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Optimization of the experimental parameters of the ligase cycling reaction

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ABSTRACT

The ligase cycling reaction (LCR) is a scarless and efficient method to assemble plasmids from fragments of DNA. This assembly method is based on the hybridization of DNA fragments with complementary oligonucleotides, so-called bridging oligos (BOs), and an experimental procedure of thermal denaturation, annealing and ligation. In this study, we explore the effect of molecular crosstalk of BOs and various experimental parameters on the LCR by utilizing a fluorescence-based screening system. The results indicate an impact of the melting temperatures of BOs on the overall success of the LCR assembly. Secondary structure inhibitors, such as DMSO and betaine, are necessary for assemblies made of large parts ($>350\,\mathrm{bp}$) whereas they show negative effects for assemblies with mixtures of small and large parts. Adjustments of the annealing, ligation and BO-melting temperature are useful but depend on the sizes of the DNA fragments. Based on this, a step-by-step protocol is offered within this study to ensure a transferable routine for high efficient LCR assemblies.

INTRODUCTION

It is the goal of synthetic biology to specify, design, build and test genetic circuits, and this goal requires rapid prototyping approaches to facilitate assembly and testing of a wide variety of circuits. To this end, many assembly methods were used in the last decades to build DNA constructs, e.g., Gibson assembly (1), Golden Gate assembly (2, 3), circular polymerase extension cloning (CPEC, 4), biopart assembly standard for idempotent cloning (BASIC, 5) and yeast homologous recombination (YHR, 6). Some of these methods require specific modifications of the DNA parts such as overhangs or restriction sites, which hamper the reusability, while other methods are slow or leave scars. Additionally, DNA part standardization approaches, e. g. the BioBrick-system, result in sequence redundancies which have a negative impact on assembly efficiencies (7).

By contrast, the ligase cycling reaction (LCR) fulfills the prerequisites for automated assemblies and uses phosphorylated DNA parts (8, 9, 10, 11). The assembly order is determined by single-stranded oligonucleotides building a bridge (so-called bridging oligonucleotides, BOs) between adjacent parts. Bridging oligos are typically designed by the scientists, based on general rules provided by the literature (8, 9). One important parameter for the LCR-assembly is the melting temperature (T_m) of the BOs at around $70\,^{\circ}\mathrm{C}$ for each half to facilitate optimal hybridization of template and oligonucleotide for given cycling parameters. Closely related is the free energy ΔG, which is assumed as the more important quantity for oligonucleotide-based biological experiments (12, 13). In the LCR, this impact is counteracted by using dimethyl sulfoxide (DMSO) and betaine to increase ΔG and thus to reduce secondary structures (8, 9). Nevertheless, the role of ΔG-related crosstalk and the potential of ΔG-optimized BOs in the LCR has not been investigated so far.

The literature offers several tools regarding LCR optimization. Nowak et al. (14) provides a tool for the assembly of DNA that codes for a protein, where they return both the DNA fragments as well as the BOs to minimize unwanted effects. The tool considers codon mutations, as long as they encode the same amino acid, and is intended to be applied for LCR-based gene synthesis. Bode et al. (15) offers similar functionality. Another web-application includes the design of primers and performs T_m and ΔG cross-checks for the oligonucleotide sequences against themselves, their DNA probes and whole genomes (12) but is not applied for the LCR. Robinson et al. (16) use a BO-design-tool with an adjustable target melting temperature but without optimizing the crosstalk. An experimental perspective is given by de Kok et al. (9), where a design-of-experiment approach and multivariate data analysis were used to assess the impact of a wide range of parameters including the concentrations of the secondary structure inhibitors DMSO and betaine. The following study starts with these baseline-conditions. The LCR is investigated with the scope on the impact of the choice of BOs, their intramolecular and intermolecular crosstalk and the context of the experimental temperatures. For this, a toy-model plasmid and fluorescence-based readout is utilized (graphical abstract: Figure 1) to detect and validate the influence of all parameters and to generate new rules for an optimized LCR-assembly.

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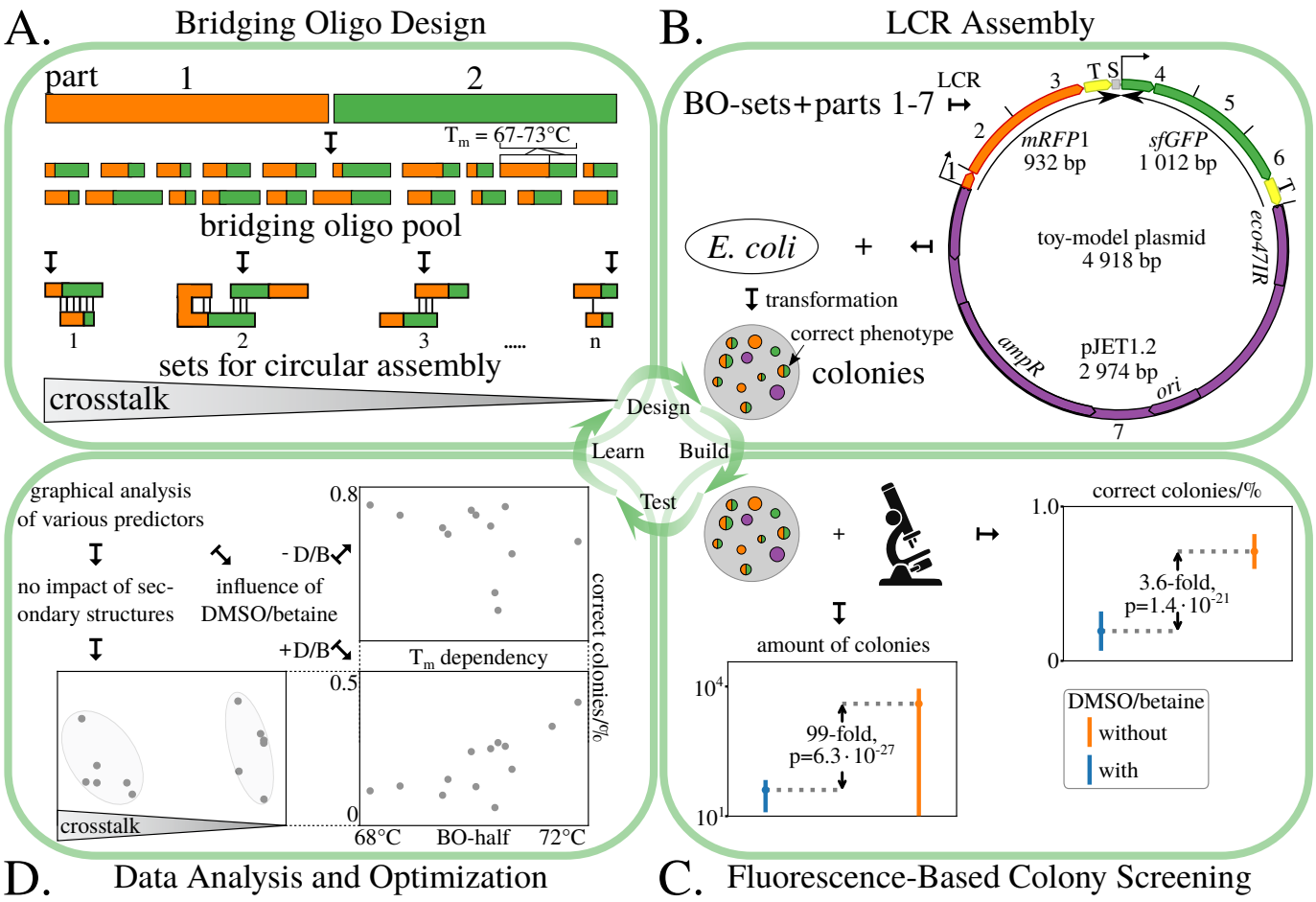

Figure 1. Workflow for the LCR optimization. **A**. Bridging oligo-sets (BO-sets) were designed using general design rules with the focus on ΔG-related BO-crosstalk while maintaining a T_m of 70 ± 3°C. **B**. In total, 25 sets were designed, including one manually designed set using the software `Primer3`, and utilized for the LCR-assembly of a toy-model plasmid. The plasmid consists of seven parts with lengths in the range of 79 bp to 2974 bp and a total length of 4918 bp. It contains two genes for fluorescent proteins, *mRFP1* and *sfGFP*, and a vector. Both fluorescent protein genes were split into three subparts (*mRFP1*: parts 1-3, *sfGFP*: parts 4-6). The same terminator *BBa_B0015* (T) was used twice to simulate sequence redundancies. A DNA-spacer S (37 bp) was added at the end of part 3 to avoid hybridization of the BO utilized for the ligation of parts 6 and 7. The sequences of all parts are shown in Supplementary Table S1. **C**. The toy-model plasmid enables a fast and reliable fluorescence-based readout to observe the LCR efficiency and the total amount of colonies to investigate various LCR conditions. Based on this method, a significant negative impact by using the baseline-LCR conditions (8 % − v/v DMSO, 0.45 M betaine) was revealed for the seven-part toy-plasmid. The p-values were derived from a Kolmogorov-Smirnov test between the two sets of data shown in Figure Supplementary Figure S1. **D**. With the focus on all BO-sets, no ΔG-related impact was detected in the baseline and DMSO/betaine-free LCR conditions. Further graphical analysis of the BO-sets revealed an association of the average BO-T_m, the efficiency and total amount of colonies. Finally, the optimizations were applied on another split of the toy-plasmid (three-part design: 1. *mRFP1*, 2. *sfGFP*, 3. vector). *ampR*: gene for ampicillin resistance, *±D/B*: with or without DMSO and betaine, *eco47IR*: gene for a restriction enzyme (reduction of religations of the vector), *mRFP1*: monomeric red fluorescent protein 1, *ori*: origin of replication, *sfGFP*: superfolder green fluorescent protein, T_m: melting temperature.

MATERIALS AND METHODS

Toy-model plasmid

All LCRs were performed with a toy-model plasmid made of seven fragments (Figure 1B), with a total length of 4918 bp. This plasmid consists of six inserts and the plasmid cloning vector CloneJet pJET1.2/blunt (2974 bp; Thermo Fischer Scientific, Massachusetts, USA). For the inserts, two genes of fluorescent proteins, *sfGFP*, *mRFP1*, were ordered at Addgene (www.addgene.org: pYTK001, pYTK090; 17) and split into three subparts each to increase size heterogenity, so that the plasmid fragment length ranges from 79 bp to 2974 bp. Additionally, the same terminator *BBa_B0015* was used in both fluorescent protein genes to further increase assembly complexity. Due to this sequence homology, a spacer sequence of 37 bp was added at the 3'-end of part 3 to prevent the ligation with part 7. For all *in silico* cloning, the software `Geneious` was utilized (v. 11.0.5, http://www.geneious.com, 18).

Part amplification

Primers for the amplification (Eurofins Genomics, Ebersberg, Germany) were phosphorylated by the T4-polynucleotidekinase/-buffer (New England Biolabs, Ipswich, USA) prior to amplification *via* PCR (Q5® High-Fidelity Polymerase, New England Biolabs, Ipswich, USA). Forward and reverse primers were phosphorylated separately in a total volume of 50 µL, a primer-concentration of 1 µM, 2 mM ATP and 10 U of T4-PNK for 1 h at 37 °C and 20 min at 65 °C for the denaturation. The low primer concentration was chosen

because it is beneficial for the enzymatic phosphorylation. The 79 bp promotor of *mRFP1* (part 1, Figure 1B) was ordered as forward and reverse strand (lyophilized, salt-free). Both strands were phosphorylated separately, as described for the amplification primers followed by an annealing procedure to obtain double-stranded DNA (3 min at 95 °C and 70 cycles of 20 s with an incremental decrease of 1 °C). The vector pJET1.2/blunt was PCR-amplified using a template suitable to detect plasmid-carryover by blue-white screening (pJET1.2/blunt+*lacZ*). Prior to amplification of the vector, the pJET1.2/blunt-*lacZ* plasmid was linearized by restriction digestion (restriction site in the *lacZ*). Afterwards, all PCR products were DpnI-digested (60 min at 37 °C; inactivation: 20 min at 80 °C), purified (column-based NEB Monarch® PCR & DNA Cleanup Kit; New England Biolabs, Ipswich, USA) and the DNA quantity/quality was measured by photometry (Spectrophotometer UV5Nano, Mettler Toledo, Columbus, USA). Afterwards, the phosphorylated primers and DNA parts were stored at −20 °C. The sequences of all amplification primers are shown in Supplementary Table S2. The sequences of the toy-model parts are shown in Supplementary Table S1.

Bridging oligo design

Bridging oligos were predicted according to the rules given in de Kok et al. (9): they were all orientated in forward direction and designed at specific ingredient concentrations of 10 mM Mg^{2+}, 50 mM Na^{+}, 3 nM plasmid parts, 30 nM BOs and 0 mM dNTPs. All BOs were ordered lyophilized from Eurofins Genomics (Ebersberg, Germany) as salt-free custom DNA oligonucleotides. Quality was checked by Eurofins Genomics *via* matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) or capillary electrophoresis (CE).

One BO-set was designed manually with the melting temperature tool of `Primer3` (19), which is distributed with the software suite `Geneious`. For the T_m calculation and salt correction, the nearest-neighbor algorithm and the corresponding salt correction by SantaLucia (20) were utilized to design a BO-set with melting temperatures of 70 °C for each half-BO. `Primer3` only accepts a single DNA concentration, despite the experiment using different concentrations of parts and BOs. As prompted by `Geneious`, only the BO concentration of 30 nM was inputted. This manual set is denoted by an "M".

To investigate the crosstalk of BOs, more sets were designed by minimizing or maximizing ΔG-dependent crosstalk between oligonucleotides while maintaining a T_m between 67 °C and 73 °C. Crosstalk is defined as the sum of all minimum free energies (MFEs) when cofolding each oligonucleotide of a BO-set with each oligonucleotide in that set and with itself. As a reference temperature for the crosstalk calculations, the annealing temperature of the multi-step LCR protocol was used (55 °C, 9). As with the manual set, the SantaLucia parameters were utilized for the T_m calculations. Additionally, the DNA part concentration was adjusted to 3 nM to match the experiment. The impact of DMSO and betaine were not considered for the calculations of the MFEs. Sets with minimized crosstalk are denoted by "L" for low crosstalk and sets with maximized crosstalk by a "H" for high crosstalk. All BO-sets, sequences and melting temperatures are provided in Supplementary Table S3.

Ligase cycling reaction

For the assembly, the purified PCR products were mixed with supplements with the following concentrations: 1× ligase buffer, 0.5 mM NAD^{+}, 3 nM of toy-plasmid parts 1-6, 0.45 M betaine and 8 %−v/v DMSO. In contrast to de Kok et al. (9), the concentration of the vector pJET1.2/blunt (part 7) was reduced to 0.3 nM to achieve fewer religations of the vector. LCRs using these experimental concentrations are called baseline-conditions. The 10×-Ampligase® reaction buffer was self-made with the concentrations described by the manufacturer of the Ampligase® (Lucigen, Wisconsin, USA). NAD^{+} was supplied separately by using a self-prepared 10 mM stock solution. Bridging oligo sets were premixed in nuclease-free water with 1.5 µM of each BO, heated up for 10 min at 70 °C and cooled down on ice before adding them to the split master-mixes. Subsequently 10 U of Ampligase® (Lucigen, Wisconsin, USA) was added. After rigorous mixing and centrifugation, each LCR was split in at least three ($n=3$) reactions with a cycling-volume of 3 µL. The results presented in Figure 2 and Supplementary Figure S7 were based on LCR-quintuplets ($n=5$) and show the impacts of utilizing or omitting DMSO and betaine. After the cycling, all LCRs were cooled down on ice to recondense evaporated liquid in the PCR-tubes, and centrifuged.

For the cycling, a DNA Engine Tetrad®2 thermal cycler, 96-well Alpha™ Unit cycling block and low-profile PCR stripes (Bio-Rad Laboratories GmbH, Muenchen, Germany) were utilized. The speed of ramping used for all LCRs was 3 °C s^{-1}. The cycling was initiated by a denaturation step at 94 °C for 2 min, followed by 25 cycles at 94 °C for 10 s, 55 °C for 30 s and 66 °C for 1 min. In contrast to the published protocols (8, 9), 25 cycles instead of 50 cycles were used due to the low total LCR volume of 3 µL. Afterwards, 30 µL electro-competent or chemically-competent NEB® 10-β *E. coli* cells (self-made batches; New England Biolabs, Ipswich, USA) were mixed with each LCR and the total volume was transferred to cuvettes for electroporation with a diameter of 1 mm. The transformations were performed by applying an electric pulse of 2.5 kV or by using 96-well PCR-plates and a cycling block for the heat-shock. For the heat-shock, the 96-well plates were put on ice for 30 min, then heat-shocked for 30 s at 42 °C, then put on ice again for 10 min. After the recovery at 37 °C for 1 h in 470 µL SOC medium (chemical transformation: 170 µL), an appropriate volume of the transformation mix was plated on Lysogeny Broth (Miller) plates containing 1 %−m/v agar and 100 µg mL^{-1} ampicillin, which were grown for 15 h at 37 °C.

Electroporations were performed serially for each single LCR: 1. cells were mixed from a master-aliquot for each experiment with one LCR-replicate of one LCR condition, 2. suspension was transferred to a cuvette, 3. an electric pulse was applied, 4. SOC-medium was added, 5. the cell-suspension was transferred to a tube, 6. the tube was put in a thermal cycler for recovery and 7. finally the suspension was plated on agar plates. Each LCR condition was transformed in a non-batch-wise manner to reduce workflow-derived influences, so that, e.g., each condition was transformed once

before proceeding with the second replicates. For chemical transformations, the experiments were performed in a more parallel manner: 1. cells were mixed from a master-aliquot for each experiment with all replicates of all LCRs in a 96-well plate using a multi-channel pipette, 2. the suspensions were incubated on ice, 3. the heat-shock was applied in a PCR-cycler, 4. the suspensions were put on ice, 5. SOC-medium was added to all LCR-cell-mixes followed by the recovery and plating.

Colony screening and plasmid sequencing

By transforming the LCRs into *E. coli*, the toy-model plasmid enables a fluorescence-based discrimination of correct (red-fluorescent and green-fluorescent CFUs) and misligated plasmids (red or green or non-fluorescent CFUs). To investigate the LCR assembly, the efficiencies of all LCRs were determined by observing the phenotype of ampicillin-resistant CFUs *via* fluorescence microscopy (microscope: Axio Vert.A1, Carl Zeiss Microscopy GmbH, Jena, Germany, 50×-magnification; LEDs for sfGFP/mRFP1: $470/540-580\,\mathrm{nm}$). To calculate the LCR efficiency, the phenotypes of all CFUs per LCR were screened. For plates with more than 100 CFUs, 100 colonies were screened randomly by first picking the spot of interest macroscopically followed by the observation with the microscope. The efficiency was obtained by dividing the number of correct CFUs by the total number of observed CFUs. The amount of CFUs per 3 µL was obtained from the total CFUs per plate and the used plating volume and dilution.

To validate this fluorescence-based system, plasmids from 120 CFUs with different phenotypes were isolated with the Monarch® Plasmid Miniprep Kit (New England Biolabs, Ipswich, USA) and analyzed *via* Sanger-sequencing (Eurofins Genomics, Ebersberg, Germany) to correlate them with the genotypes.

Graphical analysis

Unless specified otherwise, T_m calculations in the following sections were performed with the thermodynamic parameters and salt correction by SantaLucia (20) and the divalent salt conversion by von Ahsen et al. (21). The shown T_ms are of half-BOs. Each half-BO is complementary to the DNA part that is designed to attach to. A BO is the direct concatenation of two half-BOs. Dangling ends and coaxial stacking were not included in the calculation. Error bars are the standard deviation with Bessel's correction. The average T_m of a BO-set is obtained from all half-BOs of the set.

RESULTS AND DISCUSSION

Toy-Model Plasmid Offers Robust System to Investigate LCR Assemblies

To simulate a challenging SynBio-construct for the investigations of the LCR, a toy-model plasmid was designed (Figure 1B). This plasmid consists of seven parts of varying size (79 bp to 2974 bp) and the same BioBrick terminator sequence *BBa_B0015* in both fluorescent protein genes. To investigate the LCR, different bridging oligo sets, experimental parameters, a toy-model plasmid and fluorescence-based analysis were used. For the analysis and optimization, ∼100 CFUs/LCR (if available) were screened *via* fluorescence microscopy to calculate the efficiency of each assembly reaction. The amount of CFUs per 3 µL LCR was determined by macroscopic counting of all colonies per agar plate and extrapolation by using the dilution-factor. In total, 61 different experimental conditions were tested and more than 15000 colonies were screened by fluorescence-microscopy to obtain the assembly efficiencies. In contrast to the vector concentration of $3\,\mathrm{nM}$ in the literature (8, 9), the concentration was decreased to $0.3\,\mathrm{nM}$ to counteract religations. Positive effects of increasing the molar insert-to-vector ratio were already described for other cloning methods (22, 23) and were confirmed in preliminary LCRs (data not shown).

No fluorescent CFUs were obtained in the control reactions without the ligase (Figure 2). Additionally, blue-white screening of about 1000 non-fluorescent CFUs revealed no carry-over of the template used for the amplification of the vector pJET1.2/blunt. A carry-over of the templates pYTK001 or pYTK090 used for the amplification of *sfGFP* and *mRFP1* was not possible due to a change of the antibiotic resistance marker gene.

To validate the fluorescence-based readout, the observed phenotypes of 120 CFUs with different phenotypes were correlated with the corresponding plasmids *via* Sanger-sequencing. The analyzed plasmids from 60 CFUs with a bicolored fluorescence (red and green) contained all seven DNA parts in the correct order/orientation when compared with the *in silico* sequence of the toy-plasmid. Sequencing results of 60 plasmids with a different phenotype (20 plasmids from only green fluorescent CFUs, 20 plasmids from only red fluorescent CFUs and 20 plasmids from non-fluorescent CFUs) indicated that they lacked at least one *sfGFP*-subpart or *mRFP1*-subpart (still red or green) or were religated vector (no fluorescence). The latter phenotype was also observed for plasmids with missing subparts of both fluorescent protein genes in preliminary experiments. Point-mutations in the LCR products were regarded as errors introduced by amplification primers, PCRs or *E. coli* and were not treated as LCR-misassemblies.

Within our experiments, the misassembled plasmids from green fluorescent colonies lacked at least one subpart of the *mRFP1*. Interestingly, about 10 bp to 100 bp of both ends of the missing subparts were still existent. For the 20 analyzed CFUs with only the red fluorescent phenotype, the plasmids lacked the spacer sequence at the 3'-end of the *mRFP1* and the entire *sfGFP*. This suggests that *E. coli* can recognize the 129 bp *BBa_B0015* terminator that is used for both *sfGFP* and *mRFP1* and delete it by *recA*-independent recombination (24, 25). Related to this, the BO that spans parts 6 and 7 can partly hybridize with the terminator in part 3 and may negatively influence the ligation.

Another issue is related to the heterogeneous LCR-mixture, which contains the desired DNA fragments, debris of these fragments from PCRs, amplification primers and PCR templates (even if they are digested and purified). *E. coli* may circularize any linear DNA in the mixture. The subsequent transformation of *E. coli* enables a ring closure by endogenous mechanisms and the growth of CFUs containing the religated vector or plasmids with missing parts. This was observed in

the control reaction without ligase in Figure 2. Thus the carry-over of DNA in combination with the ability of *E. coli* to ligate linear DNA contributes both to the amount of misassembled plasmids and correct plasmids.

In summary, the fluorescence-based screening of colonies employed here is a valid and fast (manually: $\sim 500\,\mathrm{CFUs\,h^{-1}}$) method to determine the assembly efficiencies and to investigate the influence of changing parameters in the LCR. A correlation of the phenotype and genotype is a useful tool without the need for next generation sequencing and offers an objective true-false readout by microscopy (as utilized for these studies) or photometric analysis of images (using e.g. `OpenCFU`, 26; `CellProfiler`, 27).

Influence of DMSO, betaine and the T_m of bridging oligos on LCR assemblies

Thirteen different BO-sets, with sequences given in Supplementary Table S4, were used to assemble the toy-model plasmid made of seven fragments. Two separate experiments were performed with different conditions. The baseline-LCR used $8\,\%-\mathrm{v/v}$ DMSO and $0.45\,\mathrm{M}$ betaine, whereas the crosstalk-increased LCR did not use any DMSO or betaine.

On average, the LCRs with both detergents revealed $3.6\times$ lower efficiencies and $99\times$ fewer total CFUs per $3\,\mu\mathrm{L}$ in comparison to assemblies without DMSO and betaine ($p<0.001$, Figure 1C; raw data in Supplementary Figure S1). Further confirmation of these results can be seen in Supplementary Figure S7 and Supplementary Figure S9 in more consistent experiments using the same batches of DNA-parts and competent cells. Graphical analysis of various predictors revealed no ΔG-related effects for assembling the toy-plasmid for both experimental setups (Figure 3A and 3B; more predictors in Supplementary Figure S2, Supplementary Figure S3, Supplementary Figure S4 and Supplementary Figure S5). Guanosine or cytosine were not found to be necessary at the 3'-end of BOs.

For LCRs with DMSO and betaine, BO-related differences were detected, e.g., the LCR using BO-set L1 was less efficient than using H1 (Figure 2A). Further analysis of the used BO-sets revealed an impact of the melting temperature despite the T_ms of all sets being similar and found in the range from $68\,^{\circ}\mathrm{C}$ to $72\,^{\circ}\mathrm{C}$ (Figure 3C). Sets with a BO-T_m of $68\,^{\circ}\mathrm{C}$ were found to have 10% efficiency whereas sets with a BO-T_m of $72\,^{\circ}\mathrm{C}$ were found to have 50% efficiency. This suggests that a melting temperature higher than $72\,^{\circ}\mathrm{C}$ may result in even better assemblies for LCRs with DMSO and betaine. This is confirmed by recalculating the melting temperatures of the set of sequences designed to have a T_m of $70\,^{\circ}\mathrm{C}$ by de Kok et al. (9), who used the SantaLucia parameters with the salt correction by Owczarzy et al. (28). We have found the average T_m of these half-BOs to be $72.2\,^{\circ}\mathrm{C}$ when calculated using these parameters. When using the SantaLucia parameters and SantaLucia salt correction as has been done so far in this study, the T_m is found at $74.8\,^{\circ}\mathrm{C}$. The impact of different salt corrections is illustrated in Supplementary Figure S6. Overall, for LCRs with $8\,\%-\mathrm{v/v}$ DMSO and $0.45\,\mathrm{M}$ betaine, a BO target temperature above $70\,^{\circ}\mathrm{C}$ was found to be beneficial.

Investigation of the `Primer3` source code used for the design of the manual BO-set and comparison with SantaLucia (20) revealed that the code expects both concentrations to be identical and to sum up to the input amount. Thus the prompted BO concentration of $30\,\mathrm{nM}$ corresponds to part and BO concentrations of $15\,\mathrm{nM}$. Due to this, the manual BO-set had an average T_m of $71.5\,^{\circ}\mathrm{C}$ (Figure 3) when evaluated in full accordance with the SantaLucia formula instead of the targeted $70\,^{\circ}\mathrm{C}$.

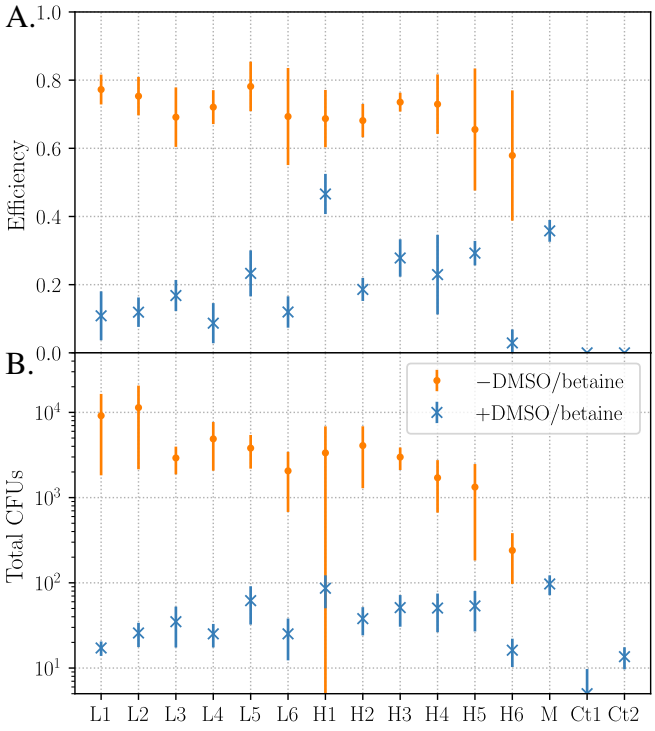

Figure 2. LCRs of a seven-part toy-plasmid by utilizing bridging oligo-sets (BO-sets) with high crosstalk (sets H1-H6) and low crosstalk (sets L1-L6). Each LCR was performed as a quintuplet. The standard deviation for each LCR is indicated by error bars. In addition to these twelve sets, a 13th set ("manual" set M) was utilized for the baseline-LCR ($8\,\%-\mathrm{v/v}$ DMSO and $0.45\,\mathrm{M}$ betaine). The total amount of colonies are plotted on a logarithmic scale. **A.** All LCRs without DMSO/betaine resulted in higher efficiencies in comparison to the LCRs with both detergents. No correlation between crosstalk and BO performance was found. Additionally, BO-set dependent differences were found for the baseline-LCRs, e.g., BO-set H1 resulted in a higher efficiency than BO-set L1. The negative control reactions with BOs without ligase (Ct1) and without BOs and ligase (Ct2) resulted in no fluorescent colonies. **B.** LCRs without DMSO/betaine resulted in more colonies in comparison to the baseline-conditions. The raw data of the LCRs presented here are shown in Supplementary Figure S1. The sequences of all BO-sets are shown in Supplementary Table S3. BO: bridging oligo, CFU: colony forming unit, DMSO: dimethyl sulfoxide.

An alternative approach to obtain higher assembly efficiencies without rising costs due to synthesizing longer oligonucleotides is to omit the liquids DMSO and betaine. This omission also aids automated liquid handling approaches because both detergents have unfavorable properties like extreme viscosities, hygroscopic characteristics and acting as surfactants. These new experimental conditions greatly improved both the efficiency and total number of CFUs (Figure 2). Consistent with the results of the LCRs with DMSO/betaine, the melting temperature is the most influencing parameter. As a result of the omission of DMSO and betaine, BO-sets with lower T_ms were favorable, yielding the greatest total amount of colonies at high efficiency

(Figure 3D). This behavior is also observed by comparing the total CFUs derived by utilizing the BO-sets L2, H1 and the manual one (Supplementary Figure S7). Similar to this seven-part toy-plasmid, another LCR-design with a T_m-independent BO-design (20 bp for each BO-half) was published by (11) where six parts in a range of 79 bp to 2061 bp were assembled with high efficiencies. The *Taq*-ligase and a two-step LCR-protocol with annealing and ligation at the same temperature of 60 °C were utilized.

An explanation for low efficiencies in the baseline-LCR is related to the large difference between the annealing temperature of 55 °C and ligation temperature of 66 °C in combination with the utilization of DMSO and betaine. Both detergents reduce the energies required for strand separations (29, 30). Together with the experimental temperatures, this may result in an extensive reduction of template for the ligase by separating already hybridized BO/probe double strands. Bridging oligo-sets with lower T_ms are theoretically more affected than sets with higher melting temperatures and should benefit from decreasing the temperature interval. Related to this, a reduction of the ligation temperature is expected to be advantageous. This was validated by using three BO-sets with different melting temperatures (Supplementary Figure S8) despite the ligation temperature of 60 °C being lower than the optimum temperature of the ligase (according to the manufacturer: 70 °C). Increasing the annealing temperature, which would also decrease the interval, was assumed to be disadvantageous due to accelerated BO/template-separation and was not investigated.

Several mechanisms are suspected to cause the total CFU decrease in LCRs with DMSO/betaine. First, lower LCR efficiencies result in fewer fully assembled plasmids and fewer colonies. The effects of DMSO and betaine were also investigated separately to prove these results (Supplementary Figure S9). Second, DMSO/betaine negatively influence the electroporation process. This can also be seen in Supplementary Figure S9, where an LCR without DMSO/betaine was mixed with both detergents before the electroporation and 3-4× fewer CFUs were obtained in comparison to DMSO/betaine-free controls. In contrast to the literature (8, 9), a lower volume-ratio of LCR and cells was used for the transformations (1:10; 2× higher concentration of DMSO/betaine). These conditions are not toxic for the *E. coli* strain NEB® 10-β because chemical transformations of the same strain with the plasmid pUC19 revealed no negative effects of utilizing a combination of DMSO and betaine (Supplementary Figure S12). A negative impact of DMSO and a positive impact of betaine was observed. A strain-dependent influence of DMSO in chemical transformations was described in (31) and such an influence is also plausible for betaine and a combination of both reagents. To counteract the CFU-reducing effects of detergents, a 30 min dialysis using *aq. dest.* and a nitrocellulose-membrane after the LCR was found to increase the amount of colonies (data not shown).

Performing LCRs without DMSO/betaine is recommended for the assembly of the seven fragments. To investigate optimal experimental temperatures for the new conditions without DMSO and betaine, the interaction of the annealing, ligation and BO-melting temperatures need to be considered.

Increased annealing temperature is beneficial for LCRs without DMSO and betaine

To optimize the LCR without DMSO/betaine, several experiments were performed. Figure 3D shows that the BO-sets with low T_ms yield the most correct CFUs, which suggests that the BOs may bind too strongly to unwanted sites and be unavailable for ligation. To counteract this, the optimal annealing temperature was determined by performing a gradient-LCR, i.e. LCRs at different cycling conditions. For this, three new BO-sets were composed from the existing pool of BOs used in LCRs shown in Figure 2 to obtain sets with average melting temperatures of 67.8 °C, 69.9 °C and 71.8 °C (sequences in Supplementary Table S4). Within an annealing temperature range of 56.5 °C to 75.6 °C these sets were analyzed by chemically transforming the corresponding LCRs in chemically competent NEB® 10-β *E. coli*. This resulted in roughly 100× lower transformation efficiency in comparison to the electrocompetent cells utilized in previous experiments.

For all sets, the efficiency was similar throughout the entire temperature range (Figure 4A). Consistent with previous results, the total amount of CFUs increased with lower BO-T_ms (comparing the results shown in Figures 3H and 4B). Furthermore, all sets have a global CFU maximum in the annealing temperature range of ∼66-71 °C. This range contains the ligase optimum of 70 °C, which suggests positive effects of prolonging the total ligation time to 1.5 min (30 s annealing at 66-68 °C, 1 min ligation at 66 °C) in comparison to the baseline-condition of 30 s annealing at 55 °C and 1 min ligation at 66 °C.

In total, increasing the annealing temperature from 55 °C to 66 °C improved the CFU yield of every BO-set by a factor of two without a loss of efficiency. The BO-set "67.8 °C" performed better than the other sets. Supplementary Figure S11 shows that at the optimum annealing temperature of 67.8 °C, the BO-set "67.8 °C" also showed slightly greater efficiency than the other sets.

Lower BO melting temperatures may have additional benefits for LCR assemblies without DMSO and betaine. To validate this, new BO-sets with lower melting temperatures of 62 °C to 67.3 °C were designed and applied for ligations at 66 °C (Figure 4C+D, sequences in Supplementary Table S5). Surprisingly, a drop-off in efficiency and CFUs were observed for BO-sets with equal or lower melting temperatures than 66 °C. In comparison to the results shown in Figure 4A the efficiency of the 69.9 °C-set is 30% lower. This result is most likely related to a loss of function by additional freeze-thaw cycles or contamination of the DNA parts, BO-sets and/or supplements. Negative impacts of repeated freeze-thaw cycles on the DNA parts and BOs were already mentioned for the LCR (16) and for single-stranded oligonucleotides (32). Nevertheless, the total amount of CFUs was further optimized by increasing the annealing temperature in the range of the ligase optimum without decreasing the efficiency. Together with previous optimizations by performing the LCR without DMSO and betaine the assembly of the seven-part was highly improved.

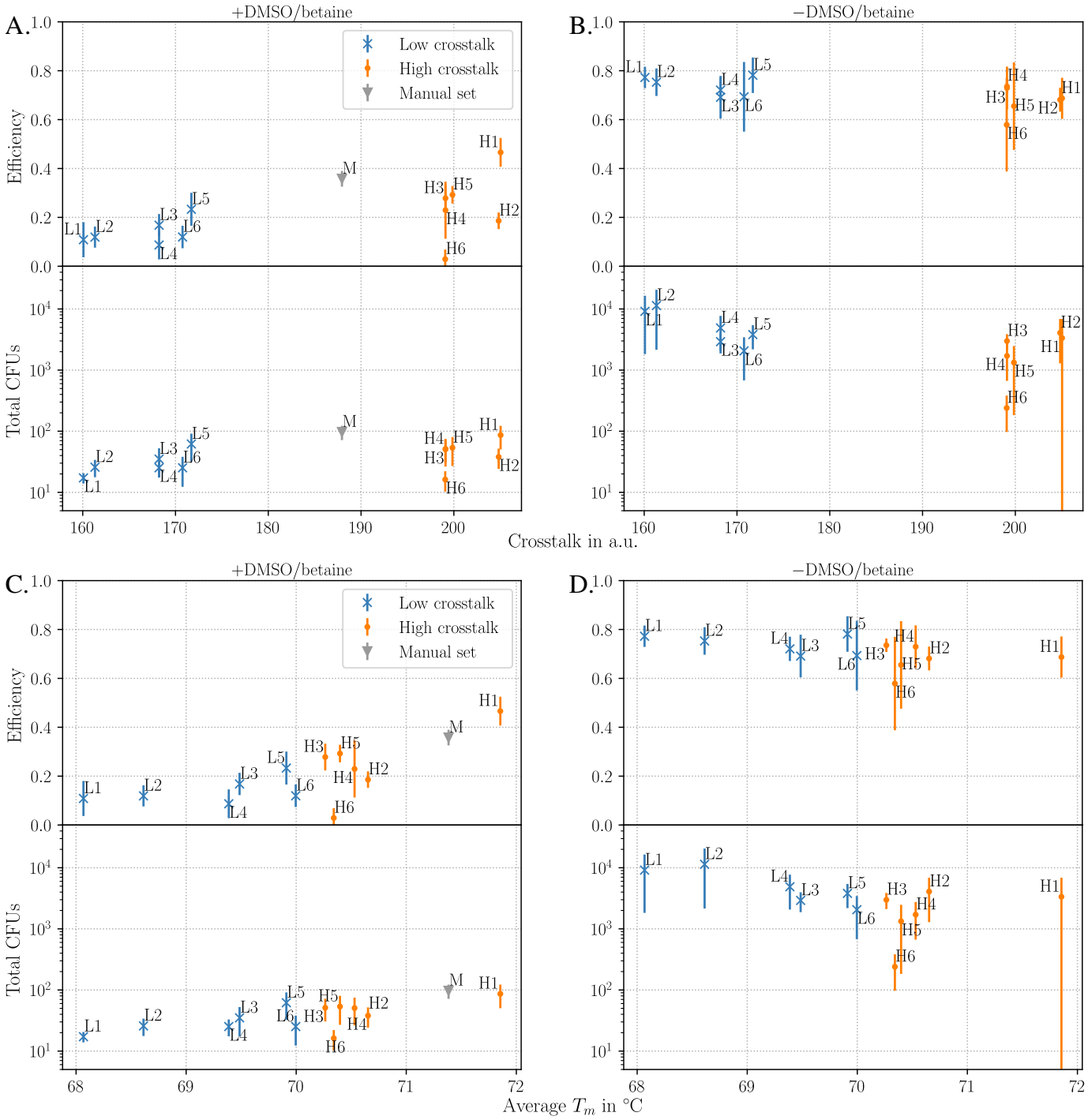

Figure 3. Graphical analysis of the bridging oligo sets (BO-sets) utilized for the assembly of a seven-part toy-plasmid, based on the results shown in Figure 2. The total colony forming units (CFUs) are plotted on a logarithmic scale. The standard deviation for each LCR is indicated by error bars. **A+B.** No crosstalk-dependent effects of the BOs were observed for the LCR with (A) and without (B) DMSO/betaine. Both clusters (L1-L6 and H1-H6) were distinguishable by the crosstalk but without affecting the LCR efficiency and total amount of colonies. Slightly higher efficiencies and more colonies were observed for the cluster of the sets H1-H6 when DMSO/betaine was used. **C+D.**: The average melting temperature of the BO-sets influenced the LCRs. Higher T_ms resulted in higher efficiencies and more colonies when DMSO and betaine were used (C). Without DMSO and betaine, all BO-sets resulted in similar efficiencies suggesting no impact of crosstalk of BOs with the DNA parts in the LCR-assembly of the seven-part toy-plasmid. More colonies were observed for sets L1-L6. In contrast to LCRs with DMSO and betaine (C), the total amount of colonies was found to be increasing with decreasing melting temperatures. All T_ms presented here were calculated by using the formula of (20) for the T_m calculation and salt correction. The manual set ("M", used for LCRs with DMSO and betaine) was designed in `Geneious` with the same algorithms and a target temperature of 70 °C. The difference of ∼1.5 °C in comparison to the target T_m of 70 °C is due to the lack of an option to specify the DNA-part concentration in the software. The raw data of the LCRs presented here are shown in Supplementary Figure S1. a. u. arbitrary unit, BO: bridging oligo, CFU: colony forming unit, DMSO: dimethyl sulfoxide, M: manually designed BO-set, T_m: melting temperature of a BO-half.

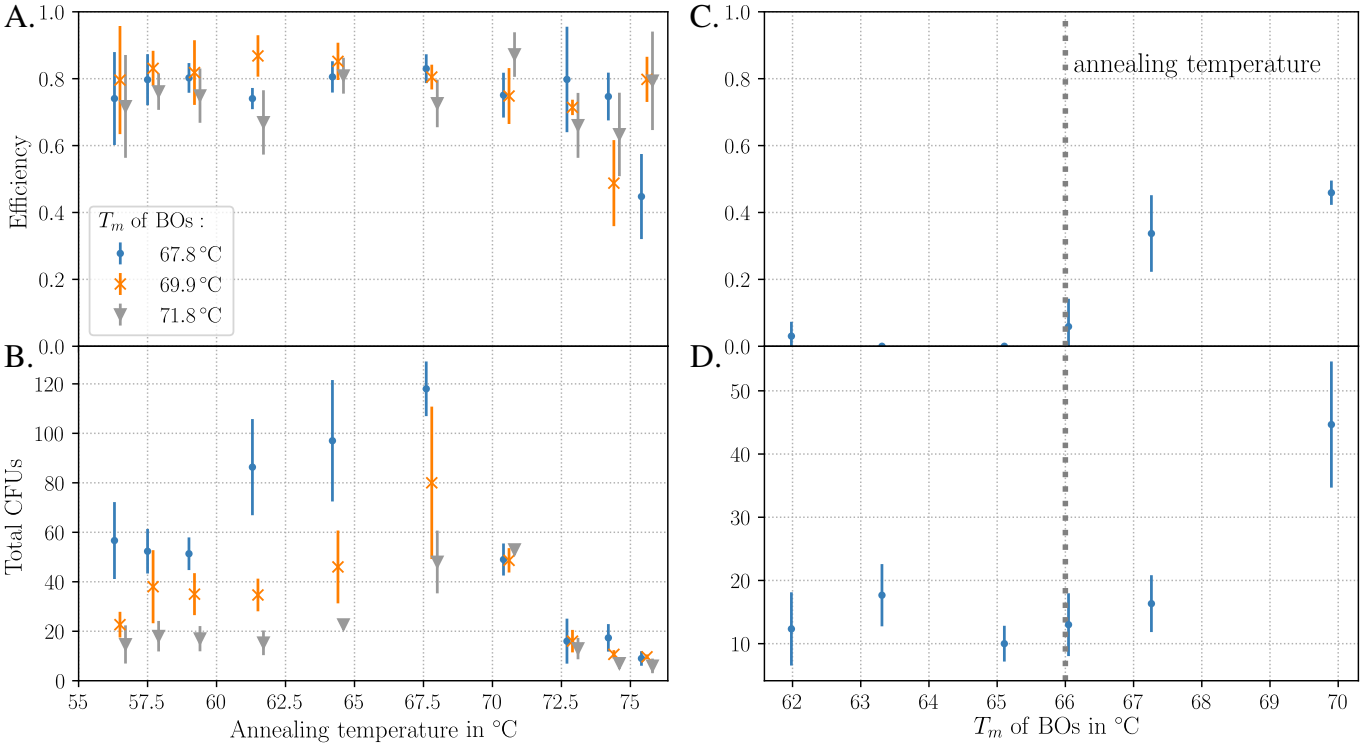

Figure 4. Adjustment of the annealing temperature and melting temperature of bridging oligo halves of DMSO/betaine-free LCR. The LCRs were performed as triplets using the same DNA-parts and chemically competent cells. The standard deviation for each LCR is indicated by error bars. **A.** For a better visibility the bars of the results were shifted with an offset of the annealing temperatures shown on the x-axis. Optimization of the annealing temperature of the seven-part plasmid via gradient-LCR. The temperature range for the annealing was 56.5 °C to 75.6 °C. Three BO-sets with different melting temperatures were used (67.8 °C, 69.9 °C and 71.8 °C). For the three BO-sets the LCR-efficiency was at a similar level. It started decreasing at an annealing temperature of more than 70.6 °C. **B.** As already observed in B (and Figure 3H), the total amount of colonies was increased with a lower BO-T_m. Overall, the LCRs using these BO-sets resulted in a global maximum of colonies in a range of ∼66-71 °C with 2× more colonies (also shown in Supplementary Figure S10). The BO-set seuqences of the three sets are shown in Supplementary Table S4. **C+D.** Based on the optimization shown in A+B, the annealing temperature of 66 °C was used to investigate the influence of BO-halves with lower T_ms of 62.0 °C to 67.3 °C. As reference, the BO-set "71.8 °C" from A+B was used. Further decrease of the BO-T_m did not improve the LCR at the optimized annealing temperature of 66 °C. In comparison to A the LCR with the BO-set "69.9 °C" was less efficient due to loss of function by repeated freeze-thaw cycles of the DNA parts and/or BOs. The sequences of the BO-sets "62.0 °C" to "67.3 °C" are shown in Supplementary Table S5. BO: bridging oligo, CFU: colony forming unit, DMSO: dimethyl sulfoxide, T_m: melting temperature of a BO-half.

Optimizations are dependent on the size of DNA parts

In order to further validate the positive effects of omitting DMSO/betaine and to use a higher annealing temperature of 66 °C in DMSO/betaine free conditions, the sequence of the seven-part plasmid was used for a three-part split consisting of *mRFP1* as part 1, *sfGFP* as part 2 and pJET1.2/blunt as part 3. The BOs used for this assembly were externally resynthesized and new aliquots of all supplements. This plasmid was assembled by using the baseline and improved LCR conditions. For the baseline condition, the LCR was performed using the manual BO-set with a T_m of 71.4 °C for each half, 8 %−v/v DMSO, 0.45 M betaine and the annealing at 55 °C. For the improved LCR condition, the BO-set had a T_m of 67.8 °C for each half (set was already used for the gradient-LCR in Figure 4), no DMSO/betaine were used and the annealing was at 66 °C. The sequences of both BO-sets are shown in Supplementary Table S6. In general, the three-part plasmid was built by the two different LCR-protocols with no differences in the total plasmid size (4918 bp), sequence and BOs in comparison to the seven-part version.

Unexpectedly, the results for the three-part plasmid were contrary although the sequences and context remained the same (Supplementary Figure S13). The use of DMSO/betaine and an annealing temperature of 55 °C are recommended and corroborate the literature (8, 9) but with a potential for further optimization. The efficiency was around 40 %, similar to the assembly of the seven-part plasmid using the manual set (Figure 2A). As observed for LCRs with DMSO and betaine (Figure 3B), BOs with a higher T_m of 74.8 °C may improve the efficiency. Using 8 %−v/v DMSO and 0.45 M betaine has shown a positive impact for three-part plasmid, but proved detrimental for the seven-part plasmid that contains a very short part with 79 bp. To avoid higher costs for larger oligonucleotides, another approach for the optimization was to omit the T_m-decreasing substances DMSO and betaine. For the assembly of the seven-part plasmid, the efficiency and amount of colonies were highly increased (2-3× higher efficiency, Figure 2). The total amount of CFUs was further increased by adjusting the annealing temperature to 66 °C (Figure 4).

Besides general factors like the genetic context, total plasmid size, amount of parts, purification grade and freeze-thaw-cycles, the successful optimization of the LCR depends on the part size distribution. As hypothesized by de Kok et al.

(9), positive effects of DMSO/betaine utilization are related to accelerated strand separation during the denaturation step, so that the addition of both detergents should be advantageous for all LCR assemblies. However, the assembly of the seven-fragment plasmid showed a negative impact when adding both DMSO and betaine. Smaller subparts of a sequence denature faster into single strands than larger parts at the same thermodynamic conditions (33). A maximal denaturation of the DNA parts may be disadvantageous for the ligation, because BOs might anneal unspecifically to sites on completely single-stranded parts. A partially denaturated DNA part might have single-stranded ends but a double-stranded central region, which is not available for unwanted BO hybridization. Assemblies of smaller parts are thus negatively affected by the utilization of DMSO and betaine whereas DMSO and betaine are necessary for larger parts to unwind the double-stranded ends for the annealing of the BO.

CONCLUSION

To our knowledge, the presented assembly of a plasmid with *sfGFP* and *mRFP1* is the first documented experimental LCR-design that includes a direct and fast readout to investigate the influence of various plasmid designs and experimental conditions. The utilized toy-model plasmid in combination with the fluorescence-based analysis enables a robust and easy-to-adapt *in vivo* system to get valuable insights into the LCR and is also adaptable for investigations of other assembly techniques.

Based on this workflow, the impact of intramolecular and intermolecular crosstalk between BOs is assumed to be negligible for the assembly of the seven-part plasmid, whereas a strong sensitivity in regards to the BO T_m was observed. We revealed that a higher target-T_m than $70\,^{\circ}\mathrm{C}$ for the BO-design of LCRs utilizing DMSO and betaine is highly beneficial when compared with the published conditions. Related to this, it is of crucial importance to be consistent in the choice of the algorithms for the T_m calculation. Sets with a melting temperature of $74.8\,^{\circ}\mathrm{C}$ when using the formula of SantaLucia (20) are recommended. A melting temperature of $72.2\,^{\circ}\mathrm{C}$ is recommended when using the algorithms of SantaLucia (20) and the salt correction of Owczarzy et al. (28). Using guanosine or cytosine at the 3'-end of the BOs was not found to be necessary. If BO-sets with a target T_m of less than $70\,^{\circ}\mathrm{C}$ are used, it is advantageous to decrease the ligation temperature from $66\,^{\circ}\mathrm{C}$ to $60\,^{\circ}\mathrm{C}$.

For LCRs where smaller parts than $350\,\mathrm{bp}$ are included, the addition of $8\,\%-\mathrm{v/v}$ DMSO and $0.45\,\mathrm{M}$ betaine is not necessary. Increasing the annealing temperature from $55\,^{\circ}\mathrm{C}$ to $66\,^{\circ}\mathrm{C}$ is useful for DMSO/betaine-free LCRs. To ensure high efficient assemblies, we provide an optimized step-by-step LCR-protocol for constructs with small parts ($<350\mathrm{bp}$) and long parts (protocol in the supplement).

Altogether, these observations offer improved protocols for LCRs with and without $8\,\%-\mathrm{v/v}$ DMSO and $0.45\,\mathrm{M}$ betaine to achieve highly efficient DNA assemblies. To further design more experimental rules for the LCR, the combination of *in vitro* studies using cell-free systems (34) and the toy-plasmid-based screening offers another approach to gain further insights of the LCR.

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Conflict of interest statement. None declared.

SUPPLEMENTARY DATA

Supplementary data in pdf-file.

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