Note added after publication: This version replaces the supporting information published on 26th September 2023 as there was an error in the experimental details for the RCA reactions.

Supporting Information

Accurate quantification of DNA content in DNA hydrogels prepared by rolling circle amplification

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1 Experimental Section

Synthesis of the circular DNA templates

For the preparation of the circular templates, 5 µL of linear template (100 µM) was mixed with 5 µL of the corresponding ligation primer (100 µM) in TE buffer (20 mM Tris-Base, 1 mM EDTA, pH 7,6) supplemented with 100 µM NaCl resulting in a final reaction volume of 100 µL. The reaction mixture was then subjected to a temperature cycle, starting with heating to 85 °C in a thermocycler (Master cycler pro, Eppendorf) and gradually cooling down to 25 °C at a rate of 0.01 °C/s. The ligation process was initiated by adding 20 µL of a commercial 10x T4 ligase buffer, 70 µL of ddH_2O, and 10 µL of T4 ligase (NEB, 2 U/µL) to the reaction mixture, followed by an incubation at room temperature for 3 hours. The enzyme was then inactivated by heating the mixture at 70 °C for 20 minutes. Subsequently, an enzymatic digestion was carried out using 10 µL of Exonuclease I (40 U/µL) and 10 µL of Exonuclease III (200 U/µL), with overnight incubation at 37 °C. The enzymes were deactivated by heating the reaction mixture at 80 °C for 10 minutes. To purify the circularized template from digested DNA fragments of the linear template and primer DNA, the mixture was subjected to three washes with 2 mL of TE buffer using an Amicon Ultra centrifugal filter with a 10 kDa cut-off (Merck Millipore). The purity of the circularized template was determined using denaturing urea-PAGE, and the concentration was measured using UV-VIS spectroscopy. The circularized template was diluted to a concentration of 1 µM in TE buffer and stored at -20 °C.

RCA reactions

To prepare a 250 µL reaction mixture, 190 µL of ddH_2O, 12.5 µL of the circular DNA template (1 µM), 25 µL of commercial 10x phi29-polymerase buffer, 2.5 µL of RCA primer (10 µM) and 5 µL of the phi29-polymerase (10 U/µL) were mixed. Subsequently, 12.5 µL of an adjusted dNTP solution (100 mM) was added, tailored to match the base composition of the amplicon for each circular DNA template (G:C:T:A for Template A: 26:18:16:12, Template B: 23:24:16:9, Template C: 23:27:21:22). The reaction was carried out for defined time periods at 30 °C before the enzyme was inactivated through heat-denaturation at 80 °C for 10 minutes.

Fluorometric Sybr Green I assay

For the Sybr Green I Assay, the RCA was terminated after various reaction times (0, 1, 6, 24, 30, 48, 72 hours) through heat denaturation of the phi29-polymerase (80 °C, 10 min). Subsequently, 2.5 µL of a 1:10 dilution of Sybr™ Green I (10,000x concentrate in DMSO, Invitrogen) was added to the samples and mixed until a homogeneous distribution of the dye was observed. After the addition of the dye, the samples were incubated on an orbital shaker (Unimax 1010, Heidolph Instruments) at 4 °C for 3 hours, followed by fluorescence measurement in a microplate (F96 Polysorb Nunc Plate, Thermo Fisher Scientific) using a Synergy H1 instrument (BioTek Instruments GmbH) with an excitation wavelength of λ_{EX} = 490 nm and an emission wavelength of λ_{EM} = 520 nm. The stained DNA was quantified using a calibration curve of UltraPure™ herring sperm DNA (Invitrogen). The quantification of dsDNA

during RCA using the fluorometric Sybr Green I assay was performed in three independent experiments.

Photometric Supernatant Depletion Assay

In the Supernatant Depletion Assay, the concentration of free nucleosides (dNTPs) in the supernatant was determined to quantify the amplified DNA during RCA. For this, RCA reactions were terminated after various reaction times (0, 1, 6, 24, 30, 48, 72 hours) through heat denaturation of the phi29-polymerase (80 °C, 10 min). Subsequently, the entire reaction mixture was transferred to a filter unit with a 10 kDa cut-off* (Amicon® Ultra, 2 mL, Merck Millipore) and washed three times with 1 mL of TE buffer. The flow-through was collected and completely evaporated using a SpeedVac (Eppendorf, Concentrator plus). The resulting pellet was resuspended in the initial volume (250 µL) in TE buffer, and the absorption at λ = 260 nm was measured using a Synergy H1 instrument (BioTek Instruments GmbH) with a micro-volume plate (Take3 Plate, BioTek Instruments GmbH). The concentration was subsequently determined using the averaged molar extinction coefficients in regard to the nucleotide composition in the adjusted dNTP mixtures of the templates (A: 11980,5 $M^{-1}cm^{-1}$, B: 11501,4 $M^{-1}cm^{-1}$, C: 12023,9 $M^{-1}cm^{-1}$). The quantification of amplified DNA using the supernatant depletion assay was performed in three independent experiments. To determine the total amount of amplified DNA during RCA, the number of incorporated nucleotides in the DNA hydrogel was calculated. For this, the individual data points from the dNTP concentrations in the supernatant, that were measured after a defined time of RCA (t = x h), were subtracted from the starting dNTP concentration measured in the beginning (t = 0 h). Then, using the molecular weight of nucleotides in the dNTP mixtures (A: 485,93 g/mol, B: 483,26 g/mol, C: 486,23 g/mol) and the reaction volume (250 µL), the total DNA mass of the RCA product was calculated.

The used cut-off value of the membrane of 10 kDa only allows the passage of single dNTPs and DNA fragments up to 30 bases. In the context of DNA amplification by rolling circle amplification, it is highly unlikely that phi29 polymerase would stop DNA elongation even before replication (>72 nt) of the circular template, since it is known that phi29 polymerase can generate up to 70,000 nt before detaching from the circular template.[1] Therefore, we can assume that the filtrate sample contains mainly single dNTPs and no short fragments generated by incomplete polymerization.

qPCR Assay

For the DNA quantification using qPCR, an RCA reaction mixture of 250 µL was prepared and subsequently aliquoted into smaller samples of 10 µL. After various reaction times (0, 1, 6, 24, 30, 48, 56, 72 hours), the samples were diluted with 90 µL of ddH_2O and the polymerase was inactivated at 80 °C for 10 min. The amplicon concentration in the samples was determined by using a calibration curve of the amplicon sequence for each circular template (20 nM to 156.3 pM). Depending on the reaction time, different dilutions (1:10-1:2000) were prepared for qPCR measurements to ensure that the concentrations of amplified DNA were within the values of the calibration curve. The dilutions were thoroughly mixed and treated with ultrasound (VWR Ultrasonic cleaner, Incident Power: 30 W) for 5 s to induce fragmentation of the ultralong ssDNA chains generated during RCA.* The samples were stored at 4 °C until usage and sonicated again immediately before qPCR experiments.

For the preparation of a 10 mL qPCR mix, 1 mL of 10x qPCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 25 mM KCl, 25 mM $MgCl_2$, 0,1% Tween- 20), 200 µL of dNTPs (10 mM each), 100 µL of forward primer (100 µM), 100 µL of reverse primer (100 µM), 20 µL of the TaqMan probe (100 µM), and 100 µL of Taq DNA polymerase (5 U/µL, New England Biolabs) were combined in autoclaved ddH2O. For the qPCR, 20 µL of the qPCR mix was mixed with 1.5 µL

of the calibration standards (amplicon sequence of A, B or C) or the diluted RCA samples. Measurements were performed in technical triplicates using a real-time thermocycler (Corbett Research). The cycle threshold (Ct) value was subtracted from the maximum number of cycles (C_{max}). The ΔCt values were plotted against the logarithmic concentration of the calibration standards to obtain DNA concentrations using linear regression. The total DNA mass amplified during RCA was determined using the experimentally determined amplicon concentration, the reaction volume (250 µL) and the molecular weight of the amplicons (A': 21139 g/mol, B': 22136,3 g/mol C': 30242,5 g/mol). The quantification of amplified DNA using qPCR was performed in at least three independent experiments.

* With regard to possible artifacts in qPCR analysis due to fragmentation and sequence-dependent differences in PCR amplification, we note that the interference caused by fragmentation should be negligible under the conditions used. In fact, at the incident power of 30 W and a short treatment time of 5 s used here, no significant negative effects on amplified genomic DNA fragments during qPCR have been observed.[2] Regarding sequence-induced bias during qPCR amplification, we note that it is known from genomic analysis that sequence-dependent biases based mainly on the kinetics of annealing and denaturation can arise from factors such as amplicon sequence length, GC content, and primer annealing temperature. While in our case the length of the DNA fragments of all sequences should be similar due to the equal ultrasound treatment (30 W, 5 s), and the annealing temperatures of the primers (T_m = 57.2-60.0 °C, Table S2) are all very similar, the matrix sequences have a slight variation in their GC content (A: 61.1%, B: 65.2%, C: 56.1%). However, since in this work each amplicon-specific qPCR was calibrated using the respective template (see Figure S8), we assume that the sequence bias does not affect the qPCR-based DNA quantification.

Secondary structure analysis

The prediction of the amplicon secondary structures for template A, B and C was performed using Mfold web server, based on free energy calculations.[3]

Rheology and viscosity measurements

A rotational rheometer (Physica MCR 501 from Anton Paar) equipped with concentric cylinder (CC10) and cone-plate (diameter 50 mm, cone-angle 1°) measuring cells was used to perform steady as well as small amplitude oscillatory shear experiments, respectively. The frequency range covered was from 0.05 to 15 Hz. Strain sweep experiments performed prior to frequency sweeps ensure that the strain amplitude used was sufficiently small to provide a linear material response. From these modulus curves, the elastic plateau modulus G_0 was determined as the value of the storage modulus G´ at = 0.2 Hz where it exhibits a constant plateau. For steady shear measurement, the viscosity was obtained as a function of shear rate in an interval of 10 to 1000 s^{-1}.

2 Supplemental Figures

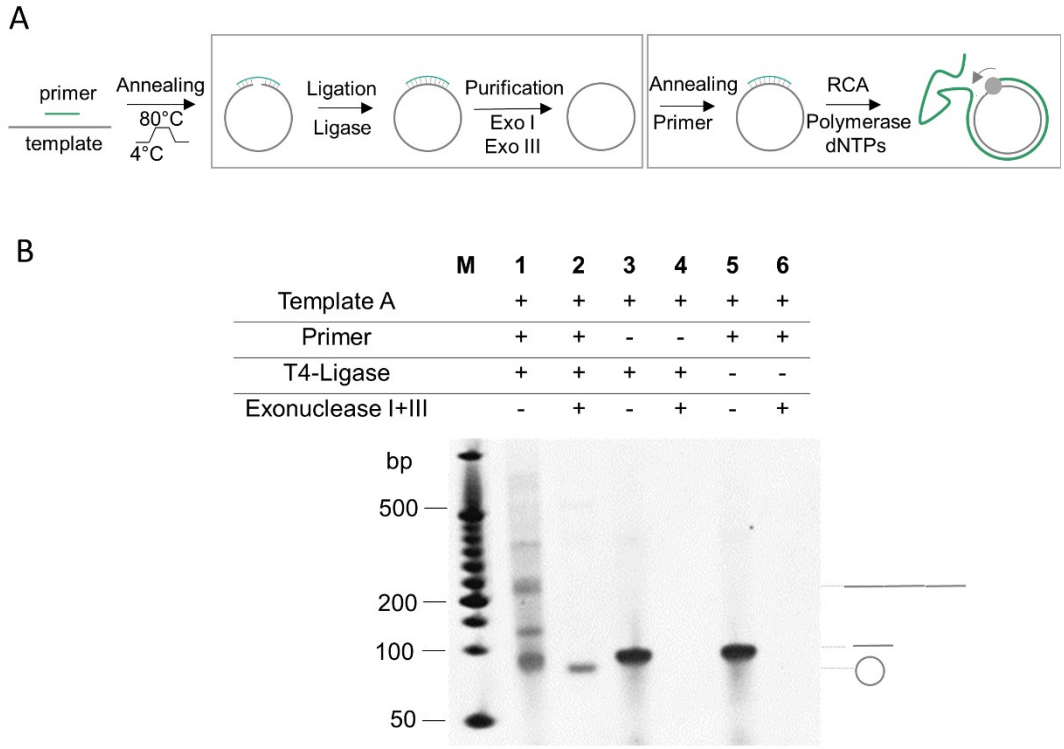


Figure S1. Synthesis and characterisation of the circular template used as starting material for RCA. **A)** Schematic representation of the workflow starting from the respective linear template strand. The annealing of the primer induces the formation of a nicked circularized structure that is subsequently ligated through the T4-ligase. To purify the circularized template from linear starting material and primer DNA an enzymatic digestion with exonuclease I and III was performed. The purified circular template was then used as starting material for the RCA process. **B)** Electrophoretic analysis of the synthesis and purification of the circular template using Urea-PAGE (12 %). In addition to intramolecular primer annealing which facilitates the circularization of one linear template molecule, intermolecular primer annealing gives rise to the formation of high-molecular weight products (lane 1). The subsequent enzymatic digestion by exonucleases I and III degrades all linear DNA, including the starting material and non-circularized products formed through intermolecular primer annealing (lane 2). As expected, in the absence of ligase, circularization did not occur, and all DNA oligomers were susceptible to degradation during the enzymatic digestion process (lane 3 and 4).

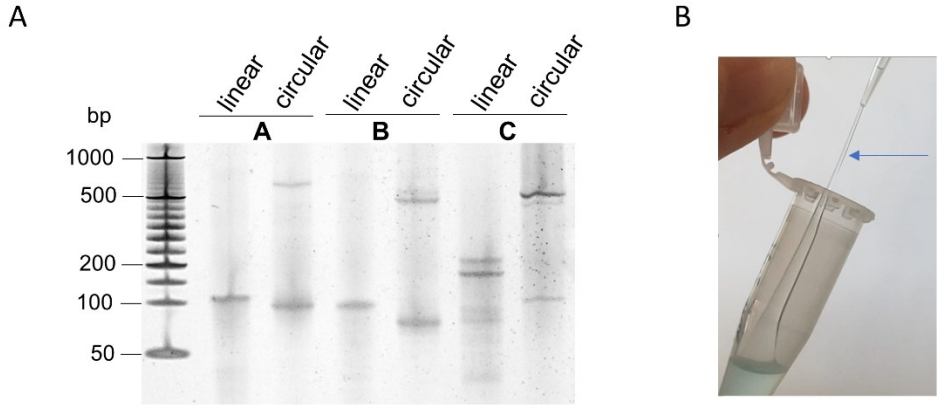


Figure S2. Characterisation of the circular templates and product formation of the RCA process. **A)** Electrophoretic analysis of the linear and circular templates A, B and C after enzymatic digestion and purification. The circular template exhibits a slightly higher electrophoretic mobility than the linear starting material as seen for all three templates. Note that the formation of an additional high-molecular circular product was observed for all three templates. However, this does not interfere with the RCA process facilitated by the phi29-polymerase, as it also enables the continuous amplification of DNA. **B)** Image of the RCA mixture after 72 h of DNA amplification by the phi29-polymerase. The formation of a viscous hydrogel was observed, indicated by the formation of long filaments during pipetting.

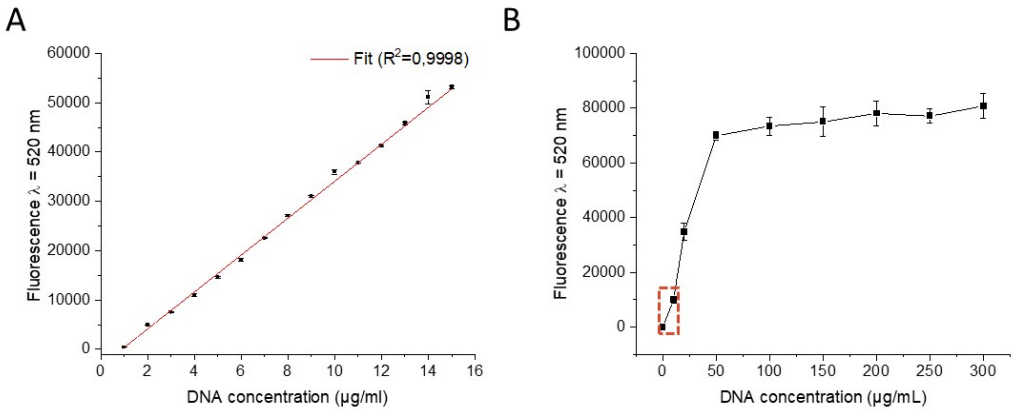


Figure S3. A) Calibration curve of Sybr Green fluorescence using solutions with known concentrations of herring sperm DNA. Error bars indicate the standard deviation from technical triplicates. **B)** Determination of the upper detection limit of the method. Note that the upper detection limit of Sybr Green I quantification is approximately 50 µg/ml, which far exceeds the DNA concentration in the analytical samples from the RCA studies (dashed outlined area). It should be noted that the measured fluorescence intensities deviate for (A) due to a different gain setting during the fluorescence measurement. Error bars indicate the standard deviation from technical triplicates.

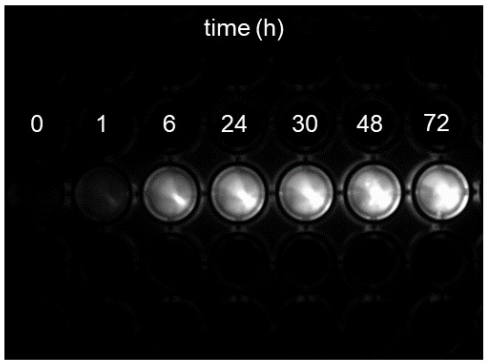


Figure S4. Representative image showcasing the fluorescence (λ_{EM} = 520 nm) emitted by the dsDNA-intercalating dye Sybr Green I during RC, shown here for template C. The fluorescence of Sybr Green I increases upon its intercalation into double-stranded DNA. The fluorescence intensity observed in the image illustrates the increasing amount of amplified DNA and consequently the accumulation of secondary structures within the solution as the RCA progresses.

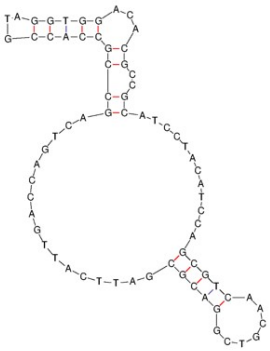
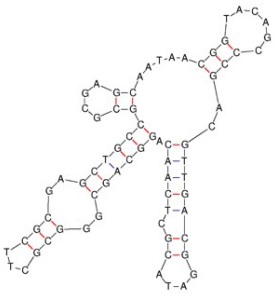
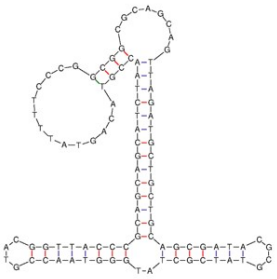
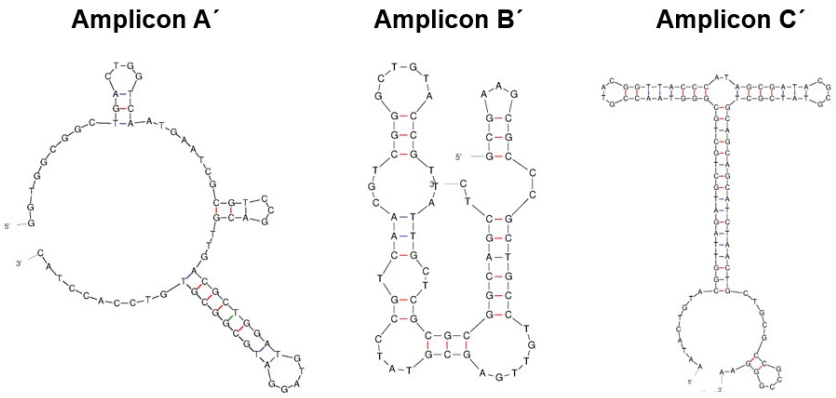
	Template A	**Template B**	**Template C**
			
Free energy	ΔG= - 2.83 kcal/mol	ΔG= - 10.97 kcal/mol	ΔG= - 24.29 kcal/mol
Intramolecular base pairing	39.4 %	58.3 %	65.3 %

Figure S5. Secondary structure prediction of the circular DNA templates A, B and C based on free energy calculations using mfold software, showing the structure predictions with the lowest free energy. Note that the free energy of the predicted structures and the number of bases involved in intramolecular base pairing varies significantly for the analyzed sequences.



Free energy	ΔG= - 7.83 kcal/mol	ΔG= - 10.07 kcal/mol	ΔG= - 32.38 kcal/mol
Intramolecular base pairing	38.9 %	55.6 %	69.4 %

Figure S6. Secondary structure prediction of the amplicon sequences A', B' and C' based on free energy calculations using mfold software, showing the structure predictions with the lowest free energy. Note that the free energy of the predicted structures and the number of bases involved in intramolecular base pairing varies significantly for the analyzed sequences.

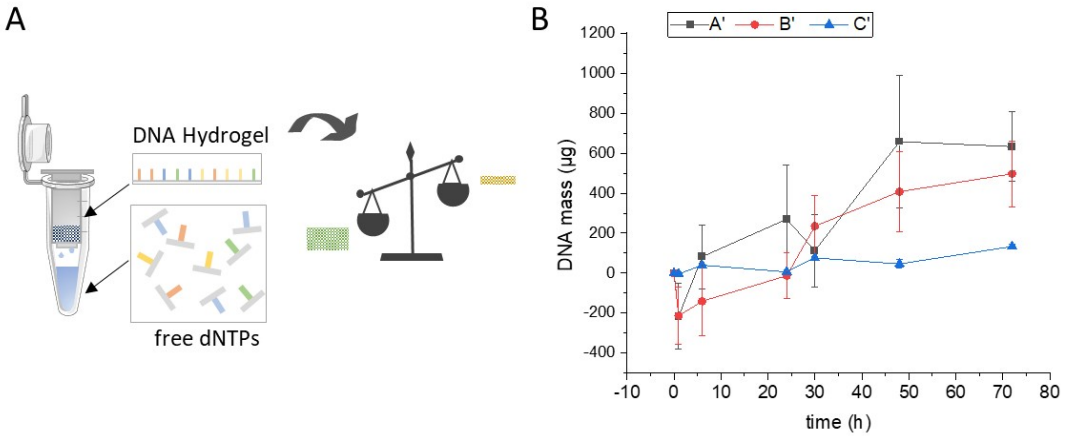


Figure S7. Gravimetric quantification of amplified DNA by weighing the RCA product remaining in the filter membrane after centrifugation. **A)** Schematic illustration of the workflow. **B)** For this purpose, the filtered macromolecular RCA products were washed, dried under vacuum centrifugation, and weighed. Note that although the data have inconsistent negative values and very large error bars, a general trend of time-dependent increase in RCA products formed. The high error rate is presumably due to the low absolute masses of the RCA products in comparison to the net weight of the Eppendorf centrifugation cup. Error bars indicate the standard deviation from three independent experiments.

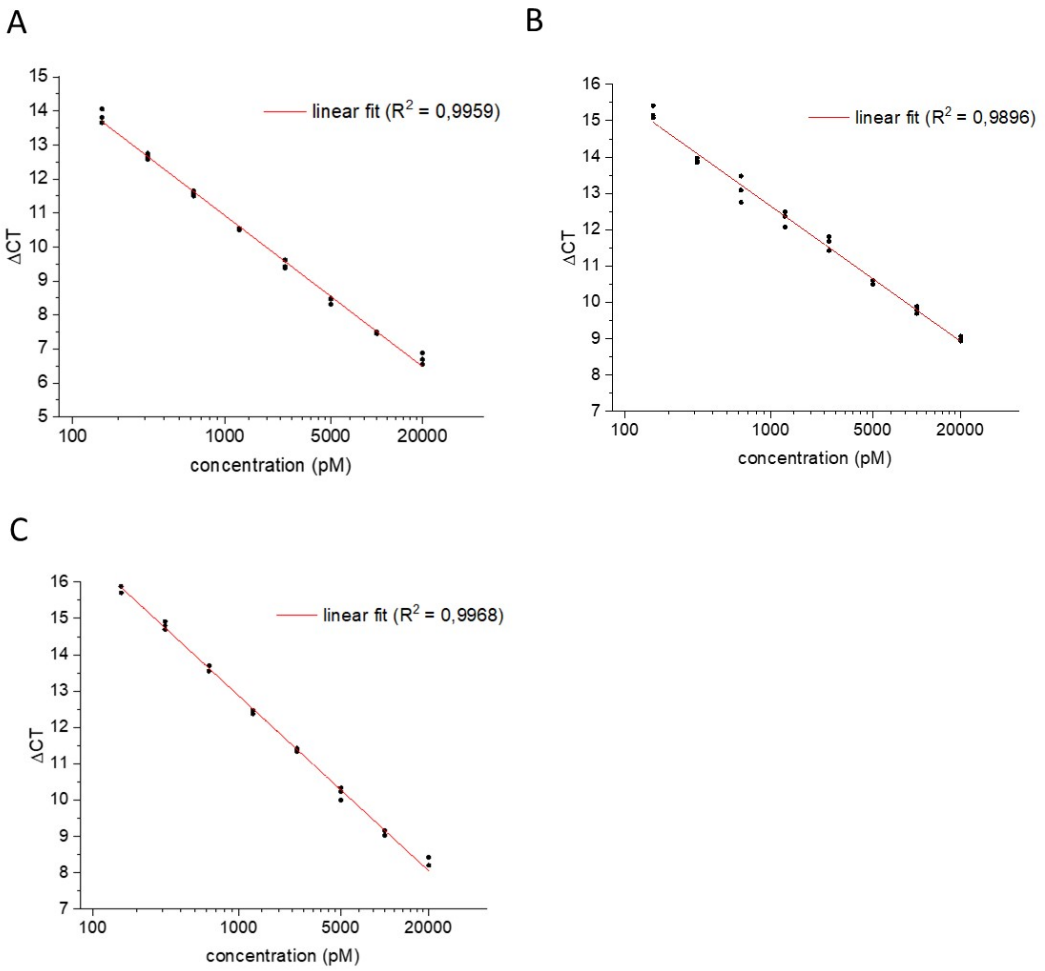


Figure S8. Amplicon calibration for the DNA quantification during RCA using qPCR. Calibration curves of a linear single-stranded DNA oligonucleotide representing one amplicon of the circular template are shown for template A **(A)** B **(B)** and template C **(C)**. All data points of technical triplicates are shown.

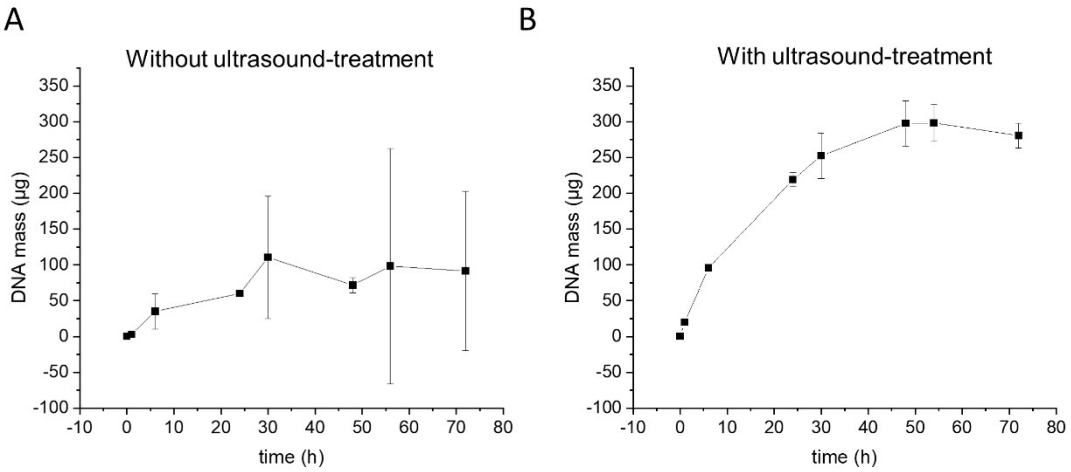


Figure S9. Effect of ultrasound treatment on the quantification of DNA during RCA using qPCR. The RCA samples obtained with template A were terminated by heat-denaturation, diluted at different concentrations (1:10-1:2000) depending on the reaction time and treated without **(A)** or with **(B)** an ultrasound-sonication of 5 seconds before being used as template for qPCR. Error bars in (A) and (B) indicate the standard deviation from three independent experiments.

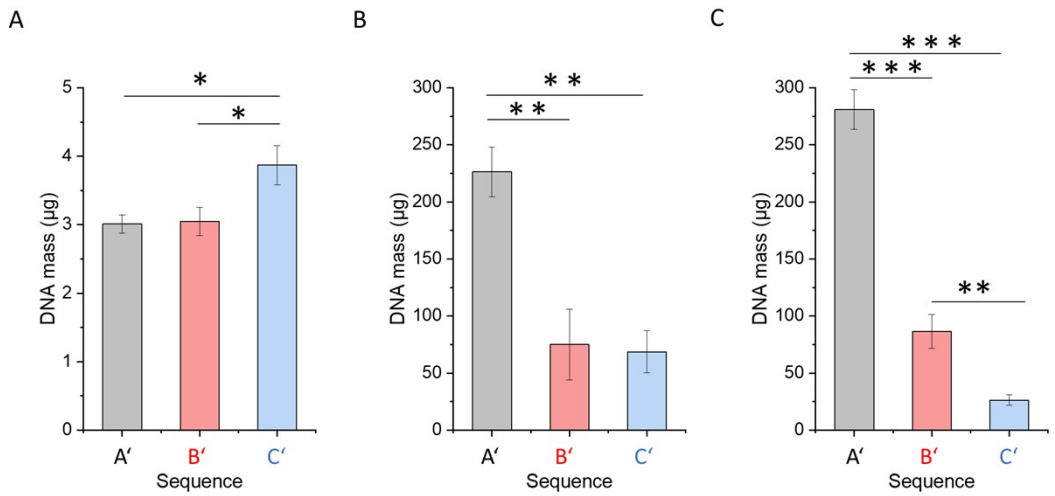


Figure S10. Results of the statistical significance testing of differences observed in the amount of amplified DNA after 72 h of RCA for sequence A', B' and C' quantified by means of **(A)** Sybr Green I assay, **(B)** Supernatant depletion and **(C)** qPCR. Data were analyzed using one-way ANOVA followed by Tukey´s multiple comparisons test. * P < 0.05, ** P < 0.01 and **** P < 0.001* for comparisons between sequence A', B' and C'.

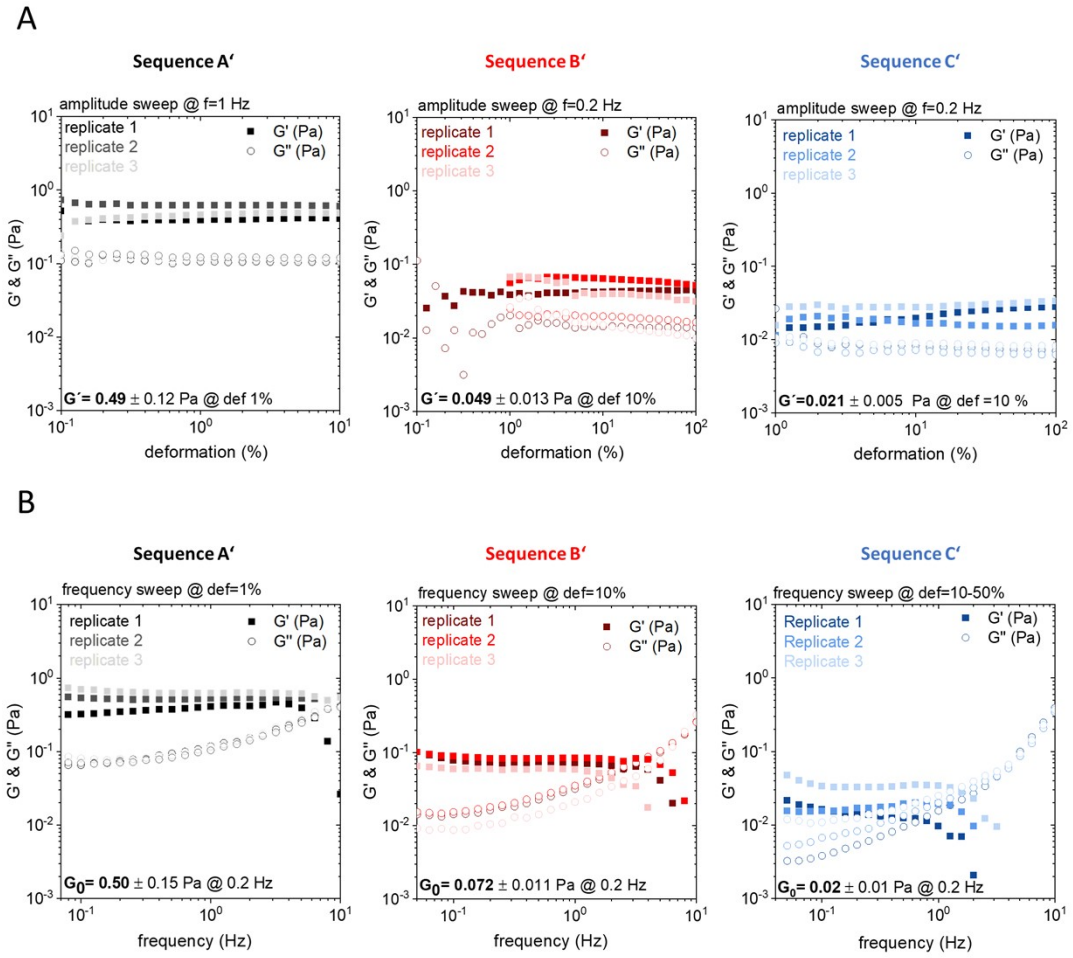


Figure S11. Rheological characterization of the mechanical properties of DNA hydrogels with different sequences (A', B', C') after 72 h of RCA. Amplitude sweep data (A) and frequency sweep data (B) of three representative hydrogel samples of each sequence are shown to demonstrate storage (G', filled symbols) and loss modulus (G'', empty symbols). The elasticity of the DNA hydrogels, characterized by the elastic plateau modulus G_0, decreases continuously for hydrogels with sequences A', B' and C', respectively. The sequence-dependent trend in the mechanical properties of the three materials is also supported by steady shear viscosity measurements (Figure S11) where a decrease of the absolute values of the viscosity at a low shear rate of 10 s^{-1} is observed following the sequence hydrogel A', B' and C'.

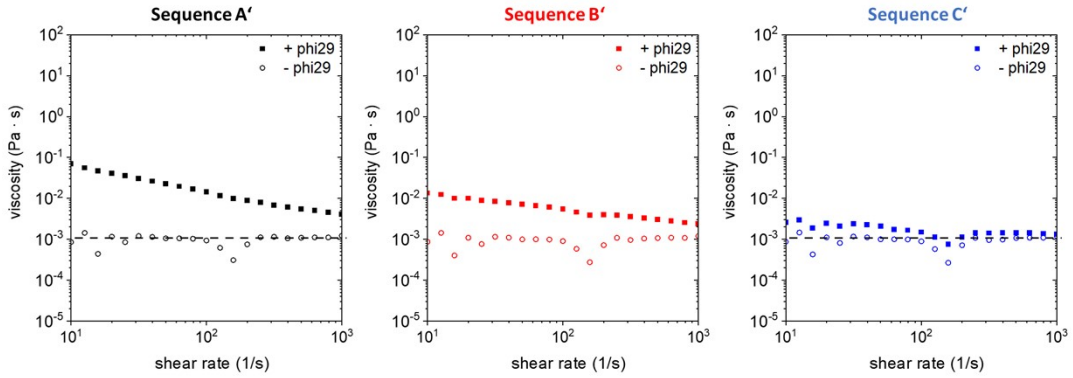


Figure S12. Viscosity of DNA hydrogels with different sequences (A', B', C') after 72 h of RCA. The viscosity is shown as a function of shear rate (1/s) for the hydrogel samples (filled symbols) and negative controls where the phi29-polymerase was omitted. The absolute values of the viscosities at low shear rates show a decrease in the viscosity for the hydrogels A', B' and C', respectively. The same sequence-dependent trend was observed in the rheological measurements (Figure S10).

3 Supplemental Tables

Table S1. DNA sequences of the templates, amplicons and primers used for RCA.

	sequence (5´→ 3´)
template A	[Phosphate]-GTAGGTGGACACGCCGCATCCTACATCCAGCGTCAACGTCGGA CGCGATTCATTGACCAGTCAGCCGCCACC
template B	[Phosphate]-GAGCTGCCGCGCGAGCAATAACGGTACAGCCCGACGTTGACGG ATACGCTCAACAGGCAGCGGGCGCTTCGC
template C	[Phosphate]-TTCCCGGCGGCGCAGCAGTTAGATGCTGCTGCAGCGATACGCG TATCGCTATGGGTAACCGTACGGTTACCCGCAGCAGCATCTAACCGTACAGT ATT
amplicon A’	GGTGGCGGCTGACTGGTCAATGAATCGCGTCCGACGTTGACGCTGGATGTA GGATGCGGCGTGTCCACCTAC
amplicon B ‘	GCGAAGCGCCCGCTGCCTGTTGAGCGTATCCGTCAACGTCGGGCTGTACCG TTATTGCTCGCGCGGCAGCTC
amplicon C’	AATACTGTACGGTTAGATGCTGCTGCGGGTAACCGTACGGTTACCCATAGCG ATACGCGTATCGCTGCAGCAGCATCTAACTGCTGCGCCGCCGGGAA
ligation-Primer A	CCACCTACGGTGGCGGCTGA
ligation-Primer B	GGCAGCTCGCGAAGCGCCCG
ligation-Primer C	TCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA
RCA-Primer A	CCGACGTTGACG
RCA-Primer B	CGTCAACGTCGG
RCA-Primer C	TCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA

Table S2. DNA sequences of the primers and probes used for qPCR.

qPCR primer / probe	**sequence (5´→ 3´)**	**T_m (°C)**
primer_fwd Amplicon A’	GTGGCGGCTGACTGGTCAATG	58.3
primer_rev Amplicon A’	GGACACGCCGCATCCTACATC	58.3
TaqMan probe_Amplicon A‘	[FAM]-CGCGTCCGACGTTGACGC-[TAMRA]	57.2
primer_fwd Amplicon B’	GAAGCGCCCGCTGCCTGTTG	60.0
primer_rev Amplicon B’	GCCGCGCGAGCAATAACGG	57.6
TaqMan probe_Amplicon B‘	[FAM]-GCGTATCCGTCAACGTCGGG-[TAMRA]	57.9
primer_fwd Amplicon C’	GATGCTGCTGCGGGTAACCG	57.9
primer_rev Amplicon C’	CCGGCGGCGCAGCAGTTAG	59.7
TaqMan probe_Amplicon C‘	GATACGCGTATCGCTGCAGCAG	58.6

4 References

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[3] M. Zuker, *Nucleic Acids Res.* **2003**, *31*, 3406-3415.