Synthesizing an RNA control for RT-LAMP using GeneArt Strings DNA Fragments

Introduction

Synthetic RNAs are used for a variety of research applications, such as *in vitro* translation, RNase protection assays, isolation of RNA-binding proteins, microarray studies, northern blotting, and *in situ* hybridization. Custom synthetic RNAs can also be used as positive controls for reverse transcription quantitative PCR (RT-qPCR) and reverse transcription loop-mediated isothermal amplification (RT-LAMP). To generate custom synthetic RNA, a typical workflow includes cloning of a double-stranded DNA insert into an expression plasmid downstream of a T7 RNA polymerase promoter. Subsequently, the plasmid is linearized by restriction enzyme digestion downstream of the insert to be transcribed, purified, and used as a template for *in vitro* transcription (IVT). The greatest transcription yield is typically achieved with the highest-purity plasmid templates. The full workflow including cloning, linearization, purification, and IVT steps can take up to several days.

This application note optimizes generation of synthetic RNA by utilizing custom double-stranded Invitrogen™ GeneArt™ Strings™ DNA Fragments designed to contain the T7 RNA polymerase promoter sequence upstream of the insert to be transcribed. These fragments were used directly for the IVT reaction without the need for cloning into an expression vector. Transcribed RNA was used as a positive control in RT-LAMP reactions alongside clinical purified RNA using Invitrogen™ SuperScript™ IV RT-LAMP Master Mix (2X) and demonstrated equal performance to commercially available transcripts. GeneArt Strings DNA Fragments are supplied highly pure, which helps ensure high transcriptional yields. This approach significantly simplified the workflow and generated high-quality, high-yield synthetic RNA fragments within a few hours, instead of days.

Important notes

To minimize the risk of environment-borne contamination, thoroughly clean the laboratory workspace and all equipment using the following reagents in the provided order:

1. Invitrogen™ DNA*Zap*™ PCR DNA Degradation Solutions (Cat. No. AM9890)
2. Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water (Cat. No. 10977023 and Cat. No. 10977049)
3. 70% ethanol solution
4. Invitrogen™ RNase*Zap*™ RNase Decontamination Solution (Cat. No. AM9780)
5. 70% ethanol solution

Prior to handling RNA samples, spray RNase*Zap* RNase Decontamination Solution on gloved hands, and wipe down instruments, pipettors, and other surfaces using Invitrogen™ RNase*Zap*™ RNase Decontamination Wipes (Cat. No. AM9786) to avoid liquid contact with sensitive parts (e.g., electronic control modules).

Materials

- Thermo Scientific™ TranscriptAid™ T7 High Yield Transcription Kit (Cat. No. K0441)
- Invitrogen™ MEGAclear™ Transcription Clean-Up Kit (Cat. No. AM1908)
- Applied Biosystems™ *Power* SYBR™ Green RNA-to-C_T™ *1-Step* Kit (Cat. No. 4391178)
- 96% ethanol
- Custom GeneArt Strings DNA Fragments
- Invitrogen™ SuperScript™ IV RT-LAMP Master Mix (2X) (Cat. No. A51801)
- Invitrogen™ SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Cat. No. S34854)
- Primer sets (from Park et al. [1])
- Thermo Scientific™ Water, nuclease-free (Cat. No. R0581)

Methods

Design of GeneArt Strings DNA Fragments

Custom GeneArt Strings DNA Fragments were designed as shown in Figure 1. Briefly, three different SARS-CoV-2 genomic regions of varying lengths were selected for gene synthesis (Figure 1, green, purple, and orange). The T7 RNA polymerase promoter sequence was included upstream of the selected SARS-CoV-2 genomic sequence to be transcribed. Two restriction sites were included at the start (SacI) and end (KpnI) of the fragment, with additional bases on each end of the restriction site (to ensure maximum restriction enzyme efficiency), for utility in downstream cloning applications. Double-stranded, uncloned DNA fragments were resuspended in nuclease-free water, and 1 µg of each were used directly for IVT reactions.

IVT directly from GeneArt Strings DNA Fragments

Truncated SARS-CoV-2 RNA fragments were synthesized using the TranscriptAid T7 High Yield Transcription Kit according to the user manual. The reaction mix was set up at room temperature in the order listed in Table 1, and incubated at 37°C for 2 hr. A control reaction was set up in parallel using 2 µL of the control template DNA (0.5 µg/µL) provided with the kit.

Table 1. IVT reaction setup.

Component	Amount
DEPC-treated water	To 20 µL
5X TranscriptAid Reaction Buffer	4 µL
ATP/CTP/GTP/UTP mix*	8 µL
Linear template DNA	1 µg
TranscriptAid Enzyme Mix	2 µL
Total volume	**20 µL**

* Equal volumes of the four provided NTP solutions combined in one tube.

RNA transcripts were purified using the MEGAclear Transcription Clean-Up Kit according to the user manual. Each RNA sample was processed using a microcentrifuge and recovered with 100 µL of elution solution.

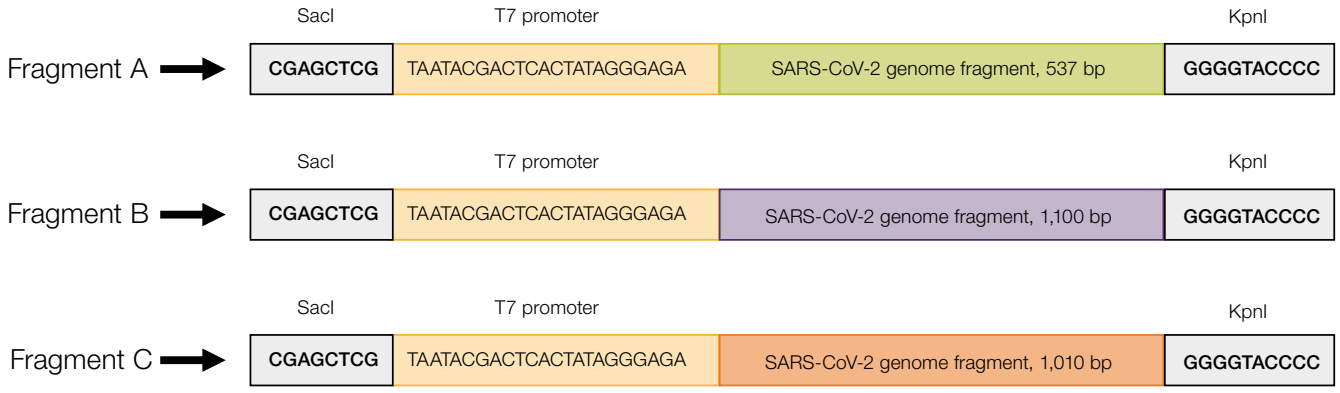


Figure 1. Design of GeneArt Strings DNA Fragments for direct use in IVT reactions to generate synthetic RNA. Selected SARS-CoV-2 genomic fragments (green, purple, and orange) were placed directly after the T7 RNA polymerase promoter sequence (yellow). Each construct has restriction sites on both ends that can be used for downstream cloning applications.

Results

Quality evaluation

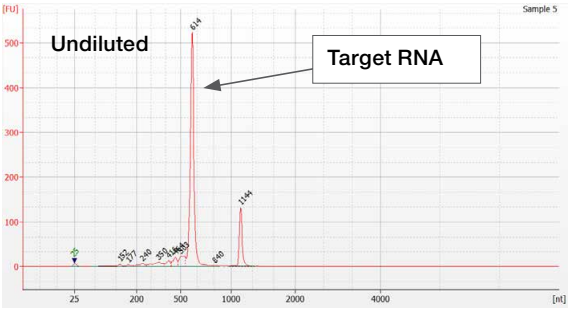
Concentrations of synthetic RNA generated from the IVT reaction were measured using the Thermo Scientific™ NanoDrop™ spectrophotometer, and results are shown in Table 2.

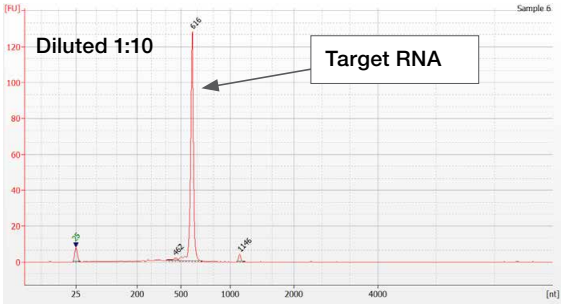
The integrity of RNA transcripts was verified using the Agilent™ RNA 6000 Nano Kit and Agilent™ 2100 Bioanalyzer™ system according to the manufacturer's user guides. Undiluted and 10-fold diluted samples were used for analysis. Sharp peaks of expected sizes were observed on the electropherograms confirming successful transcription of the full RNA fragment (Figure 2).

Table 2. Amount of synthetic RNA generated directly from GeneArt Strings DNA Fragments.

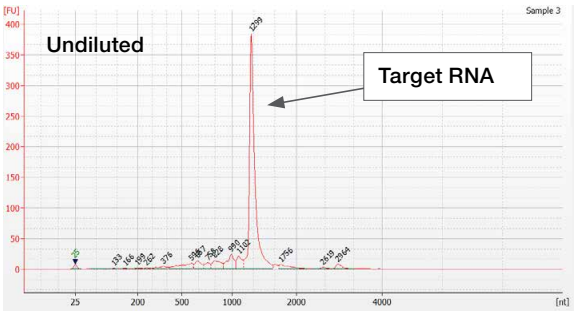
Fragment used	Concentration (µg/µL)	Total amount generated (µg)
A	1.72	172
B	2	200
C	1.7	170

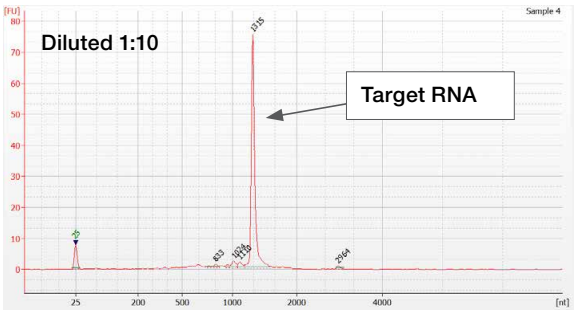
Synthetic RNA from fragment A



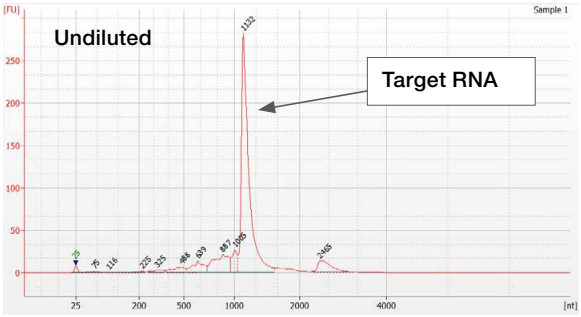


Synthetic RNA from fragment B





Synthetic RNA from fragment C



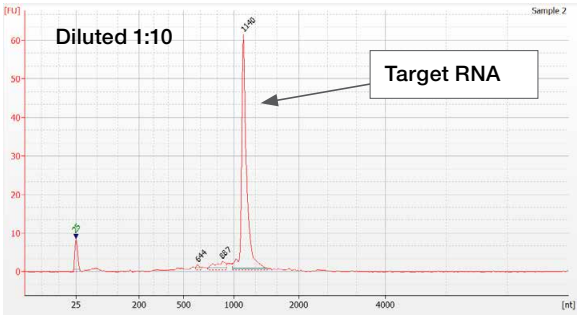


Figure 2. Electropherograms of synthesized RNA fragments. Integrity of RNA was verified using undiluted (left) and 10-fold diluted (right) purified RNA samples; sharp peaks of expected sizes represent complete RNA transcripts. Note: Size of synthetic RNA measured by the Bioanalyzer system differs slightly from the theoretical size due to the influence of settings used during system setup.

RNA quantification with RT-qPCR to determine copy number

Synthetic RNA generated from fragments B and C were quantified using the *Power* SYBR Green RNA-to-C_T *1-Step* Kit and verified primers [1] in order to measure precise copy number. Only fragments B and C were quantified because of their planned use in downstream RT-LAMP applications. The reaction mix was set up in the order listed in Table 3. SARS-CoV-2 RNA Control 1 (Twist Bioscience, Cat. No. 102019) was used for standard curve preparation, ranging from 32 to 100,000 copies. Serial dilutions of RNA transcript were prepared in water, and 3 technical replicates of each reaction were performed.

Table 3. RT-PCR reaction setup.

Component	Final conc.	Amount
Power SYBR Green RT-PCR Mix (2X)	1X	10 µL
10 µM forward primer	100 nM	0.2 µL
10 µM reverse primer	100 nM	0.2 µL
RT Enzyme Mix (125X)	1X	0.16 µL
Template RNA	–	4 µL
Water, nuclease-free	–	5.44 µL
Total volume		**20 µL**

RT-qPCR was performed on an Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System using the cycling protocol described in Table 4. A representation of RT-qPCR data for fragment C is shown in Figure 3. A total of 6.73 x 10^{12} (fragment B) and 6.19 x 10^{12} (fragment C) copies of synthetic RNA were synthesized directly from 1 µg of custom GeneArt Strings DNA Fragments as calculated from the standard curve.

Table 4. Cycling protocol.

Number of cycles	Step	Temp.	Time
1	Reverse transcription	48˚C	30 min
1	Activation	95˚C	10 min
40	Denaturation	95˚C	15 sec
	Annealing and extension	60˚C	1 min
–	Melt curve (optional)	60–95˚C	15 sec

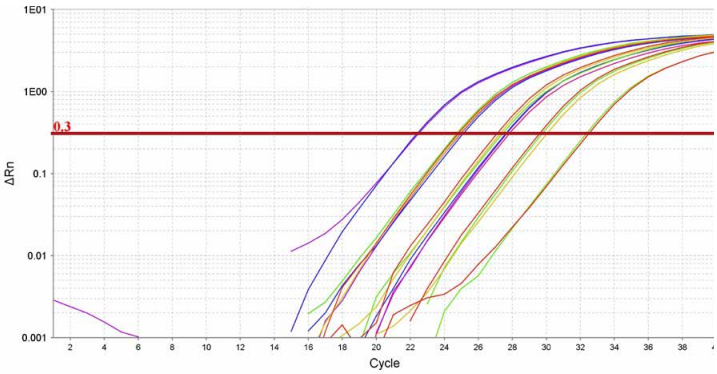


Figure 3. Quantification of transcribed RNA by RT-qPCR. Red, yellow, and green lines are standard curves. Blue, purple, and pink lines are amplification of RNA transcript from different dilutions of fragment C.

Downstream applications

Generated synthetic SARS-CoV-2 RNA fragment was used as a positive control in RT-LAMP reactions with SuperScript IV RT-LAMP Master Mix and verified primers [1]. The reaction mix was set up in the order listed in Table 5.

Generated synthetic RNA fragment was compared to a commercially available SARS-CoV-2 RNA control alongside purified RNA from SARS-CoV-2 positive clinical samples. One-step RT-LAMP was performed using 10,000 copies of RNA synthesized from fragment C, 10,000 copies of SARS-CoV-2 RNA Control 1 (Twist Bioscience), and positive clinical samples from infected patients (one sample each with high or low SARS-CoV-2 RNA copy number).

SARS-CoV-2 RNA was amplified in 5–10 min depending on template and copy number input (Figure 4). The exact quantity of high copy number RNA purified from clinical samples was unknown, and could have been higher than 10,000 copies, which explains slightly faster amplification (dark green curve). RNA transcript (red curve) performed as efficiently as commercial RNA control (blue curve) with only 5.8 and 5.2 minutes to result, respectively (Table 6). These results confirm that RNA controls generated via IVT using GeneArt Strings DNA Fragments are of high quality and can be used for downstream applications.

Table 5. RT-LAMP reaction setup using SuperScript IV RT-LAMP Master Mix.

Component	Detection of RNA	No-template control (NTC)	Final concentration
SuperScript IV RT-LAMP Master Mix (2X)	12.5 µL	12.5 µL	1X
40 µM FIP/BIP primers	1 µL each	1 µL each	1.6 µM
10 µM F3/B3 primers	0.5 µL each	0.5 µL each	0.2 µM
10 µM LoopF/LoopB primers	1 µL each	1 µL each	0.4 µM
50 µM fresh stock solution of SYTO 9 Green Fluorescent Nucleic Acid Stain	2.5 µL	2.5 µL	5 µM
Water, nuclease-free	4 µL	5 µL	—
Close NTC tubes or wells prior to adding target RNA			
Target RNA	1 µL	—	—
Total volume	**25 µL**	**25 µL**	**—**

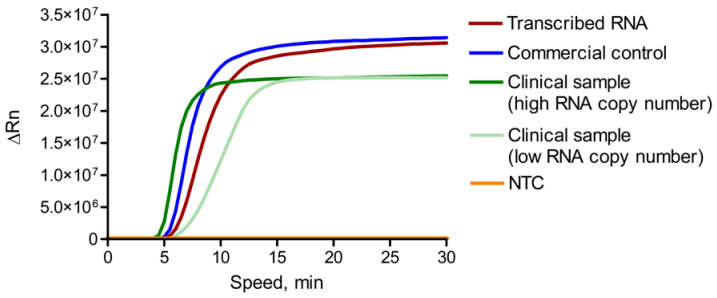


Figure 4. SARS-CoV-2 RNA amplification via real-time RT-LAMP using SYTO 9 Green Fluorescent Nucleic Acid Stain. RNA transcribed from fragment C, SARS-CoV-2 RNA Control 1 (Twist Bioscience), and positive clinical samples of high and low RNA copy numbers were used as templates. Multiple technical repeats (n = 4) for each sample were performed. No nonspecific amplification signal from the NTC was observed.

Table 6. RT-LAMP results.

Template	Time-to-results
NTC	Undetermined
Transcribed RNA (fragment C)	**5.8 min**
Commercial control	5.2 min
Clinical sample (high RNA copy number)	4.5 min
Clinical sample (low RNA copy number)	7.0 min

invitrogen

Summary

Custom double-stranded GeneArt String DNA Fragments can be used directly for IVT reactions to generate large quantities of high-quality synthethic RNA in just a few hours. Synthetic RNA controls generated via this approach can be used in RT-qPCR, RT-LAMP, next-generation sequencing, and many other applications, and provide a safe and effective alternative to nucleic acids extracted from active viruses.

References

1. Park GS et al. (2020) Development of reverse transcription loop-mediated isothermal amplification assays targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *J Mol Diagn* 22:729–735.
2. Thermo Fisher Scientific (2021) Optimized real-time and endpoint RT-LAMP for SARS-CoV-2 using Bsm DNA Polymerase and SuperScript IV Reverse Transcriptase. Application note available at thermofisher.com/lamp

Find out more at **thermofisher.com/rtlampmastermix**

ThermoFisher SCIENTIFIC