APPLICATION NOTE No. 489

Boosting Yield and Purity of DNA Templates for mRNA Production in a Single PCR Run

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Abstract

In vitro production of high-quality mRNA is crucial for numerous applications such as vaccine development and cell differentiation experiments. mRNAs are produced using *in vitro* transcription (IVT) which relies on high-quality template DNA. Here, we show how template DNA production can be significantly improved in a single PCR run. Using the 2D-temperature gradient function (2D-Gradient) of the Mastercycler® X50a, we tested 96 combinations of annealing and denaturing temperatures in a single PCR run of 76 minutes. We identified a combination of annealing and denaturing temperatures that provided both an IVT template with a high purity and ~fourfold increased yield compared to the temperatures which are similar to the original protocol. Hence, using the 2D-Gradient sped up the optimization of the template DNA up to ~8-fold compared to a conventional 1D gradient.

Introduction

Production of pure mRNAs is required in many modern areas of life science. One example is mRNA vaccines [1]. Another example of rising importance is the induction of pluripotent stem cells; here *in vitro*-transcribed mRNAs encoding five different transcription factors are transfected into human fibroblast to induce pluripotency [2]. It is anticipated that patient-derived induced pluripotent stem cells (iPSCs) will be used increasingly to generate cell types for autologous therapies or to model disease [2].

Here we demonstrate how the production of a template DNA, required for the IVT of the reprogramming factor LIN28A-encoding mRNA [3], is enhanced efficiently using a PCR 2D-Gradient.

mRNAs are preferentially produced *in vitro* as they are fragile molecules that can easily be degraded by enzymes within the living cell. Producing an mRNA *in vitro* further allows the incorporation of modified bases to enhance mRNA stability or *in vivo* efficacy. The quality of the DNA template is crucial for mRNA production as e.g., inhomogeneous template lengths lead to a mixture of truncated mRNA products, requiring time-consuming additional purification steps. Polymerase chain reaction (PCR) is a standard technique used in every life science lab, allowing to amplify DNA templates on a large scale [4]. During a PCR, a mixture of template DNA, primers, nucleotides, and DNA polymerase is subjected to repetitive temperature cycles in a thermal cycler.

Specific temperatures are required to separate the template DNA strands (Denaturation temperature – T_D) but also for primer hybridization to the template DNA (Annealing temperature – T_A). The ideal temperatures depend on the length and base composition of the DNA template and primers, as well as the desired specificity. Optimizing these temperatures is therefore an important step [5].

Here, we show how the Mastercycler® X50a ("a" stands for aluminum thermoblock) saves time and effort during the identification of the ideal combination of T_D and T_A to generate a pure template DNA with a high yield for mRNA production.

To this end, we harvested a bacterial culture containing a LIN28A-carrying plasmid grown in an Innova® S44i shaker (Figure 1). The LIN28A template DNA was purified and amplified using the 2D-Gradient of the Mastercycler® X50. This feature allows setting up eight denaturation temperatures and twelve annealing temperatures or vice versa. This way, in a single run, 96 PCR-conditions were tested, and the best T_D/T_A combination was chosen based on the obtained DNA fragment size, homogeneity, and concentration. Once the best T_A and T_D were identified, a larger-scale PCR was performed with the optimized parameters to generate sufficient DNA for several IVT reactions.

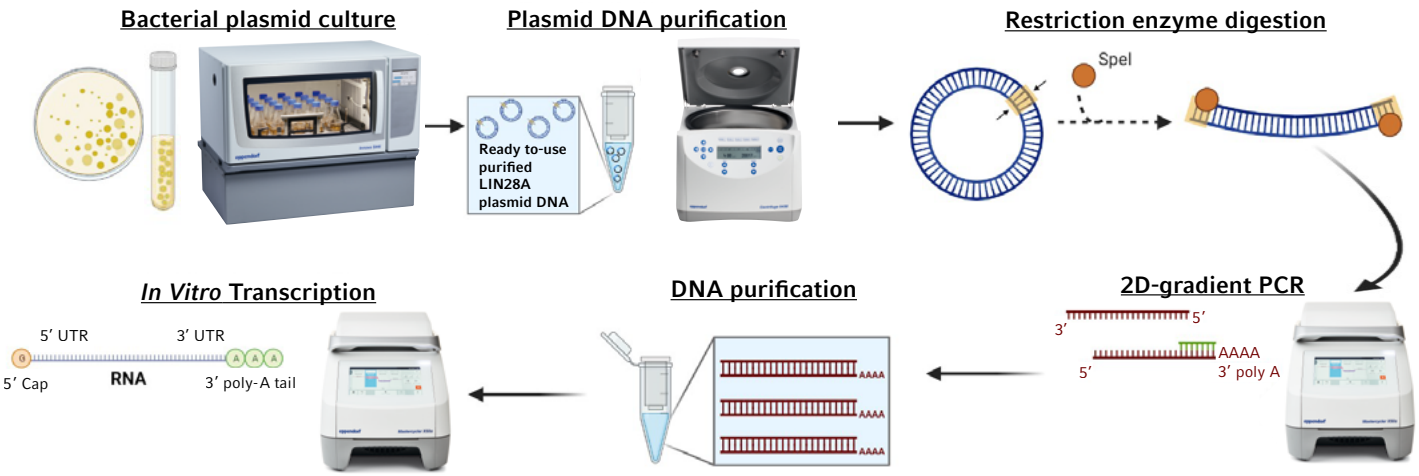


Figure 1: Workflow from the bacterial culture to mRNA IVT using the 2D-Gradient of the Mastercycler® X50a for the optimization of the template DNA (PCR product).

Result and Discussion

Plasmid linearization and purification
The restriction digest of the plasmid with SpeI resulted in short fragments (684 bp) and long fragments containing inserted genes. After purification, digestion was verified by the Agilent® D5000 ScreenTape System at the tape station. The expected size band confirmed the enzyme's proper digestion (Figure 2).

Optimization of the amplification of the target gene by 2D-Gradient PCR
The LIN28A template DNA (target gene) was amplified by tail-PCR using primers Xu-F1 and Xu-T120 [3] in the Mastercycler® X50a. The expected length of PCR products, based on data generated previously by Mandal and Rossi [3], was confirmed using Agilent D5000 ScreenTape® System of the TapeStation (Figure 3A). The PCR parameters which are similar to the ones used by Mandal and Rossi [3] (T_A= 60.4 °C, T_D= 98.5 °C, red rectangle in Figure 3A) gave the right product length even though a smear was observed (Figure 3 A). The presence of impurities could also be shown by the high peak bandwidth in the electropherogram generated by the D5000 ScreenTape System (Figure 3B). Furthermore, the DNA concentration was comparably low (20.4 ng/µl). Several factors, such as the specifications of thermal cyclers, the ramp rates, the chosen consumables, and the reagents affect the results of a PCR, even established protocols frequently require reoptimization when transferred to a new laboratory.

The optimization of the PCR allowed us to identify the best combination of temperatures to get a pure and single PCR product. A T_A of 68.7 °C and T_D of 94.6 °C (green rectangle in Figure 3 A & C) resulted in the most homogenous PCR product with a comparatively high concentration (75 ng/ml). This concentration is ~fourfold higher than the concentration which was achieved at a T_A of 60.4 °C and T_D of 98.5 (20.4 ng/µl, red rectangle in Figure 3A).

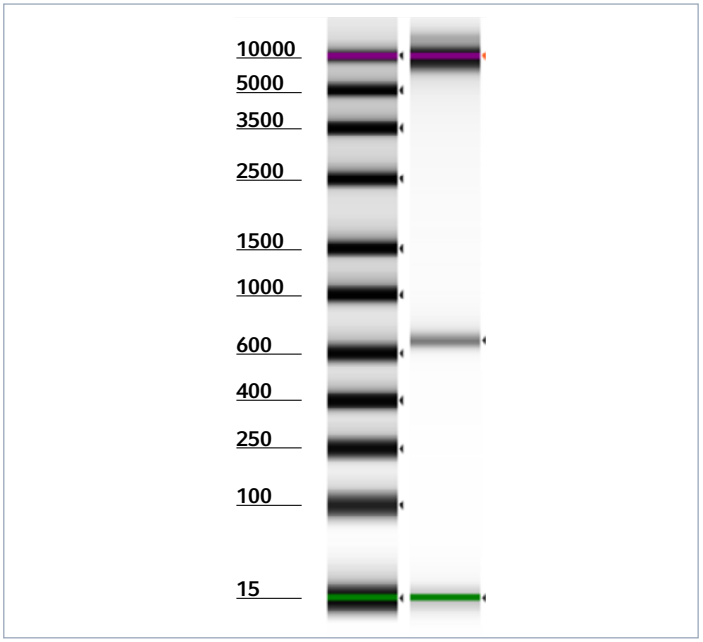
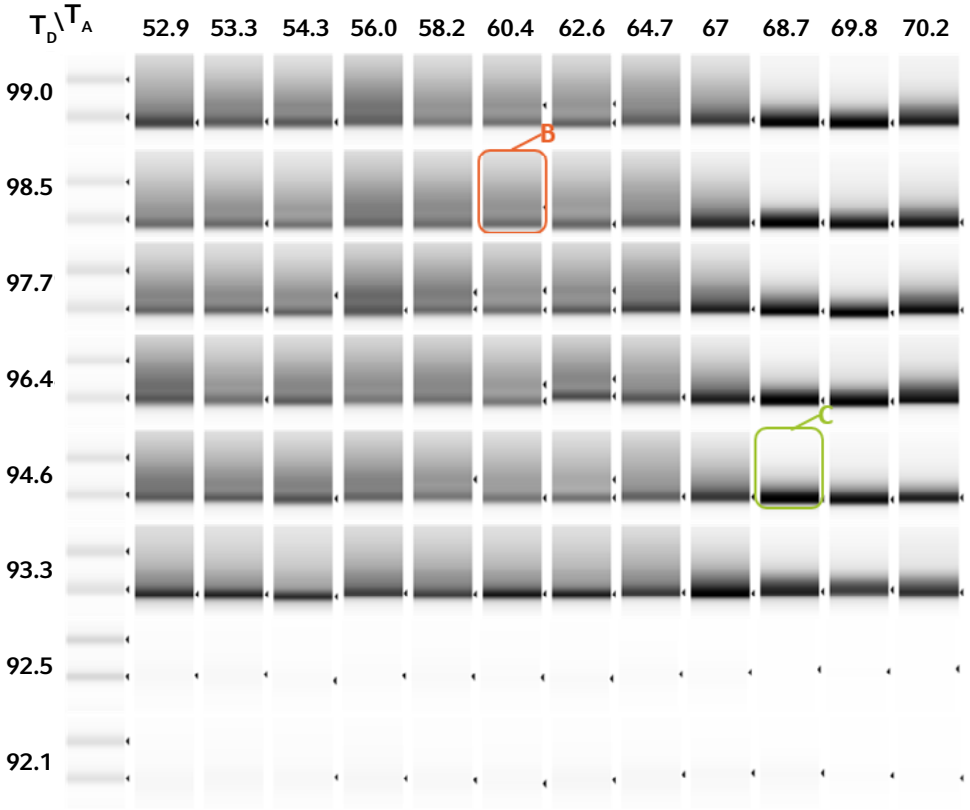


Figure 2: Linearization of the plasmid using SpeI resulted in two fragments. The analysis of these bands with the Tapestation confirmed the linearization as two different fragments are detected at the expected size. The lower and upper detection limit is indicated by the green and purple line.

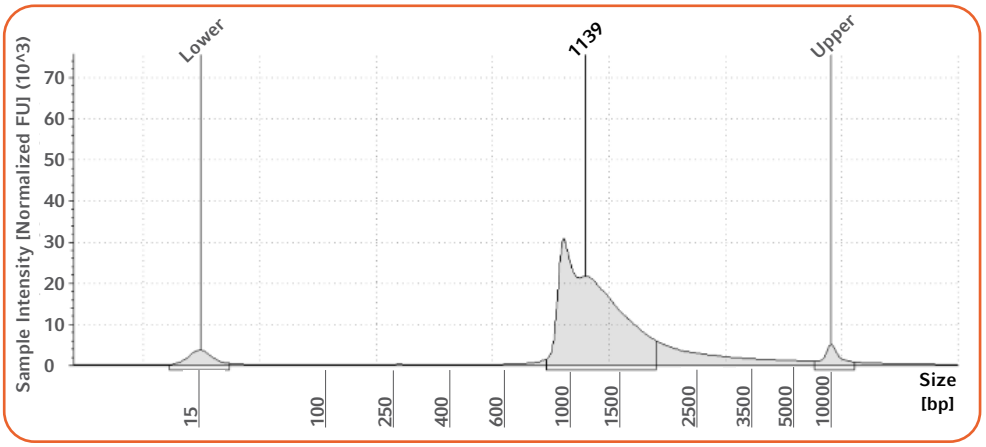
Other temperature combinations either gave lower concentrations or unspecific bands with a smear. The importance of the T_D optimization becomes evident at a T_A of 68.7 °C (Figure 3D). Here, the highest concentrations of 73.4 and 75.0 ng/µl were obtained at a T_D of 93.3 and 94.6 °C, respectively. Lower or higher T_D led to lower DNA concentrations, underlining that the optimization of the T_D in addition to the T_A optimization is useful to obtain more PCR product. In this line, the mean DNA concentration of all samples with a T_D of 94.6 (104.0 ng/µl) was more than five times higher than at 98.5 °C (18.7 ng/µl, Figure 3E).

The optimal T_D becomes higher with increasing GC content and template length. As the LIN28A template without PolyA-tail is short (807 bp) and the GC content is in the middle range (57%), a relatively low optimal T_D of 94.6 °C was determined using the 2D-Gradient.

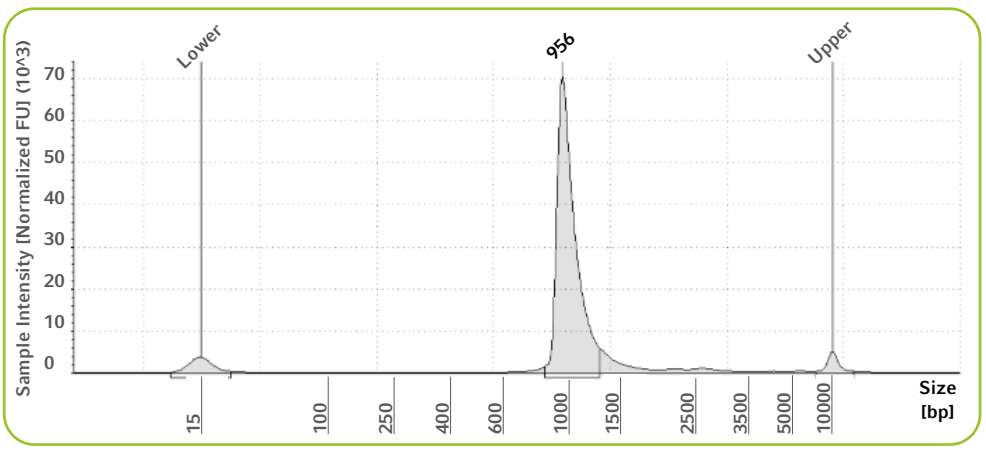
A


$T_D \backslash T_A$
52.9
53.3
54.3
56.0
58.2
60.4
62.6
64.7
67
68.7
69.8
70.2
99.0
98.5
97.7
96.4
94.6
93.3
92.5
92.1
B
C

B


Lower
1139
Upper
Sample Intensity [Normalized FU] (10^3)
70
60
50
40
30
20
10
0
15
100
250
400
600
1000
1500
2500
3500
5000
10000
Size [bp]

C


Lower
956
Upper
Sample Intensity [Normalized FU] (10^3)
70
60
50
40
30
20
10
0
15
100
250
400
600
1000
1500
2500
3500
5000
10000
Size [bp]

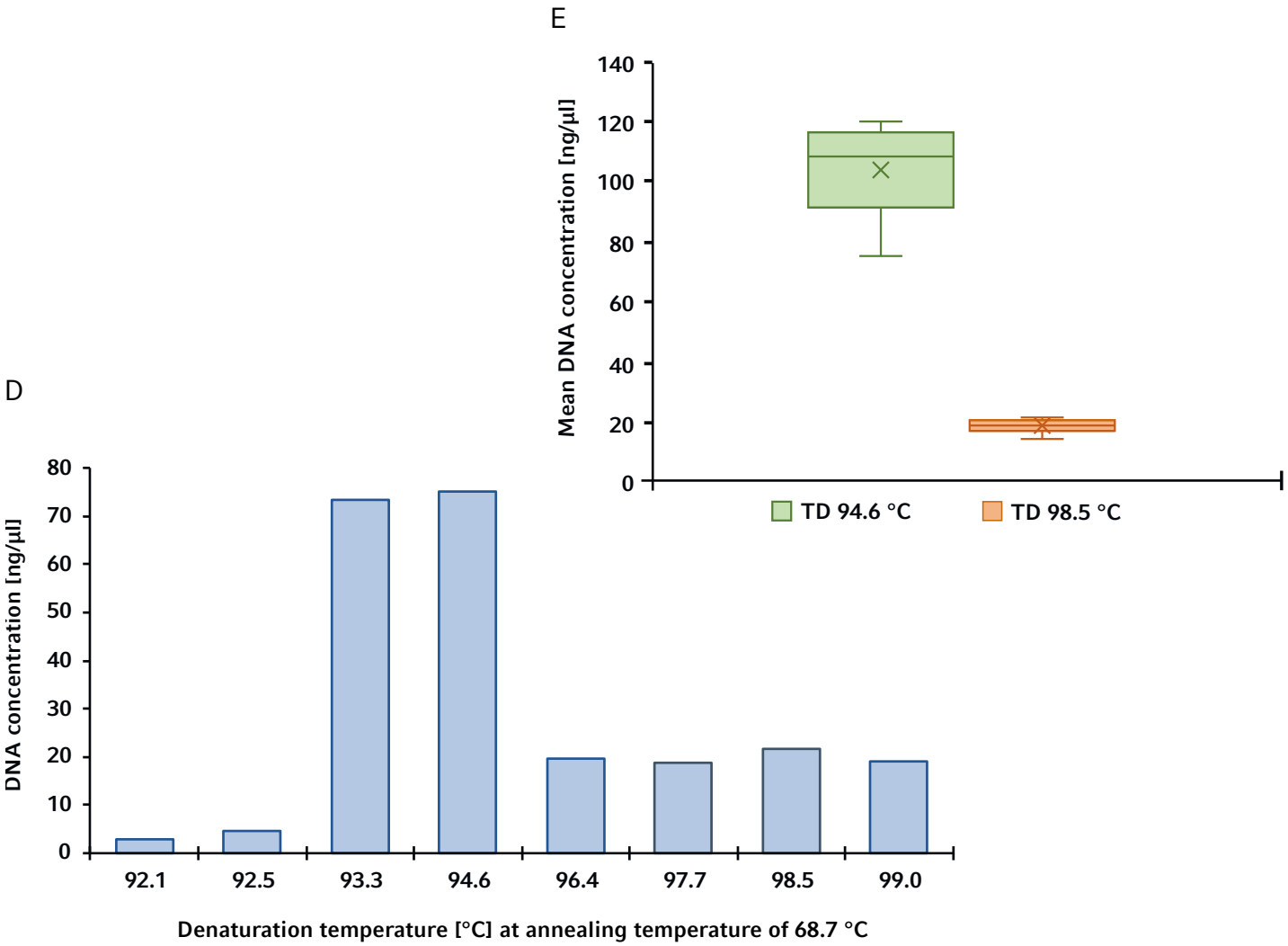


Figure 3: Optimization of the PCR program by using the 2D-Gradient of the Mastercycler® X50a.
(A) The purity and amount of the PCR product depend on T_A and T_D combinations.
(B) Electropherogram of the PCR product obtained with the parameters similar to the ones used from Rossi *et al.* [2-3] (red rectangle in A)
(C) Electropherogram of the PCR product obtained with the best T_A and T_D combination (green rectangle in A).
(D) Influence of T_D at a T_A of 68.7 °C on DNA concentration measured by Qubit 4 Fluorometer.
(E) Mean DNA concentrations measured by Qubit 4 Fluorometer of all samples with a denaturation temperature of 94.6 vs. 98.5 °C.

The 2D-Gradient allows testing of up to 96 temperature combinations in one single PCR run. The optimization shown here would have needed eight PCR runs when using a thermal cycler with a 1D gradient function. Thus, the 2D-Gradient of the Mastercycler X50 helps to reduce hands-on time as well as consumption of reagents, consumables, and energy for temperature optimization of a PCR. Furthermore, potential sources of error are reduced as only a single PCR run using one mastermix, PCR plate, and sealing is needed when utilizing the 2D-Gradient.

IVT and purification of the mRNA

In the final step, mRNA was produced by IVT using the Mastercycler® X50a. After DNase and phosphatase treatments, the mRNA was purified and analyzed with an Agilent RNA ScreenTape System. A single band just beneath 1000 nucleotides was observed, confirming the generation of mRNA molecules of the correct size. The mean concentration of mRNA was 600.3 ng/µL. The RIN score was 10, indicating that pure, intact mRNA was produced.

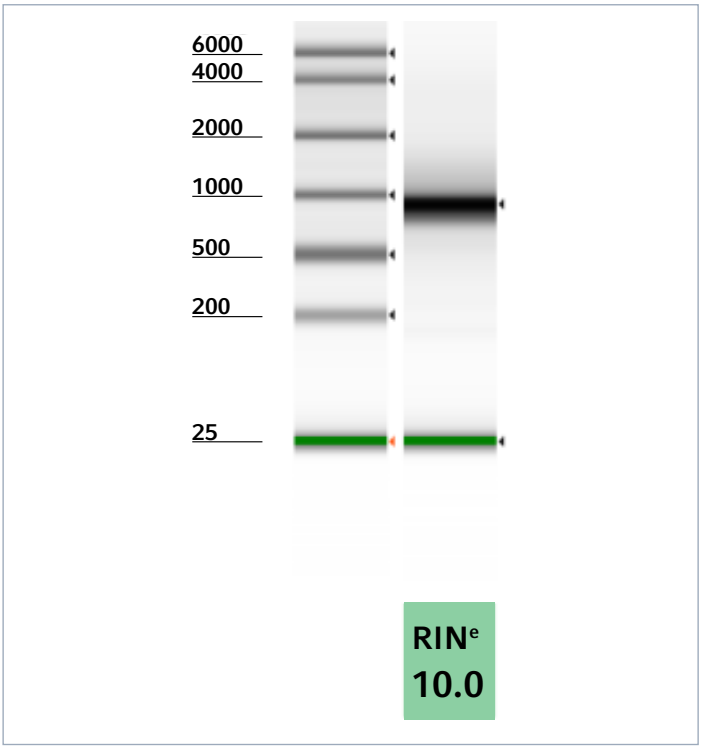


Figure 4: IVT producing single and intact mRNA. The RNA integrity number (RIN) assigns values from 1 to 10 (1 = strongly degraded, 10 = intact). The lower detection limit is indicated by the green line.

Conclusion

Diverse PCR applications are developed in different fields of life science, requiring optimized protocols. Even established protocols can require re-adjustments when transferred to a different PCR cycler or when consumables or reagents are changed. Optimized PCRs require a delicate balance between the amplification of certain products and avoiding the production of non-specific products. Each step, from the extraction of the DNA template to the cycle time and temperature, must be carefully considered. The important parameters evaluated in this Application Note, are denaturation and annealing temperature. By using the 2D-Gradient of the Mastercycler® X50a, we have been able to improve the purity and yield of the PCR product while scanning a wide range of combinations of T_A and T_D in a single PCR run. By doing so, it was straightforward and quick to choose the best T_A and T_D for producing highly pure and concentrated template DNA for the subsequent, successful LIN28A-mRNA production steps.

Materials and Methods

The procedure followed the protocol and recommendations, with minor modifications, of the research papers by Rossi *et al*. [2-3].

Plasmid
A plasmid encoding human LIN28A was purchased from the Addgene® plasmid repository (Addgene plasmid #26819; http://n2t.net/addgene:26819; RRID: Addgene_26819). The plasmid construct was designed by Derrick Rossi in the DH5alpha growth strain. It contained the LIN28A sequence (813 bp), the 5' sequencing primer "CMV Forward" and the 3' sequencing primer "TK polyA reverse" (5'UTR-LIN28A-3'UTR). The total plasmid backbone size with the insert was 6213 bp.

Culturing of bacteria
Amplification of plasmid in cell culture and cryopreservation in glycerol
Bacteria were inoculated on agar (Invitrogen™, #11518916) containing ampicillin at 100 µg/mL (Sigma-Aldrich, #A5354) in a 10 mm dish and grown for 24 h at 37 °C in an incubator. A single colony was picked and transferred to a 15 mL conical tube filled with 10 mL of LB Broth (Sigma Aldrich, #L2897) containing ampicillin at 100 µg/mL and amplified overnight at 37 °C and 200 rpm in an Innova® S44i shaker (Eppendorf, #S44I311001). Bacteria were spun at 1400 x *g* for 5 min with Centrifuge 5430 (Eppendorf, #5427000015) and the pellet was resuspended in 10 mL of LB Broth with glycerol 50% (v/v). Aliquots of 500 µL were frozen in cryovials at -80 °C for further experiments.

Plasmid culture
LB Broth (15 mL) containing ampicillin at 100 µg/mL was poured into a 15 mL tube with 15 µL of defrosted bacteria in glycerol. Bacteria were then cultured at 37 °C for 24 h with 200 rpm agitation in an Innova® S44i shaker.

Plasmid purification
Plasmid DNA was extracted using the QIAprep Spin Miniprep kit (Qiagen, # 27104) following the manufacturer's instructions.

Plasmid linearization and purification
The plasmid was linearized using the restriction enzyme SpeI (Promega, #R6591). Briefly, a single 50 µL restriction digest, containing the plasmid (500 ng/µL), 5 µL of buffer (100x), 0.5 µL of BSA (10x), SpeI (0.1 U/µL), and water, was incubated in a ThermoMixer® C (Eppendorf, #5382000015) for 2 h at 37 °C. The enzyme was then inactivated for 20 min at 80 °C in the ThermoMixer® C. The linearized plasmid was purified using the QIAquick PCR Purification Kit (Qiagen, #28106) following the manufacturer's instructions. The nucleic acid concentration was measured using the Nanodrop™ (Thermo Fisher, #ND-2000) and stored at -20 °C for further analysis.

Addition of the poly-(A) tail by PCR
The primers were purchased from Integrated DNA Technologies. Primer Xu-T120 was synthesized as an Ultramer oligos at a 4-nmol scale.
Xu-F1: 5'-TTGGACCCTCGTACAGAAGCTAATACG-3'
Xu-T120 : 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTCTTCCTACTCAGGCTTTATTCAAAGACCA-3'

The PCR master mix contained 100 µL of KAPA HiFi HotStart ReadyMix (Roche, #KK2602), 6 µL of each primer (10 µM), and 8 µL of digested plasmid (400 pg/µl) and 80 µL of water to reach final volume of 200 µL. The master mix was aliquoted into Twin.tec® Trace PCR plate 96 (Eppendorf, #0030129776) and run on a Mastercycler® X50a (Eppendorf, #6313000018) using the 2D-Gradient. The twelve annealing temperatures were set up ranging from 52.9 to 70.2 °C. The eighth denaturation temperatures from 92.1 to 99 °C (Table 1).

Eppendorf Header Settings	Lid	105 °C
	Energy-saving mode	ON
	Temperature mode	Standard
Initial Denaturation	95 °C/2 min	
3-step PCR program 30 cycles	Denaturation	92.1-99.0 °C/20 s
	Annealing	52.9 to 70.2 °C/15 s
	Elongation	72 °C/60 s
Post-Cycle Elongation	72 °C/2 min	
Storage	Hold Step	10 °C

Table 1: Settings of the used PCR program which was run on a Mastercycler X50a.

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, #28106) as described above. Tailed PCR products were measured using the Qubit 4 Fluorometer (Thermo Fisher, #Q33238) and stored at -20 °C for further analysis.

In vitro transcription

The working area and pipettes were cleaned with RNaseZap™ RNase Decontamination Solution (Invitrogen™, #10708345) to ensure the elimination of RNase. RNA was synthesized with the MEGAscript™ T7 Transcription Kit (Invitrogen™, #10065754), with 40 ng/µL (final concentration) of purified tailed PCR product to template each 40 µL reaction. A custom ribonucleoside blend was added comprising 4 µL of 3´-O-Me-m7G(5')ppp(5')G RNA Cap Structure (New England Biolabs, #S1411S), 4 µL of adenosine triphosphate, and 0.8 µL of guanosine triphosphate (from the MEGAscript T7 kit), 3 µL of each 5-methylcytidine triphosphate and pseudouridine triphosphate (TriLink Biotechnologies, #N-1014 and #N-1019). The custom ribonucleoside blend was vortexed and spun briefly before adding the tailed PCR product, 4 µL of each the T7 Buffer and T7 enzyme (from the MEGAscript T7 kit). Water was added to reach 40 µL final volume. The solution was incubated for 4 h at 37 °C in a Mastercycler® X50a (Eppendorf, #6313000018). After incubation, 2 µL of Turbo DNase (from the MEGAscript T7 kit) was added to the sample and incubated for 15 min at 37 °C. RNA was purified using the MEGAclear™ Transcription Clean-Up Kit (Invitrogen™, #AM1908) following the manufacturer's instructions. Briefly, samples were mixed with 60 µL of Elution buffer, 350 µL of Binding solution, and 250 µL of 100 % ethanol, transferred to a cartridge, and centrifuged at 10,000 x *g* for 1 min at 4 °C using the Centrifuge 5702 R (Eppendorf, #5703000010). Cartridges were washed in 2 steps consisting of 500 µL of wash solution and centrifugation at 10,000 x *g* for 1 min at 4 °C. RNA was collected in 2 steps consisting of 50 µL of pre-heated (95 °C) elution solution and centrifugation at 10,000 x g for 1 min at 4 °C. Purified RNA was treated with 11 µl of 10× Antarctic phosphatase buffer and 2 µl of Antarctic phosphatase (New England Biolabs, #M0289S) for 1 h at 37 °C using a ThermoMixer® C (Eppendorf, #5382000015). Phosphatase-treated RNA was then purified again using the MEGAclear™ Transcription Clean-Up Kit (Invitrogen™, #AM1908) as described above. RNA was measured with the Nanodrop™ (Thermo Fisher, #ND-2000) and stored at -80 °C for further analysis.

Electrophoresis

The analysis of linearized plasmid, PCR products, and RNA was performed using the automated platform TapeStation 4150 System (Agilent, #G2992AA) according to the manufacturer's instructions. Digested plasmid and PCR products were run through the D5000 ScreenTape System (Agilent, #5067-5588, #5067-5589). The RNA ScreenTape System (Agilent, #5067-5576, #5067-5577, #5067-5578) was used to visualize the RNA.

Literature

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[4] Green MR, Sambrook J. The basic polymerase chain reaction (PCR). Cold Spring Harb Protoc. 2018;5:338-345. doi:10.1101/pdb.prot095117

[5] Analytical Methods Committee Amctb No. PCR - the polymerase chain reaction. Anal Methods. 2013;6(2):333-336. doi: 10.1039/c3ay90101g. PMID: 33985286.

Ordering Information

Ordering information

Description	Manufacturer	Order No.
Centrifuge 5430	Eppendorf	5427000015
Centrifuge 5702 R	Eppendorf	5703000010
Innova® S44i shaker	Eppendorf	S44I300001
Mastercycler® X50a	Eppendorf	6313000018
ThermoMixer® C	Eppendorf	5382000015
Twin.tec® Trace PCR plate 96	Eppendorf	0030129776

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