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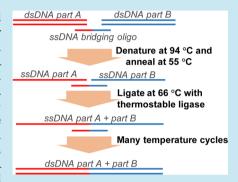
Rapid and Reliable DNA Assembly via Ligase Cycling Reaction

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Supporting Information

ABSTRACT: Assembly of DNA parts into DNA constructs is a foundational technology in the emerging field of synthetic biology. An efficient DNA assembly method is particularly important for high-throughput, automated DNA assembly in biofabrication facilities and therefore we investigated one-step, scarless DNA assembly *via* ligase cycling reaction (LCR). LCR assembly uses single-stranded bridging oligos complementary to the ends of neighboring DNA parts, a thermostable ligase to join DNA backbones, and multiple denaturation–annealing–ligation temperature cycles to assemble complex DNA constructs. The efficiency of LCR assembly was improved ca. 4-fold using designed optimization experiments and response surface methodology. Under these optimized conditions, LCR enabled one-step assembly of up to 20 DNA parts and up to 20 kb DNA constructs with very few single-nucleotide polymorphisms (<1 per 25 kb) and



insertions/deletions (<1 per 50 kb). Experimental comparison of various sequence-independent DNA assembly methods showed that circular polymerase extension cloning (CPEC) and Gibson isothermal assembly did not enable assembly of more than four DNA parts with more than 50% of clones being correct. Yeast homologous recombination and LCR both enabled reliable assembly of up to 12 DNA parts with 60-100% of individual clones being correct, but LCR assembly provides a much faster and easier workflow than yeast homologous recombination. LCR combines reliable assembly of many DNA parts via a cheap, rapid, and convenient workflow and thereby outperforms existing DNA assembly methods. LCR assembly is expected to become the method of choice for both manual and automated high-throughput assembly of DNA parts into DNA constructs.

KEYWORDS: synthetic biology, DNA assembly, high throughput, chain-reaction cloning, Gibson isothermal assembly, yeast homologous recombination

 ${\displaystyle S}$ ynthetic biologists create novel biological systems by transforming host organisms with DNA constructs. To accelerate the design-build-test engineering cycle by which such biological systems are developed and optimized, biofabrication platforms are emerging in which genetic modifications are specified via computer-aided design and the corresponding DNA constructs, typically 3-20 kb in length and consisting of 2-12 DNA parts, are assembled and transformed in an automated fashion. Such facilities routinely manufacture hundreds of DNA constructs and strains per month and therefore require efficient and robust methods at every stage of the process, including assembly of DNA constructs from modular DNA parts (e.g., regulators and genes). Despite advancements in *de novo* synthesis of complete DNA constructs,¹ assembly of DNA constructs from modular DNA parts, obtained via PCR amplification or DNA synthesis, is preferred because DNA part assembly is more cost-effective and allows storing of DNA parts and testing of variations or subsets of an idea. An ideal DNA assembly method does not place constraints on DNA sequences and enables scarless assembly of many DNA parts into DNA constructs via a convenient workflow that is amenable to high-throughput operation. In our experience, which is based on high-throughput assembly of over 30 000 DNA constructs, existing DNA assembly methods^{2,3} do not fulfill these criteria

and therefore we investigated DNA assembly *via* ligase cycling reaction (LCR).

The ligase cycling reaction was developed for the detection of single-nucleotide polymorphisms in genomic templates with high specificity^{4,5} and was pioneered for one-step DNA assembly by Pachuk and co-workers.⁶ LCR assembly utilizes singlestranded bridging oligos complementary to the ends of DNA parts to be assembled (Figure 1). After (initial) denaturation at high temperature, the upper (or lower) strands of neighboring DNA parts anneal at lower temperature on both halves of the provided bridging oligo after which a thermostable ligase joins the DNA backbones via a phosphodiester bond without introducing any scar sequences. In subsequent denaturationannealing-ligation temperature cycles, the assembled upper (or lower) strand serves as a template for assembly of the lower (or upper) strand. By applying multiple temperature cycles, many DNA parts can be assembled into complex DNA constructs. Proof-of-concept experiments showed the promise of LCR assembly,⁶ but the success rate achieved (ca. 40% of clones correct for assembly of six DNA parts) is insufficient for implementation of LCR assembly in a high-throughput DNA

Received: December 4, 2013 Published: January 9, 2014

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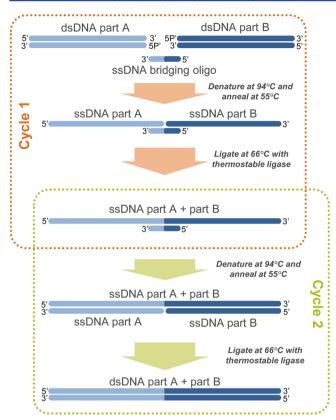


Figure 1. Mechanism of DNA assembly *via* ligase cycling reaction (LCR). Custom single-stranded bridging oligos complementary to the ends of neighboring DNA parts serve as a template to bring the upper strands of denatured (5' phosphorylated) DNA parts together, after which a thermostable ligase joins the DNA backbones. In the second and subsequent temperature cycles, the assembled upper strand serves as a template for ligation of the lower strand. Typically, 50 denaturation—annealing—ligation temperature cycles are used for assembly of many DNA parts into complex DNA constructs.⁶

assembly platform, where high success rates are a necessity because only a limited number (4-8) of colonies per DNA construct can be analyzed. In this study, we optimized and improved the conditions for DNA assembly *via* ligase cycling reaction (LCR), analyzed the main factors affecting its performance, and compared the efficiency of LCR assembly against existing methods for scarless and sequence-independent DNA assembly, including circular polymerase extension cloning (CPEC),^{7,8} Gibson isothermal assembly,⁹ and yeast homologous recombination.¹⁰⁻¹⁴

RESULTS AND DISCUSSION

Optimization of LCR Assembly. A proof of concept for LCR assembly was previously provided by assembling six DNA parts into a (circular) DNA construct.⁶ The (half) bridging oligos used for those LCR assembly reactions spanned a wide range (42–60 °C) of melting temperatures (T_m), which might have resulted in the low success rate observed (ca. 40% of individual clones were correct). Using a more consistent design of bridging oligo, targeting a T_m of 60 °C for both halves of the bridging oligo, we observed high success rates (80–100% of individual clones were correct) for the assembly of 2–6 DNA parts (0.5–1.5 kb) into a (2.2 kb) cloning vector under otherwise identical reaction conditions (data not shown).

To optimize LCR assembly, 13 factors were selected that potentially affect LCR assembly efficiency (Table 1). A full combinatorial optimization, including nonlinear and interaction effects, would require at least 313 experiments and 81 unique thermocycler conditions. To reduce the number of experiments, a design-of-experiment approach was taken.¹⁵ To reduce the number of unique thermocycler conditions, the optimization was separated into two separate experiments. One optimization experiments focused on the enzymatic ligation reaction and comprised 60 LCR conditions and three unique thermocycler conditions (Table S1). The other optimization experiments focused on factors related to denaturation and annealing of DNA parts and bridging oligos and comprised 48 LCR conditions and 27 unique thermocycler conditions (Table S2). For each condition, the efficiency of LCR was tested by assembling a circular DNA construct from five DNA parts. Subsequently, 2.5 μ L of each LCR assembly reaction was transformed into Escherichia coli to select for circular, assembled DNA constructs, and the number of colony forming units (CFUs) was counted. The integrity of the assembled DNA constructs was analyzed via restriction endonuclease-mediated DNA fragmentation and matching of the resulting DNA fragments to their expected lengths for eight randomly selected clones per LCR condition. All conditions were compared against the baseline conditions reported previously by Pachuk and co-workers.⁶

The optimization experiment focusing on factors related to the enzymatic ligation reaction yielded between 540 and 8280 CFUs per 2.5 μ L of LCR (Table S1). For every LCR condition, seven or eight out of the eight tested clones were correct and therefore the percentage of correct clones was left out of subsequent analyses. Using multivariate data analysis, a model was constructed that predicts the number of CFUs per 2.5 μ L of LCR based on the tested experimental factors (x_i) (Table S3). The model considers linear effects of factors ($Y = c_0 + c_1x_1 + ... + c_nx_n$), quadratic effects of factors (... + $c_{11}x_1^2 + ... + c_{nn}x_n^2$), and interaction between factors $(... + c_{12}x_1x_2 + ... + c_{nm}x_nx_m)$. This multivariate model predicted that relative to baseline conditions (4246 \pm 1045 CFUs/2.5 μ L of LCR; average ± 95% confidence interval) fewer temperature cycles (25; 6609 \pm 2360 CFUs/2.5 μ L of LCR) improved LCR assembly efficiency the most followed by a higher concentration of bridging oligos (30 nM; $5506 \pm 2017 \text{ CFUs}/2.5$ μ L of LCR). When fewer temperature cycles were combined with a higher concentration of bridging oligos, the highest LCR assembly efficiency was predicted ($8076 \pm 2712 \text{ CFUs}/2.5 \mu \text{L}$ of LCR), yielding ca. 2-fold more CFUs/2.5 μ L LCR than baseline conditions. Subsequent experimental verification showed that the optimized conditions indeed vielded ca. 2-fold more CFUs/ 2.5 μ L of LCR than baseline conditions, with 100% of the resulting colonies correctly assembled in both cases (Table 1). In the verification experiment, the absolute values for the number of CFUs per 2.5 μ L of LCR were consistently higher, presumably because of the use of another batch of E. coli cells.

The optimization experiment focusing on factors related to denaturation and annealing of DNA parts and bridging oligos yielded between 0 and 9725 CFUs/2.5 μ L of LCR, of which between 0 and 100% were correctly assembled (Table S2). Common to all conditions that yielded a low percentage (50% or lower) of correct clones was the presence of 10% (w/v) PEG-8000 and/or a combination of (half) bridging oligos with a low $T_{\rm m}$ (50 °C) and addition of 10% (v/v) DMSO. Using multivariate data analysis, a predictive model (Tables S4 and S5) was constructed and used to maximize the number of CFUs per 2.5 μ L of LCR with the constraint that at least 75% of the

Table 1. Optimization of DNA Assembly via Ligase Cycling Reaction $(LCR)^a$

Factor	Unit	Conditions investigated	Baseline conditions	Enzymatic assembly optimized conditions	Denaturation & annealing optimized conditions	Combined optimized conditions
[DNA parts]	nM	3, 6, 9	6	6	<u>3</u>	<u>3</u>
[Bridging oligo]	nM	0.3, 3, 30	3	<u>30</u>	<u>30</u>	<u>30</u>
[Ligase]	U/µl	0.1, 0.3, 1	0.3	0.3	0.3	0.3
[NAD ⁺]	mM	0.5, 1, 2.5	0.5	0.5	0.5	0.5
[DTT]	mM	0, 3, 10	0	0	0	0
Temperature cycles	-	25, 50, 75	50	<u>25</u>	50	<u>25</u>
Ligation time	s	30, 60, 300	60	60	60	60
Ligation temperature	°C	60, 66, 72	66	66	66	66
T_{m} of half bridging oligo	°C	50, 60, 70	60	60	<u>70</u>	<u>70</u>
Denaturation time	s	10, 30, 60	30	30	<u>10</u>	<u>10</u>
[DMSO]	% v/v	0, 5, 10	0	0	<u>8</u>	<u>8</u>
[Betaine]	М	0, 0.25, 0.5	0	0	<u>0.45</u>	<u>0.45</u>
[PEG-8000]	% w/v	0, 5, 10	0	0	0	0
Model predictions						
Relative CFUs/2.5 µl LCR (range)	-		1.0	1.9 (0.9-4.1)	4.9 (2.6-12.8)	N.A.
Colonies correct	%		>90	N.A.	>90	N.A.
Experimental data						
CFUs/2.5 µl LCR	-		5817±816	9750±551	23667±2714	20333±2944
Relative CFUs/2.5 µl LCR (range)	-		1.0	1.7 (1.4-2.1)	4.1 (3.2-5.3)	3.5 (2.6-4.7)
Colonies correct	%		100±0	100±0	100±0	100±0

^{*a*}In two optimization experiments, 13 factors were optimized. Gray shading indicates the factors that were tested in each optimization experiment. Optimized conditions that are changed relative to baseline conditions are indicated in bold and are underlined. Experimental data represents the average \pm standard deviation of six LCR assembly reactions for the number of CFUs per 2.5 μ L of LCR from two LCR assembly reactions, with each testing eight clones per LCR replicate, to obtain the percentage of colonies correct by restriction endonuclease fragment analysis. Experimental data and model parameters can be found in Tables S1–S5.

resulting colonies are correctly assembled. The best condition predicted by the model included a lower concentration of DNA parts, a higher concentration of bridging oligos, (half) bridging oligos with a higher melting temperature, a shorter denaturation time, and addition of both DMSO and betaine (Table 1). These optimized conditions were predicted to yield ca. 5-fold more CFUs per 2.5 μ L of LCR than baseline conditions (11793 ± 3467 vs 2215 \pm 1687 CFUs/2.5 μ L of LCR). Relative to baseline conditions, a shorter denaturation time (10 s; 5072 \pm 1814 CFUs/2.5 μ L of LCR) was predicted to improve LCR assembly efficiency the most followed by addition of 0.45 M betaine (3745 \pm 2741 CFUs/2.5 µL of LCR), addition of 8% (v/v) DMSO $(3126 \pm 2693 \text{ CFUs}/2.5 \,\mu\text{L of LCR})$, and (half) bridging oligos with a higher $T_{\rm m}$ (70 °C; 2657 ± 2418 CFUs/2.5 μ L of LCR). Further increasing the concentration of betaine was predicted to yield fewer than 75% of correct colonies; further increasing the concentration of DMSO was predicted to yield fewer CFUs per 2.5 μ L of LCR. Combining a shorter denaturation time (10 s) with addition of 0.45 M betaine, addition of 8% (v/v) DMSO, and (half) bridging oligos with a higher $T_{\rm m}$ (70 °C) was predicted to yield 9911 \pm 3217 CFUs/2.5 μ L of LCR. Interestingly, a lower concentration of DNA parts (3 nM; 1211 ± 2746 CFUs/2.5 μ L of LCR) or a higher concentration of bridging oligos (30 nM; 1475 ± 2634 CFUs/2.5 μ L of LCR) was predicted to lower the LCR assembly efficiency relative to baseline conditions. A positive effect of a lower concentration of DNA parts (9300 \pm 2948 CFUs/2.5 µL of LCR) or a higher concentration of bridging oligos (8873 \pm 3661 CFUs/2.5 μ L of LCR) was predicted only when a shorter denaturation time (10 s) was

combined with addition of 0.45 M betaine and 8% (v/v) DMSO (8338 ± 3067 CFUs/2.5 μ L of LCR). The highest LCR assembly efficiency (11 793 ± 3467 CFUs/2.5 μ L of LCR) was predicted for a combination of a short denaturation time (10 s), addition of 0.45 M betaine, addition of 8% (v/v) DMSO, (half) bridging oligos with a high $T_{\rm m}$ (70 °C) at a high concentration (30 nM), and a low concentration of DNA parts (3 nM). Subsequent experimental verification showed that the optimized conditions yielded ca. 4-fold more CFUs per 2.5 μ L of LCR than reference conditions, with 100% of the resulting colonies correctly assembled (Table 1), within the predicted range.

Subsequently, a combination of both sets of optimized conditions was experimentally tested (Table 1). Interestingly, the LCR assembly efficiency of the combination of both sets of optimized LCR conditions was lower than of the LCR conditions optimized in the experiment focusing on factors related to denaturation and annealing of DNA parts and bridging oligos. Apparently, two separate optima were found whose effects are not additive. The denaturation and annealing optimized conditions will be referred to as optimized conditions in the remainder of this study.

Six out of 13 factors tested in the optimization experiments contributed to improved LCR assembly efficiency, with the most pronounced effect from a shortened denaturation time. It is possible that the ligase used is not completely stable at the longer denaturation time and therefore shorter denaturation times improve the overall assembly efficiency. LCR assembly efficiency was also improved by addition of DMSO and/or betaine, which both facilitate DNA strand separation during denaturation,

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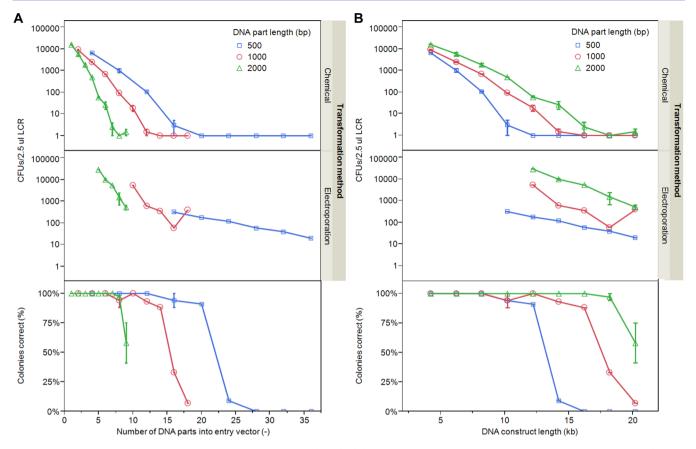


Figure 2. Efficiency of DNA assembly *via* ligase cycling reaction (LCR). (A) Efficiency of DNA assembly *via* ligase cycling reaction (LCR) using optimized conditions and organized by the number of DNA parts and their length. (B) Efficiency of DNA assembly *via* ligase cycling reaction (LCR) using optimized conditions and organized by total DNA construct length and DNA part length. The upper panels show colony forming units (CFUs) for chemical transformation $(3-5 \times 10^7 \text{ CFUs}/\mu\text{g} \text{ of pUC19})$ of 2.5 μ L of LCR reaction mixture into *E. coli*. The middle panels show colony forming units (CFUs) for transformation *via* electroporation $(10^9-10^{10} \text{ CFUs}/\mu\text{g} \text{ of pUC19})$ of 2.5 μ L of LCR reaction mixture into *E. coli*. The CFU count of LCR reactions yielding zero colonies was adjusted to one colony for plotting purposes. Points represent the mean; error bars represent the standard error of the mean of duplicate LCR assembly experiments. The lower panels show the percentage of correct colonies by restriction endonuclease DNA fragment analysis, which is based on testing eight colonies per LCR assembly experiment. Points represent the mean; error bars represent the standard error of the mean of duplicate LCR assembly experiments. DNA constructs were assembled as described in the Methods except that 2.5 nM DNA parts was used for 16 kb DNA constructs, 2.25 nM DNA parts, for 18 kb DNA constructs, and 2 nM DNA parts, for 20 kb DNA constructs because the purified DNA parts were not concentrated enough to achieve 3 nM final DNA part concentration.

especially for secondary structures, by disrupting base pairing (DMSO) or equalizing the contribution of GC pairing to AT pairing (betaine).^{16–19} It could be that the shorter denaturation time is not sufficient for complete denaturation of double-stranded DNA into single strands, which is then compensated for by addition of DMSO and/or betaine. Addition of DMSO and betaine lowers the effective $T_{\rm m}$ of DNA oligos,^{20,21} which might explain the positive effect of (half) bridging oligos with a higher target $T_{\rm m}$ and higher bridging oligo concentration on LCR assembly efficiency.²²

The optimized LCR assembly conditions contained a lower concentration of DNA parts. For LCR assembly of a circular DNA construct from three DNA parts (P1, P2, P3), bridging oligos are provided to span the following junctions: P1–P2, P2–P3, and P3–P1. LCR assembly can then lead to the desired DNA construct (circular P1–P2–P3) or formation of linear or circular multimers (P1–P2–P3–P1–P2–P3, etc.). A lower concentration of DNA parts probably favors the formation of the desired circular DNA construct. Consistent with this observation, addition of PEG-8000, a macromolecular crowding agent that effectively increases the local concentrations of all reactants,²³ including DNA parts, had a negative effect on LCR assembly

efficiency. A lower concentration of DNA parts is advantageous for high-throughput LCR assembly because it relaxes the concentration requirements for purified DNA parts.

Characterization of LCR Assembly. To test the efficiency of LCR assembly using the optimized conditions and to analyze the effect of the number of DNA parts and their length, a set of assembly reactions was designed with DNA part lengths of 500, 1000, or 2000 bp and total DNA construct lengths of up to 20 kb. Every set of four 500 bp DNA parts or two 1000 bp DNA parts represents exactly the same DNA sequence as one 2000 bp DNA part. Bridging oligos were designed with a target melting temperature of 70 °C for both halves of the bridging oligo, resulting in typical bridging oligo lengths of 60-90 bp. Transformation of LCR assembly reactions into E. coli showed that CFUs/2.5 μ L of LCR decreases with the increasing number of DNA parts and increasing DNA part length (Figure 2). For DNA constructs larger than 8-14 kb, high-efficiency E. coli transformation via electroporation was required to obtain sufficient colonies for testing. The effect of the number of DNA parts on LCR assembly efficiency can be explained by the higher complexity of assembling more junctions. The effect of the length of DNA parts could be due to a lower transformation

efficiency of larger DNA constructs.²⁴ However, transformation of purified, circular plasmids only showed a minor effect of plasmid length ($6.8 \pm 0.8 \times 10^6$ CFUs/ μ g of 4.5 kb plasmid vs 6.0 $\pm 1.2 \times 10^6$ CFUs/ μ g of 8.6 kb plasmid vs 5.0 $\pm 1.3 \times 10^6$ CFUs/ μ g of 14.2 kb plasmid), although purified plasmids are typically supercoiled and (circular) LCR-assembled plasmids are relaxed.

Restriction endonuclease DNA fragment analysis showed that LCR assembly enabled one-step assembly of up to 20 DNA parts into DNA constructs up to 20 kb (Figure 2). For DNA constructs up to 12 kb, 90–100% of clones were correct. DNA constructs larger than 12 kb assembled more efficiently from longer DNA parts. Subsequent sequencing of 245 DNA constructs (typically four clones per assembly reaction) confirmed the results obtained *via* restriction endonuclease DNA fragment analysis (high true-positive/negative rate, low false-positive/negative rate) and revealed very few single-nucleotide polymorphisms (1 per 25 kb) and insertions/deletions (1 per 395 kb) (Table 2).

Table 2. Sequencing of DNA Constructs Assembled via Ligase Cycling Reaction $(LCR)^a$

clones sequenced	245
clones correct by restriction endonuclease DNA fragment analysis	200
clones correct by sequencing (true-positive rate)	193 (96.5%)
clones incorrect by sequencing (false-positive rate)	7 (3.5%)
clones incorrect by restriction endonuclease DNA fragment analysis	45
clones correct by sequencing (false-negative rate)	1 (2.2%)
clones incorrect by sequencing (true-negative rate)	44 (97.8%)
correctly assembled DNA constructs (bp)	2 371 622
unique SNPs (SNPs per bp)	96 (4.0 \times 10 ⁻⁵)
unique Indels (Indels per bp)	$6 (2.5 \times 10^{-6})$

^aCorrect by sequencing refers to the presence of all DNA parts in the correct order and orientation. SNPs, single nucleotide polymorphisms; Indels, insertions/deletions.

Ligases join only exactly adjacent DNA backbones and therefore (unavoidable) misannealing of DNA parts on bridging oligos or on other DNA parts does not lead to (mis)assembly of DNA parts. Combined with the use of a single enzyme without errorintroducing side activities, this can explain the high fidelity of LCR assembly. DNA constructs that were incorrectly assembled via LCR typically lacked one or more complete DNA parts (data not shown), most probably via nonspecific blunt-end ligation of non-neighboring DNA parts. If such a nonspecific junction is formed during reaction setup or an early LCR cycle, then this wrongly assembled junction can act as template for further (mis)assembly and represent a significant fraction of the final assembled DNA constructs. Development of a hot-start thermostable ligase that only becomes active after initial denaturation might further improve LCR assembly accuracy. During LCR assembly, DNA parts are not amplified and bridging oligos are not incorporated in the final DNA constructs. Therefore, the errors (SNPs and Indels) observed in the assembled DNA constructs most probably originate from DNA amplification, errors present in amplification primers, and/or errors introduced during propagation of plasmids in E. coli.

This characterization of LCR assembly efficiency (Figure 2) allows for the design of optimal cloning strategies for use in a high-throughput setting. For instance, the number of colonies obtained upon transformation of a wide range of LCR reactions into *E. coli* can be predicted, which will provide information on

the amount of cells that need to be plated to obtain enough, but not too many, colonies.

Comparison of LCR Assembly against Existing DNA Assembly Methods. Next, the efficiency of LCR assembly was compared against existing DNA assembly methods, including CPEC,^{7,8} Gibson isothermal assembly,⁹ and yeast homologous recombination.^{10–14} For CPEC and yeast homologous recombination, we used conditions that were optimized in-house (optimization data not shown); for Gibson isothermal assembly, a commercial kit was used according to the manufacturer's specifications, thereby representing an optimized process for this method. To compare the efficiency of different DNA assembly methods, two sets of six DNA constructs were designed with 2-12 (insert) DNA parts (Figure 3). The DNA constructs are based on the Saccharomyces cerevisiae mevalonate pathway, and cotransformation of both 12-part DNA constructs enables onestep genomic integration of the complete mevalonate pathway for *de novo* microbial biosynthesis of isoprenoids.²⁵ Neighboring DNA parts were joined in two ways: via seamless junctions and via junctions based on in-house developed rapid yeast strain engineering (RYSE) DNA-linkers.²⁶ This resulted in a total of 24 DNA constructs that were assembled via the four DNA assembly methods being compared. To increase the diversity of the junctions assembled, the order of DNA parts and RYSE-linkers was randomized, resulting in 16 unique junctions for RYSElinkered DNA constructs and 59 unique junctions for seamless DNA constructs.

Restriction endonuclease DNA fragment analysis of up to 30 clones per assembly reaction showed that CPEC and Gibson isothermal assembly were unable to assemble more than four DNA parts into DNA inserts larger than 4.8 kb with more than 50% of individual clones being correct (Figure 4). In contrast, both LCR assembly and yeast homologous recombination enabled reliable assembly of up to 12 DNA parts. No consistent differences were observed between seamless assembly and assembly based on RYSE-linkers, despite differences in the GC content of the junctions assembled (Figure S1). Sequencing of 679 DNA constructs (typically six clones per assembly reaction) confirmed the results obtained via restriction endonuclease DNA fragment analysis and revealed few (≤ 1 per 10 kb) singlenucleotide polymorphisms and insertions/deletions for each method (Table 3). Gibson isothermal assembly yielded ca. 5-fold more single-nucleotide polymorphisms than the other DNA assembly methods, presumably because of the use of a nonproofreading DNA polymerase in the commercial kit.

An ideal high-throughput DNA assembly method enables reliable assembly of many DNA parts *via* a cheap, rapid, and convenient workflow. Experimental comparison showed that LCR assembly and yeast homologous recombination both enabled assembly of up to 12 DNA parts, whereas Gibson isothermal assembly and CPEC did not enable reliable assembly of more than four DNA parts (Figure 4). In a high-throughput setting, only a limited number of colonies can be picked and tested. When a typical number of four clones per DNA construct were analyzed, both LCR assembly and yeast homologous recombination yield an average chance to obtain at least one correct clone well above 99%, thereby clearly outperforming CPEC and Gibson isothermal assembly (Figure S2).

The workflows of LCR assembly, CPEC, and Gibson isothermal are very different from the yeast homologous recombination workflow. Whereas the *in vitro* DNA assembly methods take 1-3 h, *in vivo* DNA assembly *via* yeast homologous recombination takes 36-72 h because yeast transformants need

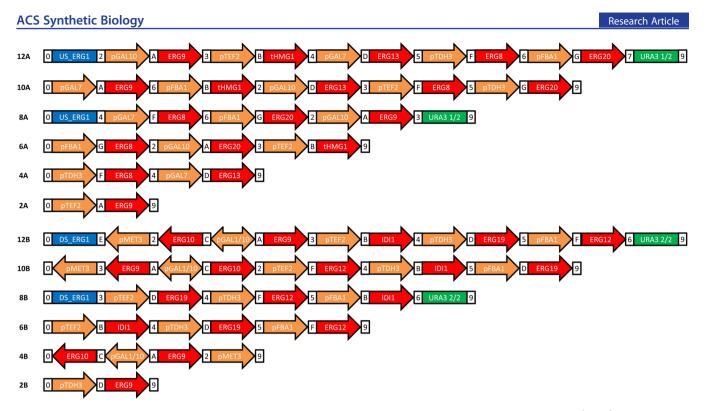


Figure 3. Design of DNA constructs to compare DNA assembly methods. Two sets, A and B, of six DNA constructs with 2-12 (insert) DNA parts were designed to compare the efficiency of different DNA assembly methods. DNA constructs are based on the *S. cerevisiae* mevalonate pathway. Both sets of DNA constructs were designed for both seamless assembly and assembly based on in-house developed rapid yeast strain engineering (RYSE) linkers²⁶ (indicated as letters and numbers in white boxes) and assembled into either a 2.3 kb vector (LCR, CPEC, and Gibson isothermal assembly) or a 4.8 kb vector (yeast homologous recombination). DNA part lengths range between 0.4 and 1.8 kb, total insets lengths range between 2.2 and 12.6 kb, and total DNA construct lengths range between 4.4 and 17.3 kb. Promoters are indicated in orange, gene-terminator combinations, in red, selection markers, in green, and homology regions for genomic integration, in blue.

to be selected and assembled DNA constructs need to be shuttled from yeast to *E. coli* to obtain sufficient amounts of purified DNA construct (Figure 4). In addition, transformation of DNA parts into yeast and isolation of assembled DNA constructs from yeast involves many labor-intensive centrifugation and liquid-handling steps, which will limit the DNA assembly throughput per person and thereby increase the cost for high-throughput assembly of DNA constructs.

Next to labor cost, the cost per DNA construct is determined by the cost of all reagents required. In contrast to other DNA assembly methods, LCR assembly requires bridging oligos to assemble DNA constructs, thereby introducing additional costs. However, the DNA parts for LCR assembly do not require terminal regions with homology to neighboring DNA parts and therefore the primers used to amplify DNA parts for LCR assembly are much shorter. For seamless assembly of 12 DNA constructs (Figure 3), LCR assembly used 1071 bp of amplification primers and 5472 bp of bridging oligos, whereas the other DNA assembly methods used 7097 bp of amplification primers. Hence, the total cost for DNA oligo synthesis is actually lower for LCR assembly. In addition, long amplification primers are difficult to synthesize at high quality and often contain errors, which are subsequently incorporated in DNA constructs assembled via CPEC, Gibson isothermal assembly, and yeast homologous recombination.¹¹ Furthermore, LCR assembly allows the use of the same DNA part in several DNA constructs because the bridging oligos specify the element to which the DNA parts are fused. For seamless assembly of 12 DNA constructs containing 2-12 DNA parts (Figure 3), LCR assembly required only 20 unique DNA parts to assemble all

DNA constructs, whereas CPEC, Gibson isothermal assembly, and yeast homologous recombination required 84 unique DNA parts. The cost of PCR amplification and DNA purification will decrease accordingly. Finally, LCR assembly requires the presence of 5' phosphate groups on DNA parts. In this study, 5'-phosphorylated primers were used to amplify DNA parts, despite high costs per primer (ca. \$10 per primer). Alternatively, 5' phosphate groups can be added to DNA parts or primers via polynucleotide kinase treatment, for which we have developed a workflow that performs the use of phosphorylated primers (see Methods; ca. \$1 per DNA construct). The exact cost of DNA assembly depends on many factors, including costs of labor, reagent cost, throughput, desired success rate, and complexity of the DNA constructs assembled. Overall, the reagent costs for LCR assembly are similar compared to other DNA assembly methods.

LCR combines reliable DNA assembly with a cheap, rapid, and convenient workflow and thereby outperforms existing DNA assembly methods. LCR assembly is expected to become the method of choice for both manual and automated highthroughput assembly of DNA parts into DNA constructs.

METHODS

Amplification Primers, Bridging Oligos, and Overlap Sequences. The 2.3 kb pUC19-based cloning vector (used for CPEC, Gibson isothermal assembly, and LCR assembly) and the 4.8 kb pRS414-based yeast shuttle vector (used for yeast homologous recombination) were both PCR-amplified with the same forward (5' CGGTGTTTAAACCCCAGCGC-CTGGCGGG) and reverse primers (5' GGCGGTTTAAACG-

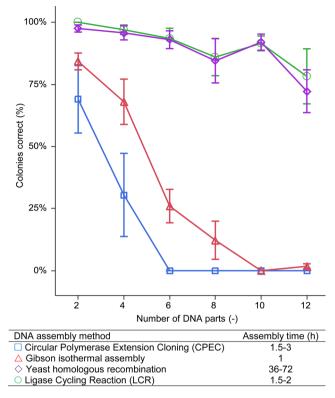


Figure 4. Experimental comparison of different DNA assembly methods. DNA assembly *via* circular polymerase extension cloning (CPEC), Gibson isothermal assembly, yeast homologous recombination, and ligase cycling reaction (LCR). The percentage of (individual) clones that are correct is based on restriction endonuclease DNA fragment analysis. For LCR assembly and yeast homologous recombination, 15 clones were tested. For CPEC and Gibson isothermal assembly, 30 clones were tested, if available. Error bars represent the standard error of four unique LCR assembly reactions. Assembly time is defined as the time required to assemble DNA parts into DNA constructs ready for transformation into *E. coli*.

CGTGGCCGTGCCGTC). Amplification primers and bridging oligos used to optimize the efficiency of LCR assembly can be found in Tables S6 and S7. Amplification primers and bridging oligos used to test the efficiency of LCR assembly can be found in Tables S8 and S9. Amplification primers and bridging oligos used to compare the efficiency of LCR assembly with other DNA assembly methods can be found in Tables S10-S13. Amplification primers used to test the efficiency of DNA assembly via CPEC, Gibson isothermal assembly, and yeast homologous recombination can be found in Tables S14 and S15. Amplification primers and bridging oligos were designed using in-house developed Genotype Specification Language (GSL) software.²⁷ Half-bridging oligos were designed with a target melting temperature (T_m) of 50, 60, or 70 °C under LCR assembly conditions (i.e., 50 mM monovalent anions, 10 mM divalent anions, and DNA concentrations as used; calculated via the nearest-neighbor method^{22,28}) and subsequently combined into a complete bridging oligo. Two types of overlap regions were used for DNA assembly via CPEC, Gibson isothermal assembly, and yeast homologous recombination: RYSE-linkers and seamless junctions. RYSE-linkers were previously designed for assembling DNA parts and have lengths of 24, 28, or 36 bp, melting temperatures of 72.5 \pm 0.9 °C (average \pm standard deviation), and GC contents of $63 \pm 9\%$.²⁶ The overlap regions for seamless assembly were designed with (i) a minimum length of 24 bp to accommodate yeast homologous recombination and Gibson isothermal assembly and (ii) a target melting temperature of 63 °C under CPEC conditions (i.e., 50 mM monovalent anion, 1.5 mM divalent anion, 0.2 mM dNTPs, and 2.5 nM DNA parts), resulting in overlap regions with lengths of 31.0 ± 7.5 bp, melting temperatures of 64.5 \pm 2.2 °C, and GC contents of 47 \pm 14%

PCR Amplification, Purification, and Quantification of DNA Parts. DNA parts were amplified from genomic DNA template or purified plasmids using Phusion Hot-Start Flex DNA Polymerase (New England Biolabs, Ipswich, MA) in an Applied Biosystems 2720 thermal cycler (Life Technologies, Foster City, CA) according to the manufacturer's instructions. DNA parts subsequently assembled via ligase cycling reaction (LCR) were amplified using desalted 5'-phosphorylated primers (Integrated DNA Technologies, Coralville, IA). Alternatively, DNA parts can be amplified using nonphosphorylated primers and subsequently phosphorylated enzymatically. To this end, 20 µL containing 90 fmol of each purified DNA part, 5 mM ATP, and 10 U T4 polynucleotide kinase in 1× Ampligase thermostable DNA ligase reaction buffer were incubated for 1 h at 37 °C and 20 min at 65 °C. DNA parts subsequently assembled via CPEC, Gibson isothermal assembly, or yeast homologous recombination were

Table 3. Sequencing of DNA Constructs Assembled via Different DNA Assembly Methods	Table 3. Sequencing	of DNA Constructs	Assembled via	Different DNA	Assembly Methods ^a
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	circular polymerase extension cloning (CPEC)	Gibson isothermal assembly	yeast homologous recombination	ligase cycling reaction (LCR)
clones sequenced	91	131	132	325
clones correct by restriction endonuclease DNA fragment analysis	25	51	109	286
clones correct by sequencing (true-positive rate)	25 (100%)	47 (92%)	109 (100%)	285 (99.7%)
clones incorrect by sequencing (false-positive rate)	0 (0%)	4 (8%)	0 (0%)	1 (0.3%)
clones incorrect by restriction endonuclease DNA fragment analysis	66	80	23	39
clones correct by sequencing (false-negative rate)	0 (0%)	2 (2.5%)	4 (17%)	6 (15%)
clones incorrect by sequencing (true-negative rate)	66 (100%)	78 (97.5%)	19 (83%)	33 (85%)
correctly assembled DNA constructs (bp)	63 389	216 251	743 569	2 096 014
unique SNPs (SNPs per bp)	$1 (1.6 \times 10^{-5})$	$22 (1.0 \times 10^{-4})$	$15 (2.0 \times 10^{-5})$	$40 (2.0 \times 10^{-5})$
unique Indels (Indels per bp)	$5(7.9 \times 10^{-5})$	$11 (5.1 \times 10^{-5})$	$32 (4.3 \times 10^{-5})$	$36(1.7 \times 10^{-5})$

^{*a*}DNA constructs were assembled *via* circular polymerase extension cloning (CPEC), Gibson isothermal assembly, yeast homologous recombination, or ligase cycling reaction (LCR). Correct by sequencing refers to the presence of all DNA parts in the correct order and orientation. SNPs, single nucleotide polymorphisms; Indels, insertions/deletions.

amplified using desalted primers (Integrated DNA Technologies, Coralville, IA). In the case of amplification from plasmid DNA, 20 U *DpnI* (New England Biolabs, Ipswich, MA) was added per 50 μ L of PCR reaction followed by incubation for 60 min at 37 °C and 20 min at 65 °C to degrade (methylated) plasmid DNA. PCR-amplified DNA parts were purified using the AxyPrep Mag PCR cleanup kit (Axygen Scientific, Union City, CA) according to the manufacturer's instructions. Typically, 150 μ L of a PCR reaction mixture was purified and concentrated by eluting into 45 μ L of 1× TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNA parts were analyzed by capillary electrophoresis on a Fragment Analyzer (Advanced Analytical, Ames, IA) for fragment size and purity. DNA concentrations were measured using a NanoDrop 8000 (Thermo Scientific, Wilmington, DE).

DNA Assembly *via* Ligase Cycling Reaction (LCR). LCR assembly reactions were performed in a 25 μ L volume using Ampligase Thermostable DNA ligase and buffer (Epicenter Biotechnologies, Madison, WI). DNA parts and bridging oligos were added in equimolar amounts at 3 and 30 nM concentrations, respectively. Desalted bridging oligos were ordered from Integrated DNA Technologies (Coralville, IA). The following temperature cycles were used: 2 min at 94 °C and then 50 cycles of 10 s at 94 °C, 30 s at 55 °C, and 60 s at 66 °C followed by incubation at 4 °C. The reaction mixture included 8% v/v DMSO (New England Biolabs, Ipswich, MA) and 0.45 M betaine (Fluka 14290, Sigma, St. Louis, MO).

DNA Assembly *via* **Gibson Isothermal Assembly.** DNA assembly *via* the Gibson Assembly master mix (New England Biolabs, Ipswich, MA) was performed according to the manufacturer's instructions in a final volume of $20 \,\mu$ L containing $10 \,\mu$ L of 6 nM DNA parts mix (i.e., 60 fmol of each DNA part). Before and after 60 min incubation at 50 °C in an Applied Biosystems 2720 thermal cycler, reactions were stored on ice or at 4 °C.

DNA Assembly via Circular Polymerase Extension Cloning (CPEC). DNA assembly via circular polymerase extension cloning (CPEC) was performed under in-house optimized conditions in an Applied Biosystems 2720 thermal cycler (Life Technologies, Foster City, CA) using Phusion Hot-Start Flex Polymerase (New England Biolabs, Ipswich, MA) in a final reaction volume of 25 μ L containing 10 μ L of 6 nM DNA parts mix (i.e., 60 fmol of each DNA part), 200 μ M dNTPS, and 0.02 U/ μ L enzyme. For RYSE-linkered assemblies, the following CPEC conditions were used: 2 min at 98 °C and then 25 cycles of 12 s at 98 °C, 30 s at 68 °C, and 20-30 s per kb total plasmid at 72 °C followed by 10 min 72 °C and incubation at 4 °C. For seamless assemblies, the following CPEC conditions were used: 2 min at 98 °C and then 25 cycles of 12 s 98 °C, 30 s 63 °C and 20-30 s per kb total plasmid 72 °C followed by 10 min 72 °C and incubation at 4 °C.

DNA Assembly *via* **Yeast Homologous Recombination.** *S. cerevisiae* strain CEN.PK2-1c,^{29,30} a tryptophan auxotroph (*MATa ura3-52 trp1-289 leu2-3,112 his3* Δ 1 *MAL2-8^C SUC2*), was used as the host for DNA assembly by yeast homologous recombination. The vector used for DNA assembly *via* yeast homologous recombination was derived from the *TRP1*-marked yeast shuttle vector pRS414³¹ and contained the RYSE-9 and RYSE-0 linkers²⁶ cloned into the open reading frame conferring chloramphenicol resistance. Yeast cells were precultured in YPD (1% yeast extract, 2% peptone, and 2% dextrose) at 30 °C and transformed with 21 μ L of DNA-part mix containing 150 fmol of each DNA part and 5 fmol entry vector using the ssDNA/LiAc/PEG protocol³² in a total transformation volume of 141 μ L. Cells were incubated in a thermal cycler for 30 min at 30 °C, heat shocked for 45 min at 42 °C, then incubated for 15–45 min at 25 °C. After heat shock, cells were washed and resuspended in 1000 μ L complete yeast synthetic medium without tryptophan. After a 2 day outgrowth period (30 °C, with shaking), assembled plasmid DNA was isolated using the Zymoprep yeast plasmid miniprep II kit (Zymo Research, Irvine, CA) and eluted in 40 μ L 1× TE.

Transformation to E. coli, Colony Counting, and **Restriction Endonuclease DNA Fragment Analysis.** After DNA assembly, an aliquot (2.5 μ L for CPEC, Gibson isothermal assembly, and LCR assembly; 10 μ L for yeast homologous recombination) of reaction mixture was transformed to either 40 µL of XL1-Blue chemically competent E. coli cells (Agilent Technologies, Santa Clara, CA; $3-5 \times 10^7$ CFUs/ μ g of pUC19) or 40 µL of TransforMax EPI300 electrocompetent E. coli cells (Epicenter Biotechnologies, Madison, WI; 10^9-10^{10} CFUs/µg of pUC19) according to the manufacturer's instructions. After transformation, cells were diluted in Luria broth (LB), and different dilutions were plated on LB agar plates containing 100 μ g/mL of carbenicillin using liquid-handling robots. After overnight incubation at 37 °C, colony forming units (CFUs) were counted. Selected colonies (up to 30 per assembly reaction, if available) were picked into liquid LB medium containing 100 μ g/mL of carbenicillin. After overnight incubation at 37 °C with shaking, plasmids were isolated via standard miniprep protocols and fragmented using restriction endonucleases. DNA fragment lengths were analyzed by capillary electrophoresis on a Fragment Analyzer (Advanced Analytical, Ames, IA) according to the manufacturer's instructions.

Sequencing of Selected Clones. Selected purified DNA constructs were prepared for multiplexed Illumina sequencing using the FC-121-1031 Nextera DNA sample preparation kit (Illumina, Hayward, CA). To tagment the DNA constructs, 8 ng of DNA in 4 μ L was combined with a mixture of 5 μ L of buffer (TD) and 1 μ L of enzyme (TDE1). After a 10 min incubation at 55 °C, 2.5 μ L of 1% (w/v) sodium dodecyl sulfate (SDS) was added to the reaction mixture followed by 5 min incubation at room temperature and dilution to 100 μ L by addition of 87.5 μ L of 1× TE. Limited-cycle PCR to add index sequences to both ends of the DNA fragments was performed in a 25 μ L volume as follows: 10 μ L of diluted tagmentation reaction mixture was combined with 2.5 μ L of 5 μ M forward barcode primer (5' AATGATACGGCGACCACCGAGATCTACACNNNN-NNNNTCGTCGGCAGCGTC) and 2.5 μ L of 5 μ M reverse barcode primer (5' CAAGCAGAAGACGGCATACGAGAT-NNNNNNNGTCTCGTGGGCTCGG), both containing unique 8 bp index sequences, and 10 μ L of PCR master mix composed of 3.75 μ L of water, 2.5 μ L of Thermopol buffer (New England Biolabs, Ipswich, MA), 0.5 µL of 100 mM MgSO₄, 0.5 μ L of 10 mM dNTPs, 0.25 μ L of Vent DNA Polymerase (New England Biolabs, Ipswich, MA), and 1.25 μ L each of 5 μ M terminal PCR primers (5' AATGATACGGCGACCACCGA and 5' CAAGCAGAAGACGGCATACGA). The following temperature cycles were applied: 3 min at 72 °C and then 10 cycles of 10 s at 98 °C, 30 s at 63 °C, and 60 s at 72 °C, with a final incubation at 10 °C. PCR reactions were cleaned up using the Agencourt Ampure XP kit (A63880, Beckman Coulter, Indianapolis, IN) according to the manufacturer's instructions using 0.6 volumes of magnetic-bead solution per volume of PCR reaction. The DNA concentration of each sample was measured using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen P11496, Life Technologies, Foster City, CA). Capillary electrophoresis (2100

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Bioanalyzer, Agilent Technologies, Santa Clara, CA) showed an average DNA fragment length of ca. 500 bp, which was subsequently used to calculate the molecular weight of the DNA fragments. For each sample, a volume equivalent to 3 fmol of DNA fragments per kilobase of plasmid template was pooled, diluted to a final concentration of 4 nM fragments in 1× TE, denatured by mixing equal volumes of DNA fragments and 200 mM NaOH, and sequenced (paired-end, 150 bp reads) using an Illumina MiSeq system, resulting in an average depth of coverage of ca. 200. Sequencing data was demultiplexed and exported as FastQ files without downstream processing using the default Ilumina MiSeq software. After sequencing, reads in FastQ files were mapped to the plasmid reference sequences using BWA $v0.6.2^{33}$ using the sampe method with default settings. Resulting alignments were stored in BAM file format using SAMTOOLS v0.1.18.34 Mapping statistics were obtained using SAMTOOLS flagstat utility. A pile-up file was generated using SAMTOOLS mpileup with default options to obtain read coverage along the reference sequence and to derive the median plasmid coverage. Variant calls were performed from the BAM files using SAMTOOLS mpileup with E (extended BAQ computation) and g (genotype likelihood computation) options followed by BCFTOOLS view with g (call genotypes) and v (output variant sites only) options. Calls for all variant sites were stored in VCF file format.³⁵ Both single-nucleotide polymorphisms (SNPs) and small insertions and deletions (Indels) could be called using this workflow. To minimize false-positive mutation calls, only SNPs and Indels with a variant quality of at least 150 were considered. As SNPs and Indels can represent errors in the reference sequence, only SNPs and Indels were considered that were not present in other clones of the same DNA construct.

Optimization of LCR Assembly Efficiency. Optimization experiments were designed and analyzed using JMP 10 statistical software (SAS Institute, Cary, NC). Experiments (D-optimal) were designed to enable response surface methodology analysis (i.e., analysis of linear, quadratic, and interaction effects). For the experiment optimizing the factors related to the enzymatic ligation reaction, 51 unique LCR conditions and nine replicates of baseline conditions were designed and tested (Table S1). For the experiment optimizing the factors related to denaturation and annealing of DNA parts and bridging oligos, 42 unique LCR conditions and six replicates of baseline conditions were designed and tested (Table S2). The order of experiments was randomized, but experiments were grouped per thermocycler condition. For all conditions, a circular DNA construct was assembled from five DNA parts with lengths of 2163 (entry vector), 1400, 673, 499, and 345 bp (Tables S6 and S7). LCR assembly reactions were transformed in duplicate into E. coli, always using the same batch of cells within one experiment. Per LCR condition, the correctness of the eight assembled DNA constructs was tested via restriction endonuclease DNA fragment analysis. For multivariate analysis and construction of the predictive model (standard least-squares personality), the concentrations of bridging oligos, ligase, NAD⁺, and the ligation time were log-transformed to facilitate differentiation between linear and quadratic effects.

ASSOCIATED CONTENT

S Supporting Information

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S.D.K., L.H.S., T.S., K.G.P., Z.S., J.D., and S.S.C. designed the research. S.D.K., L.H.S., T.S., V.F.H., K.G.P., and E.B.S. performed the research. M.D., V.F.H., E.B.S., and D.P. performed DNA sequencing and analysis. S.D.K., J.D.N., and S.S.C. wrote the paper.

Notes

The authors declare the following competing financial interest(s): All authors possess stock or stock options in Amyris, Inc.

ACKNOWLEDGMENTS

We thank Thomas Treynor for help with designed optimization experiments and Amyris's Lab Services team for general laboratory support. This work was funded by Defense Advanced Research Projects Agency (DARPA) Living Foundries grant HR001-12-3-0006.

ABBREVIATIONS

LCR, ligase cycling reaction; $T_{\rm m}$, melting temperature; CFUs, colony forming units; DMSO, dimethyl sulfoxide; SNP, single-nucleotide polymorphism; Indel, insertion or deletion

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