Scaling of High-Yield *In vitro* Transcription Reactions for Linear Increase of RNA Production

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INTRODUCTION

Synthesis of RNA, for various downstream workflows, has recently been in the spotlight due to the emergence of mRNA-based vaccines following the global COVID-19 pandemic. Sufficient amounts of RNA needed for screening studies and early-stage clinical trials cannot be produced from traditional small-scale reactions performed in the laboratory. Larger scale reactions, with RNA yields linearly scaling with reaction volume, are necessary to produce the substantial amounts of RNA required to meet the demands of these studies.

As the use of RNA as a therapeutic becomes more widespread, challenges arise for scientists to produce high-quality RNA at the significant scale required to meet the demands of vaccine production, early-stage characterization and other investigations. Cost-effectiveness, as well as minimal optimization and troubleshooting, are key considerations when scaling *in vitro* transcription (IVT) workflows. RNA yields should scale linearly as input and reaction volumes increase, an objective that is not always straightforward. Larger volume reaction vessels require optimized protocols, and precautions should be taken to ensure proper mixing and consistent temperature throughout the reaction vessel in order to maintain the abundant yields and quality of RNA that is expected.

These challenges compound as volumes of reactions increase; therefore we have tested the scalability of (IVT) reactions utilizing New England Biolabs (NEB) enzymes and kits to provide a generalized set of recommendations for synthesizing high yields of RNA, of various lengths and sequence compositions, from bench to commercial scale.

This technical note includes a set of recommendations to facilitate the scaling of workflows utilizing both the HiScribe® line of T7 and SP6 RNA Polymerase-based *in vitro* transcription kits, as well as individual enzymes (T7 RNA Polymerase, Inorganic Pyrophosphatase (*E. coli*) and RNase Inhibitor, Murine), which are available from NEB as research use only (RUO) and GMP-grade* products.

IVT Template Strategies

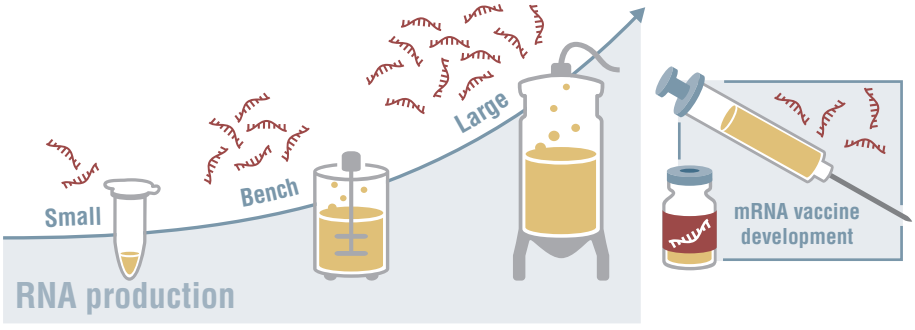
Successful *in vitro* transcription reactions are achieved by starting with high-quality input materials. First, a source of linear, double-stranded DNA (dsDNA) template that includes the T7 promoter (or other promoter sequence if using an alternative phage DNA-dependent RNA Polymerase) upstream of the sequence of interest is required. T7 RNA Polymerase recognizes and binds to the T7 promoter, transcribing in the 5′ → 3′ direction and falling off the end of the linear template, releasing the run-off transcript.

To generate dsDNA templates for IVT, two common strategies that are frequently employed are linearization of a plasmid downstream of the sequence of interest using restriction enzymes (leaving a blunt end or 5′ end overhang) or PCR amplification of a sequence of interest by utilizing high-fidelity DNA polymerases. Introduction of promoter sequences on a forward primer can be achieved through PCR, creating a complete dsDNA template. It is important that these templates are free from contaminants that can be introduced through purification steps and subsequently may inhibit the IVT process. If in-house means of generating the large amount of dsDNA required when scaling RNA synthesis is beyond capacity, outsourcing this step can be an option. However, extra planning may be required to keep projects on task and secure a supply as the time to go from plasmid expression to purified linearized DNA at scale can be a lengthy process that may be in high demand and may subsequently impact timelines.

RNA Synthesis Formats

With template design in place, *in vitro* transcription format selection is the next step in the process. NEB offers several options for RNA synthesis, including the highly optimized, high yield HiScribe IVT transcription kits, as well as standalone enzymes and reagents, which includes RNA polymerases, nucleoside triphosphates (NTPs), inorganic pyrophosphatases, RNase inhibitors and reaction buffers (Table 1, page 2).

In the following set of recommendations, features of both strategies will be highlighted. RNA synthesis in the moderate range, (up to 20 ml), can be performed with easily accessible equipment and consumables, while larger scale reactions may require more sophisticated equipment that is designed to maintain consistent temperatures throughout the vessel as well as a means for gently mixing the reactions throughout the incubation.



* "GMP-grade" is a branding term NEB uses to describe products manufactured or finished at NEB's Rowley facility. The Rowley facility was designed to manufacture products under more rigorous infrastructure and process controls to achieve more stringent product specifications and customer requirements. Products manufactured at NEB's Rowley facility are manufactured in compliance with ISO 9001 and ISO 13485 quality management system standards. However, at this time, NEB does not manufacture or sell products known as Active Pharmaceutical Ingredients (APIs), nor does NEB manufacture its products in compliance with all of the Current Good Manufacturing Practice regulations.

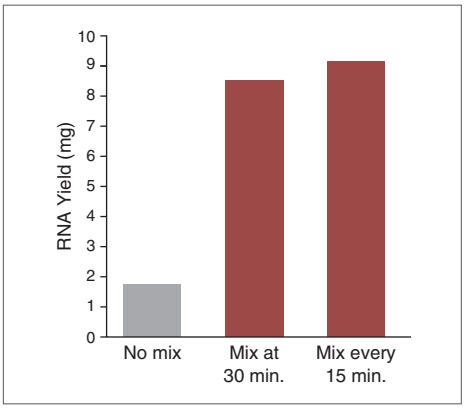
TABLE 1: **Available NEB reagents for mRNA synthesis workflows**

TEMPLATE GENERATION	*IN VITRO* TRANSCRIPTION	RNA CAPPING	POLY(A) TAILING
AMPLIFICATION:	**HISCRIBE® RNA SYNTHESIS KITS:**		
• Q5 High-Fidelity DNA Polymerase (NEB #M0491S/L) • Phi29 DNA Polymerase (NEB #M0269S/L) GMP • Phi29-XT RCA Kit (NEB #E1603S/L) • dNTPs (NEB #N0447S/L)	HiScribe® T7 ARCA mRNA Synthesis Kit (with tailing) (NEB #E2060S)		
	HiScribe® T7 ARCA mRNA Synthesis Kit (NEB #E2065S) HiScribe® T7 mRNA Kit with CleanCap® Reagent AG (NEB #E2080S)		
	HiScribe® T7 Quick High Yield RNA Synthesis Kit (NEB #E2050S) **HiScribe® T7 High Yield RNA Synthesis Kit (GMP components) (NEB #E2040S)** GMP HiScribe® SP6 High Yield RNA Synthesis Kit (NEB #E2070S)		
PLASMID LINEARIZATION:	**INDIVIDUAL ENZYMES AND REAGENTS:**		
• BspQI (NEB #R0712S/L) GMP • BsaI-HF®v2 (NEB #R3733S/L) GMP	SP6, T3, and Hi-T7® RNA Polymerases (NEB #M0207S), (NEB #M0378S), (NEB #M0658S), (NEB #M0470T)	Vaccinia Capping System (NEB #M2080S) GMP	*E. coli* Poly(A) Polymerase (NEB #M0276S/L)
DNA ASSEMBLY:	**T7 RNA Polymerase (NEB #M0251S/L), (NEB #M0460T)** GMP	Faustovirus Capping System (NEB #M2081S/L) GMP COMING SOON	
	RNase Inhibitor (Murine) (NEB #M0314S/L) GMP	mRNA Cap 2´-O-Methyltransferase (NEB #M0366S) GMP	
• NEBuilder HiFi DNA Assembly (NEB #E2621S/L/X) • NEBridge® Golden Gate Assembly Kit (BsaI-HF® v2) (NEB #E1601S/L/X) • NEBridge® Golden Gate Assembly Kit (BsmBI-v2) (NEB #E1602S/L/X)	RNase Inhibitor (Human Placental) (NEB #M0307S/L)	ARCA and other mRNA cap analogs (NEB #S1411S/L)	
	Pyrophosphatase, Inorganic (*E. coli*) (NEB #M0361S/L) GMP Pyrophosphatase, Inorganic (yeast) (NEB #M2403S/L) **NTPs (NEB #N0450S) (NEB #N0466S)** GMP DNase I (RNase-free) (NEB #M0303S/L) GMP DNase I-XT (NEB #M0570S/L)		

NEB offers reagents for template generation, *in vitro* transcription, RNA capping, and poly(A) tailing. Products listed are provided research use only grade and select reagents and kit components are also available GMP-grade, as indicated. Reagents highlighted in bold were utilized in this study.

GMP = available in GMP-grade

FIGURE 1: **Gentle, intermittent mixing during IVT incubation maximizes yields.**



HiScribe reactions (1 ml, 5 kb transcript, expected yield = 9 mg) were incubated for 1 hour at 37C in a dry air incubator and were either not mixed, mixed after 30 minutes, or mixed every 15 minutes by gentle inversion. Care was taken to ensure no bubbles were introduced during mixing.

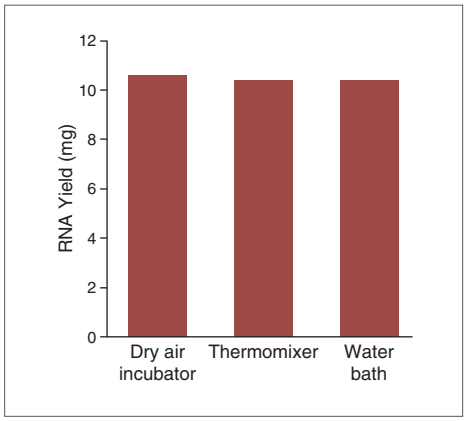
REACTION SETUP

When setting up IVT reactions, there are a few key considerations at the onset to ensure the highest yield possible. A trusted source of nuclease-free water is important — DEPC-treated water is not recommended as it is not necessary and the presence of any byproducts that remain may inhibit downstream enzymatic reactions. However, we do recommend taking precautions such as wearing gloves, using filtered tips, and working in a dedicated RNase-free area to reduce the chances of RNase contamination. When gathering the necessary enzymes and reagents, it is important to thaw the buffers, NTPs and templates to room temperature while keeping the enzyme(s) on ice. Next, thorough mixing of the buffer and NTPs is important as components tend to settle to the bottom of the tubes after thawing. The order of addition for the IVT reaction is also important and should follow the order listed in the manual, with enzyme added last. Gentle mixing of the reaction, with care taken to avoid bubble formation, is key to successful synthesis. Gentle mixing during incubation, two to three times, should be performed to achieve the highest yield possible (Figure 1). Gentle inversion of the tube five to six times is sufficient. The IVT reactions performed in this paper were incubated in Eppendorf Protein LoBind® tubes (1.5 ml snap cap (#022431081), 5 ml screw cap (#0030122356), 15 ml screw cap (#0030122216) or 50 ml screw cap (#0030122240) and incubated in a 37°C dry air incubator. Alternatively, incubation in a water bath or thermomixer can also be considered, as comparable yields were achieved (Figure 2). Whichever method is selected, it is imperative that the temperature remains consistent, and the reactions are gently mixed during incubation.

Pilot IVT Reaction Length, Template Amount and Template Purity

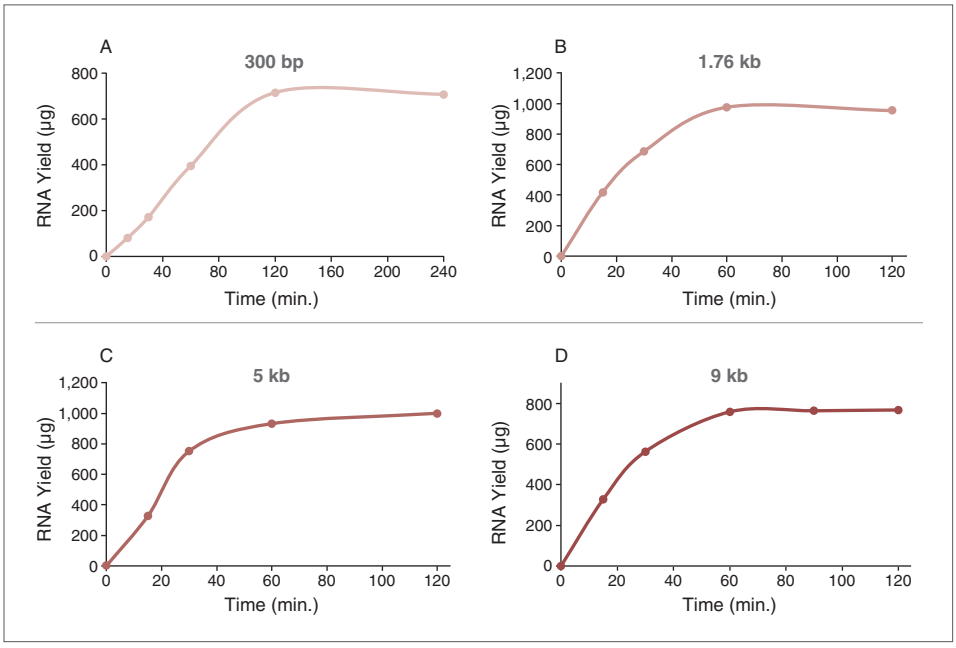
When preparing to scale IVT reactions, we would recommend testing conditions (e.g., reaction length, template amount, template purity) at a small-scale (20 – 100 µl) before scaling up to ensure conditions are optimized to achieve the highest yields. In general, transcripts that are shorter require a longer reaction incubation to allow for the highest yield (weight of RNA), as more RNA polymerase initiation events need to occur to reach the same weight of RNA compared to a longer RNA. A time course is an excellent way to determine the reaction length where maximum amount of RNA is synthesized, which may be different depending on transcript length (Figure 3). Selection of a reaction length that achieves high yield will also limit exposure of the RNA to conditions that might lead to degradation during extended incubations, especially with longer RNAs that may be more sensitive to pH changes and the presence of metals over time. The limited reaction times may also decrease the emergence of unwanted byproduct formation that can accumulate over time.

FIGURE 2: **Various incubation methods result in comparable RNA Yields for small-scale *in vitro* transcription**



HiScribe reactions were scaled to 1 ml in protein LoBind tubes using the CLuc control template and incubated for 1 hour at 37°C in a dry air incubator, a thermomixer, or a water bath. RNA yields were determined by Qubit™ (expected yield was 10 mg).

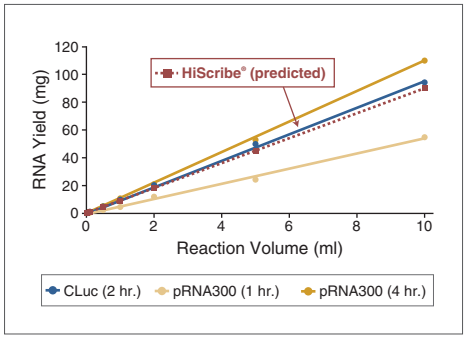
FIGURE 3: **Shorter RNAs require longer incubation times to reach maximum RNA yields.**



HiScribe reactions were scaled to 100 µl with 5 µg double-strand DNA input. RNA lengths are A (300 bp), B (1.76 kb), C (5 kb) and D (9 kb). RNA A requires a 2-hour incubation to reach maximum yields, whereas longer RNAs require approximately 1 hour.

Reaction length may need to be determined for each RNA to be synthesized, but once this is established at the small-scale, high yields can be achieved at larger volumes when all reaction components are scaled linearly (Figure 4). Comparison of incubation time for a 300 nucleotide (nt) RNA demonstrates that optimal reaction length determined in a standard small-scale reaction translates to large scale and yields can be achieved that are comparable to the scaled calculated prediction of the control template provided in the HiScribe T7 High Yield RNA Synthesis kit (NEB# E2040).

FIGURE 4: **Shorter RNAs require longer incubation times to reach maximum RNA yields in scaled IVT reactions**

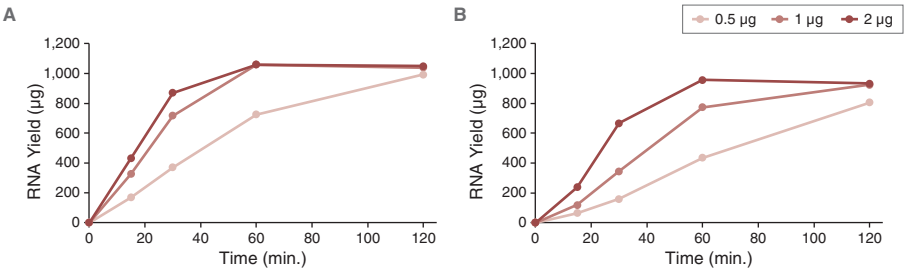


HiScribe reactions scaled linearly from 20 µl to 10 ml. Reactions were incubated in a dry air incubator in protein LoBind tubes. CLuc is 1.76 kb and pRNA300 is 300 bp. Cluc reactions were incubated for 2 hours (blue), pRNA300 was incubated for 1 (yellow) or 4 (gold) hours. Yields are compared to scaled predicted yields of NEB# E2040 (red, dotted), 180 µg from a 20 µl reaction using dsDNA CLuc control template.

An additional factor to keep in mind is the amount of input template required to reach the highest yield possible for a reaction length that limits accumulation of byproducts. This will need to be established not only for the length of the transcript, but by the type of template. As recommendations for input template are by weight (µg), comparing PCR-generated templates vs. linearized plasmids, even for transcripts of the same final length, is necessary. For long RNAs, the associated input template is also long and therefore fewer moles of that template (and promoter) exist compared to a shorter RNA or shorter PCR template that does not contain the extra plasmid sequence required for plasmid propagation. A simple template titration in a small-scale reaction will aid in the determination of the best amount of template to reach highest yields in the reaction time selected (Figure 5).

As mentioned above, template amount and reaction length are vital to guarantee that the highest yields can be reached. It is also of great importance that the dsDNA template is pure and free from any carryover salts, proteins or impurities that may interfere with transcription and result in low yields. Finally, if the plasmid is not completely linearized, transcripts of the wrong size may be generated as the polymerase will not have a defined end to fall off of for run-off transcription. Performing small-scale IVTs will identify any issue with the template before scaling up which will save time and reagents.

FIGURE 5: **Longer RNAs require more dsDNA template input, based on weight, to achieve comparable RNA yields compared to shorter transcripts.**



In vitro transcription reactions (HiScribe, 100 µl reactions) with dsDNA template titrations (2 µg, 1 µg, 0.5 µg) were incubated for 2 hours at 37°C with either CLuc (1.76 kb) or a 5 kb template. A. CLuc (1.76 kb) reached maximum RNA yields by 1 hour with either 1 or 2 µg dsDNA template and maximum yield by 120 minutes. B. 5 kb template reaction reached maximum yield by 1 hour (2 µg template) or 2 hours (1 µg template) but only reached ~80% maximum yield by 2 hours with 0.5 µg template input.

Optimization with HiScribe Kits and Standalone Enzymes

The NEB HiScribe *in vitro* transcription kits are extensively optimized to work with a broad range of RNA lengths, sequences and inputs, resulting in reproducible yields of RNA. Standalone enzymes are also available (RNA polymerases, inorganic pyrophosphatases, NTPs and RNase inhibitors) for IVT workflows when it is desired to optimize reaction conditions and components for individual sequences. Kit components and individual enzymes are offered at RUO and GMP- grade as indicated in Table 1, page 2.

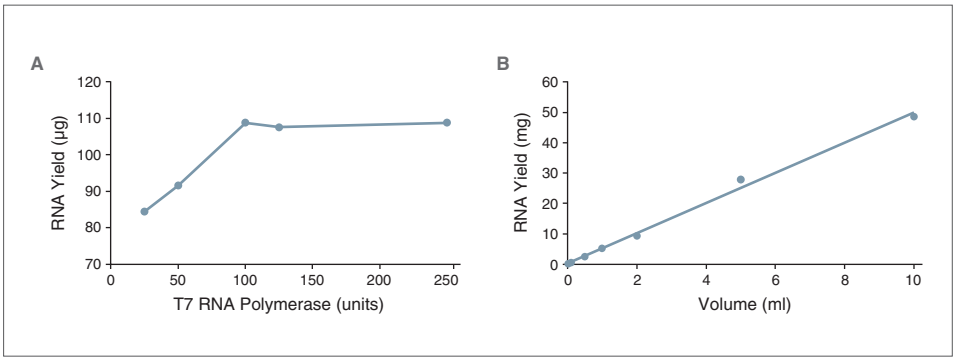
T7 RNA Polymerase is the most widely used RNA Polymerase for IVT workflows. NEB offers T7 RNA Polymerase at 50,000 U/ml (RUO and GMP-grade), 250,000 U/ml (GMP-grade) and 1,000,000 U/ml (RUO-grade). In order to determine the appropriate units of T7 RNA Polymerase needed for transcription with a certain concentration of NTPs, an enzyme titration should be performed. In Figure 6 (page 5), we show titration of T7 RNA Polymerase with 5 mM each NTP in a small-scale reaction.

For 5 mM NTPs, 100 units of T7 RNA Polymerase (5 U/ µl final in the reaction) is suitable for transcription of the control template (Figure 6A). In addition to enzyme titration, the sufficient amount of magnesium chloride ($MgCl_2$) should also be determined, being careful to avoid concentrations of $MgCl_2$ that are too high and may induce metal-dependent degradation of RNA at elevated temperatures over time.

A similar titration of Inorganic Pyrophosphatase (*E. coli*) was performed at small-scale, and 0.1 units (0.005 U/µl final) with 20 units of RNase Inhibitor (1 U/µl final) was shown to produce vast quantities of RNA and is scalable (Figure 6B). Keep in mind that the enzyme concentrations may need to be titrated based on sequence and RNA length, while keeping final glycerol concentration low, but these general guidelines provide an excellent foundation. Using high concentration enzymes, when possible, can help reduce final glycerol concentration. Once these parameters are in place, linear scaling of the reaction can be performed to synthesize the amount of RNA required for individual workflows.

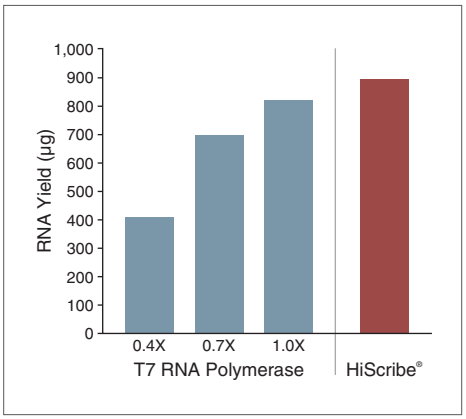
Higher yield and more convenient reaction setup can be achieved using our HiScribe kits. While these kits are available through our catalog, we can also provide bulk and GMP-grade reaction components for large-scale therapeutic manufacturing. However, as mentioned above, high-yield reactions using higher concentration enzymes to ensure low final glycerol concentration are possible. Here, yields of standard HiScribe T7 High Yield RNA Synthesis Transcription Kit (NEB #E2040) reactions were compared to IVT reactions utilizing standalone T7 RNA Polymerase titrated in the presence of Inorganic Pyrophosphatase (*E. coli*) and RNase Inhibitor (Murine). All reactions contained 10 mM each NTP; HiScribe IVT reactions had slightly higher yields (Figure 7) even though T7 RNA Polymerase was present at the same concentration (1X).

FIGURE 6: **T7 RNA Polymerase titration indicates enzyme amount required to reach maximum yields and demonstrates scalability.**



A. *In vitro* transcription reactions (per 20 µl reaction: 1X RNA Pol Buffer, 5 mM each NTP, 20 mM $MgCl_2$ (total), 0.1 units Inorganic Pyrophosphatase (*E. coli*) at 37°C for 2 hours) were performed with increasing units of T7 RNA Polymerase to determine the amount of enzyme needed to reach maximum yields. A minimum of 100 units of T7 RNA Polymerase is required to transcribe ~100 µg of CLuc RNA.
B. Reaction conditions scaled linearly from 20 µl to 10 ml results in linear RNA yields.

FIGURE 7: **Comparison of RNA yields from HiScribe *in vitro* transcription kit and individual enzymes.**



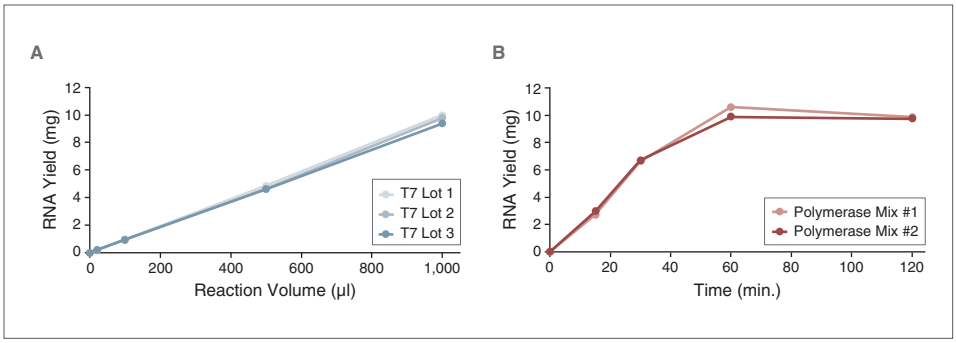
Reactions (100 µl, 10 mM each NTP) were set up following a linearly scaled HiScribe format (expected yield = 900 µg) and compared to reactions with individual enzymes (T7 RNA Polymerase, Inorganic Pyrophosphatase, and Murine RNase Inhibitor). Reactions with individual enzymes were set up with comparable concentration of T7 RNA Polymerase concentration in HiScribe format (1X), or a titration of T7 RNA Polymerase (0.7X and 0.4X) to determine the concentration of T7 RNA Polymerase required for maximum yield.

While the performance of both HiScribe kits and standalone enzymes result in high yields of RNA, it is important to know if these yields are consistent among different lots of the same enzymes. To determine this, three separate lots of T7 RNA Polymerase (50,000 U/ml) were used in IVT reactions scaling 50-fold under identical reaction conditions and linear yields were nearly identical (Figure 8A). In addition, time course experiments were carried out with multiple lots of the HiScribe T7 High Yield RNA Synthesis Transcription Kit (NEB #E2040), and the rates of the reactions were nearly identical (Figure 8B).

Finally, taking all the knowledge gained on input template amount, optimization and incubation time, reactions were scaled 1000-fold up to 20 ml following HiScribe protocols (Figure 9A) or workflows described above utilizing standalone enzymes (Figures 9B and C) and linearized dsDNA to transcribe longer RNAs (5 kb and 9 kb). When all components were scaled linearly, including templates, yields were as expected (Figure 9A) when projecting predicted small-scale yields 1000-fold with reaction volume increase.

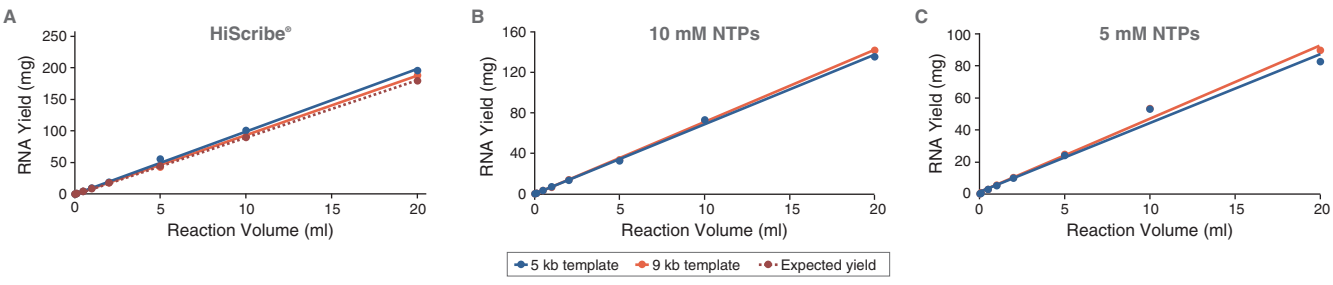
Together, these experiments demonstrate that IVT synthesis using NEB's HiScribe kits or standalone enzymes offer scalable, robust and consistent RNA synthesis, Further, this data was consistent across many manufacturing lots.

FIGURE 8: **Consistent RNA yields are obtained when using multiple lots of NEB enzymes**



Multiple lots of enzymes (T7 RNA Polymerase or HiScribe T7 RNA Polymerase Mix) were tested in side-by-side high yield IVT reactions (all other components held constant). A. High Yield linearly scaled IVT reactions (20 µl to 1 ml) with CLuc dsDNA template and 10 mM NTPs comparing three lots of T7 RNA Polymerase, RNA yield determined by Qubit™. B. HiScribe reactions (Cluc, 1 ml) comparing two lots of T7 RNA Polymerase Mix show nearly identical reaction rates and RNA yields as determined by Qubit.

FIGURE 9: **IVT reactions can be scaled linearly when using HiScribe kits or individual reactions.**



IVT reactions (5 and 9 kb transcripts) were scaled linearly from 20 µl to 20 ml with (A) HiScribe components (10 mM each NTP), (B) high yield and individual enzymes with 10 mM each NTP, and (C) moderate yield and individual enzymes with 5 mm each NTP. $R^2 > 0.99$ for all reactions.

CONCLUSION

Here we have presented a set of recommendations for scaling of *in vitro* RNA synthesis reactions for production of RNA of various sizes using both HiScribe *in vitro* transcription kits and individual enzymes (T7 RNA Polymerase, Inorganic Pyrophosphatase (*E. coli*) and RNase Inhibitor, Murine), all of which are manufactured by NEB both as research use only (RUO-grade) and GMP-grade products. The experiments described in this paper provide a foundation for scaling IVT reactions to synthesize RNAs of various lengths, sequences and structures using equipment and consumables that are readily accessible.

Scaling to even higher reaction volumes will need further planning and specialized equipment, such as bioreactors. Additionally, maintaining a consistent temperature and gentle mixing in the reaction vessel will be key to achieving optimal RNA yields in larger volume reactions. Pre-warming of reaction components was tested at the reaction scales mentioned in this paper and it did not change efficiency or yields, but it will likely be an important factor to consider when scaling up.

Finally, to increase RNA stability and generate fully functional mRNAs capable of recognition by the cellular translational machinery, additional steps will need to be taken to incorporate a 5´ cap structure and poly(A) tail. Several strategies exist for poly(A) tailing, either post-transcriptionally through addition by Poly(A) Polymerase or encoded in the dsDNA template. Additionally, numerous methods of adding a 5´ cap to the RNA can be considered, including co-transcriptional capping (ARCA, CleanCap® Reagent AG) or post-transcriptional capping using Faustovirus Capping Enzyme (FCE) or Vaccinia Capping Enzyme (VCE) and Cap 2´-O-Methyl Transferase. In addition, modifications to the methods described above also include incorporation of modified nucleotides to decrease immune response.

Subsequent downstream processing, including template removal (DNase I or DNase I-XT) can also be incorporated at scale. And finally, RNA purification to remove proteins, reaction byproducts and dsRNA that may trigger an immune response should be kept in mind and methods exist to column-purify single-stranded RNAs of the correct length.

The emergence of RNA as a therapeutic necessitates the need for larger scale production and synthesis workflows. We hope that the recommendations presented in this study can provide a framework for IVT scale up for customers interested in generating large quantities of high quality RNAs using consistent and robust reagents. NEB fully supports mRNA synthesis with a suite of tools to take you from template to transcript.