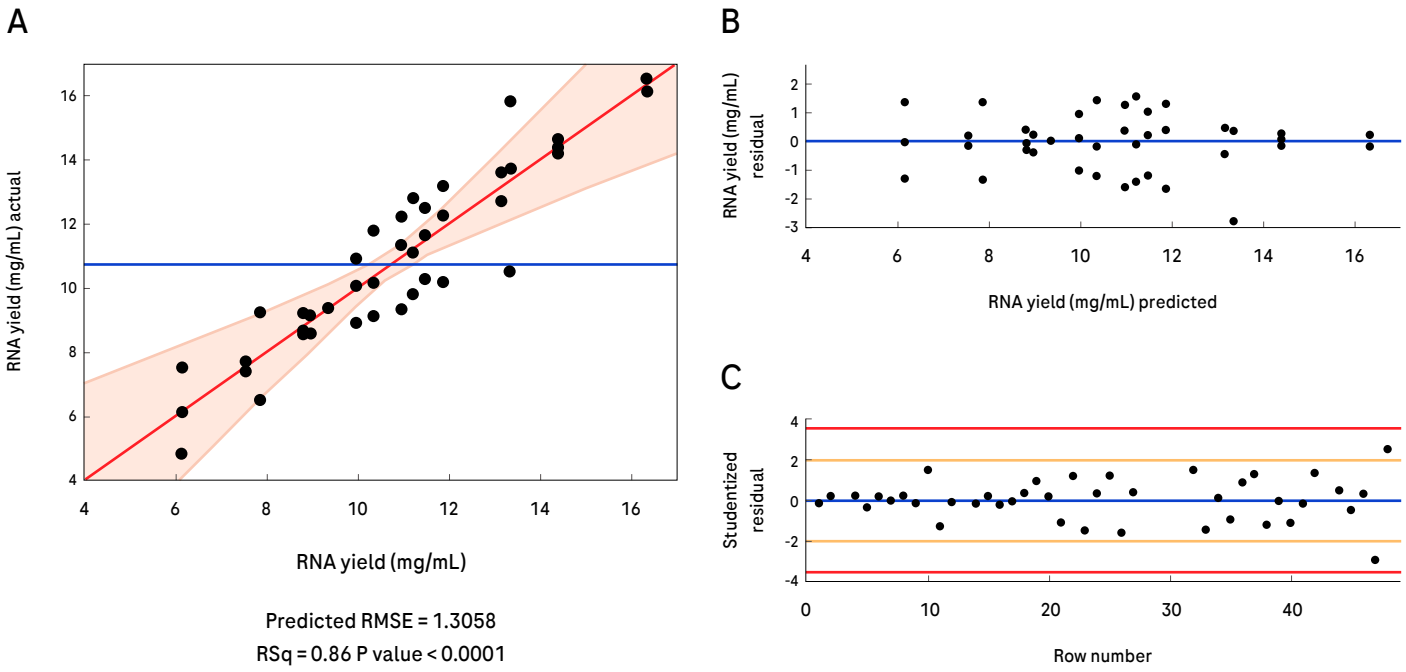


07

Prediction profiler of the least squares fit model. Predictions for the response variable are shown on the y-axis. The model factors are shown on the x-axis. These model factors were determined based on results from the DoE and used to determine how the concentration of T7 RNA polymerase, Mg:NTPs, and DNA template, as well as the use of a modified nucleotide, impact the mRNA yield and purity of the IVT reaction. The optimal inputs predicted by the DoE are indicated in red. (Roche data on file DOE 2023)

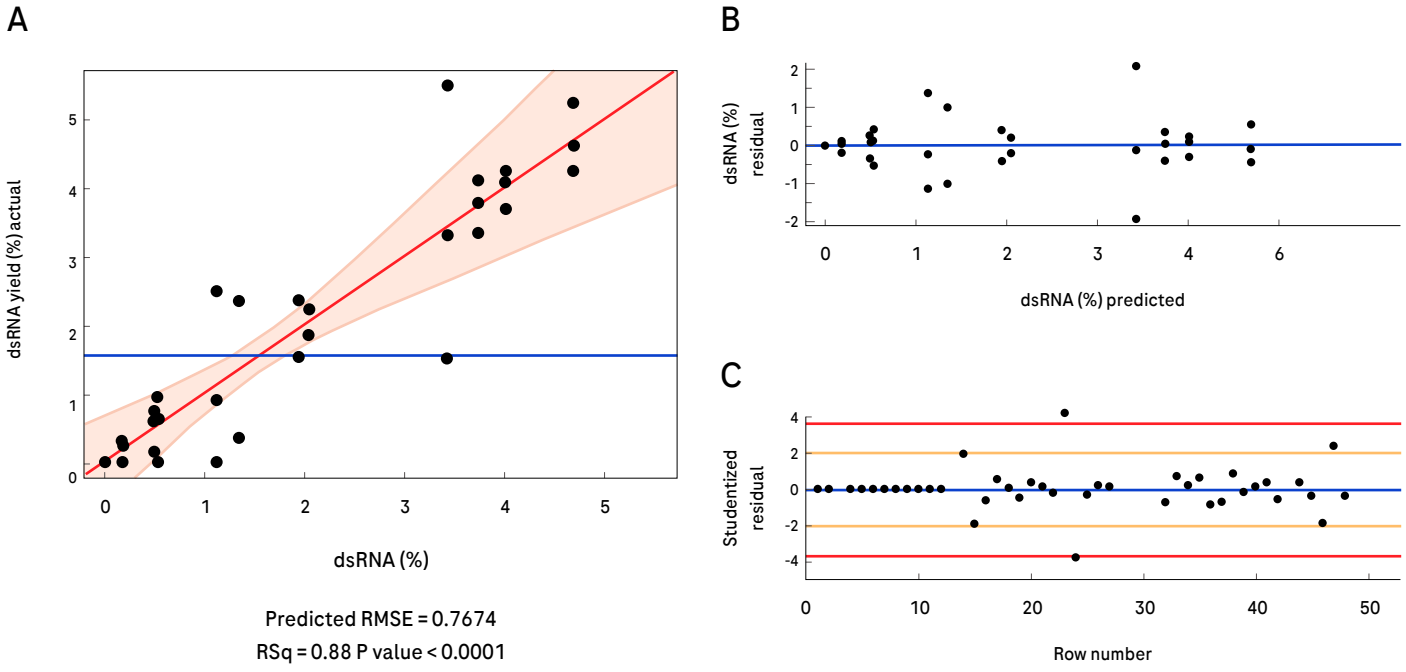
Supplementary information

The Least Fit squares model fit for yield and levels of dsRNA is shown in Figures 8 and 9, respectively. In an ideal model, all points would fall on a line in these plots. In this case, the model is not perfect due to the limited sample size of this study, yet it is still a useful prediction tool. A larger study with an expanded sample size would improve the model fit.



08

Plot summarizing the LeastFit squares statistical model fit in JMP to the RNA yield dataset (A). Plot of actual mRNA yield versus predicted yield (B). Plot of residuals (observed minus predicted mRNA yield) versus predicted yield (C). Externally studentized residuals with 95% simultaneous limits (Bonferroni) in red and individual limits in green. (Roche data on file DOE 2023)



09

Plot summarizing the LeastFit squares statistical model fit in JMP for the dsRNA titre dataset (A). Plot of actual dsRNA content as percent of mRNA yield versus predicted content (B). Plot of residuals (observed minus predicted dsRNA content) versus predicted content (C). Externally studentized residuals with 95% simultaneous limits (Bonferroni) in red and individual limits in green. (Roche data on file DOE 2023)

Key takeaways

- Compared to unmodified nucleotide, the use of N1-methyl-pseudo-UTP in the IVT reaction significantly reduced dsRNA content without reducing mRNA yield.
- The statistical analysis highlighted that interactions between the different inputs play a critical role in the IVT reaction and influence mRNA yield and purity.
- The best input conditions for a specific therapeutic need can be identified from a possible set of optimal conditions by carefully considering output targets (yield, purity), costs, and necessary trade-offs.
- Inputs identified for IVT reactions that generate high mRNA yield and low dsRNA content were 0.025 µg template DNA, 100 U/µL T7 polymerase, 30 mM magnesium:nucleotide ratio, and use of the modified nucleotide N1-methyl-pseudo-UTP. These conditions serve as a generic initial set that should be adapted to specific therapeutic needs.

Experimental Setup

IVT reactions were set up in triplicates in a 96-well plate at a final volume of 100µL containing 40 mM Tris-HCl, 2 mM spermidine, 10 mM DTT, 0.002 U/µL of pyrophosphatase (Roche CustomBiotech Cat # 08140677103), 1 U/µL of RNase inhibitor (Roche CustomBiotech Cat # 09537643103) and 4 mM CleanCap AG3'Ome (TriLink Biotechnologies Cat # N-7413-100). Master mixes were created with different T7 RNA polymerase concentrations (Roche CustomBiotech Cat # 08140669103) and magnesium acetate:nucleotide ratios. All the nucleotides (ATP Cat # 04980824103, CTP Cat # 04980875103, GTP Cat # 04980859103, UTP Cat # 04979818103 N1-methyl-pseudo-UTP Cat # 09744762103) were sourced from Roche CustomBiotech. The template DNA and modified nucleotides were added individually to each well. The template DNA encoding GFP (provided by CPI) was designed to be co-transcriptionally capped with CleanCap reagent and had a T7 promoter with modified start site. The IVT reactions were incubated for 2 h at 37°C and were subsequently treated with DNase I (Roche CustomBiotech Cat # 09873562001) at a concentration of 0.4 U/µL of the reaction to remove the template DNA prior to purification.

After the IVT reaction and DNase I treatment, each sample was transferred to a 1.5 mL Eppendorf tube and the mRNA was purified by lithium chloride precipitation. Samples were finally re-suspended in 95 µL of nuclease-free water.

RNA quantification and integrity analysis
mRNA yield was measured using a UV/Vis spectrophotometer at 260 nm (NanoDropTM) and integrity was analyzed by agarose gel electrophoresis (1% gel) as well as on a LabChip GX Touch Nucleic Acid Analyzer (PerkinElmer) with the corresponding kit for the DNA 5K / RNA / Charge Variant Assay (Perkin Elmer Cat # 760435). Capillary electrophoresis was done using a SCIEX PA800 system and kit for RNA 9000 purity and integrity (SCIEX Cat # C48231) using a pre-assembled cartridge (30.2 cm; SCIEX Cat # A55625) and an RNA Ladder (Lonza Cat # 50575).

Dot Blot setup for dsRNA detection
The J2 clone of the anti-dsRNA antibody from Exalpha (Cat # 10613002) was used to measure dsRNA content by dot blot. The protocol referenced by the vendor was followed. Reference material to generate the standard curve was purchased from Jena Bioscience (Cat # RNT-SCI-10080100; 142 bp dsRNA). Concentrations for the standard curve ranged from 32 to 0.5 µg/mL. The secondary antibody used for detection was an anti-mouse IgG (whole molecule)-peroxidase produced in rabbit (Merck Cat # A9044). The blots were imaged using the Bio-Rad gel scanner instrument and blots were quantified using the Bio-Rad ImageLab software.

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Acknowledgements
This study was performed at CPI Innovation Services Limited (CPI), United Kingdom for CustomBiotech, Roche Diagnostics GmbH. We thank the members of the CPI team who contributed to the generation and interpretation of the data.

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