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Lambda-PCR for precise DNA assembly and modification

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Abstract

Lambda-polymerase chain reaction (λ-PCR) is a novel and open-source method for DNA assembly and cloning projects. λ-PCR uses overlap extension to ultimately assemble linear and circular DNA fragments, but it allows the single-stranded DNA (ssDNA) primers of the PCR extension to first exist as double-stranded DNA (dsDNA). Having dsDNA at this step is advantageous for the stability of large insertion products, to avoid inhibitory secondary structures during direct synthesis, and to reduce costs. Three variations of λ-PCR were created to convert an initial dsDNA product into an ssDNA "megaprimer" to be used in overlap extension: (i) complete digestion by λ-exonuclease, (ii) asymmetric PCR, and (iii) partial digestion by λ-exonuclease. Four case studies are presented that demonstrate the use of λ-PCR in simple gene cloning, simultaneous multipart assemblies, gene cloning not achievable with commercial kits, and the use of thermodynamic simulations to guide λ-PCR assembly strategies. High DNA assembly and cloning efficiencies have been achieved with λ-PCR for a fraction of the cost and time associated with conventional methods and some commercial kits.

KEYWORDS

DNA assembly, DNA modifications, PCR, recombinant plasmids, λ-exonuclease

Abbreviations: *amcyan*, cyan fluorescent protein gene; dsDNA, double-stranded DNA; *gfp*, green fluorescent protein gene; *kan*^{r}, kanamycin resistance gene; ssDNA, single-stranded DNA; v1, v2, v3, variations 1, 2, and 3; λ-PCR, lambda-polymerase chain reaction.

1 | INTRODUCTION

DNA assembly, cloning, and sequence modifications are critical to the biological sciences and engineering, yet these can be prone to errors that ultimately consume considerable time and resources. Because of this, several methodologies and commercial products have been introduced to simplify and make DNA assembly a more robust process. In addition, custom DNA synthesis has matured and present solutions, but limitations still exist, and it can still be cost-prohibitive for large projects in many labs. Here, we introduce a new open-source protocol, "λ-PCR" (lambda-polymerase chain reaction), which can be used for DNA manipulations, including routine cloning and large assemblies. It is cost-effective, reliable, accommodates large DNA fragments, and allows the assembly of many DNA fragments. In addition, it requires no restriction digestions of any kind and can be designed for nearly any location in a DNA vector (single base-pair resolution). Variations of λ-PCR have been developed to allow different types of genetic manipulations including DNA insertion, substitution, deletion, and point mutations.

PCR-based DNA cloning is used routinely, and most approaches require at least one primer to contain the DNA to be cloned. In some cases, this primer set cannot be synthesized, often due to secondary structure formation or prohibitive synthesis costs. λ-PCR is a PCR-based cloning method, thus requiring a single-stranded DNA (ssDNA) primer to carry the target gene to be cloned (or DNA to be assembled). One of the significant advantages of λ-PCR is this DNA is allowed to exist as double-stranded DNA (dsDNA) before assembly. This allows it to be produced by routine PCR and provides stability during storage/incubation. In λ-PCR, the dsDNA to be cloned is converted into an ssDNA "megaprimer" immediately before assembly by PCR. This assembly PCR requires no restriction digestion of the DNA vector (or plasmid) and can occur at any location (to single base pair resolution), to our knowledge. Three different methods of converting the dsDNA to be cloned into the ssDNA megaprimer have been explored and have uses in different scenarios. We refer to these as the three variations of λ-PCR (v1-3), and they include (i) complete λ-exonuclease digestion, (ii) asymmetric PCR, and (iii) partial λ-exonuclease digestion.

Conventional methods of DNA assembly and plasmid vector construction involve restriction digestion and ligation. These strategies can be limiting due to the lack of restriction sites and inherent inefficiencies in cloning certain genes or DNA fragments. As a result, a panoply of DNA plasmid assembly and genetic manipulation techniques has emerged, and many have been commercialized. These techniques are commonly ligase-free, and several involve recombination reactions (Aslanidis & de Jong, 1990; Li & Elledge, 2007). They include the overlap extension PCR (A. Bryksin & Matsumura, 2013; A. V. Bryksin & Matsumura, 2010; Heckman & Pease, 2007; Ho et al., 1989), in vitro site-specific recombinational cloning (Gateway® cloning) (Hartley et al., 2000), In-Fusion™ assembly (Zhu et al., 2007), Gibson Assembly® (Gibson et al., 2009), Golden Gate Cloning (Engler et al., 2008; Engler & Marillonnet, 2014), and others. However, the cost of cloning kits, supplies, and DNA synthesis can limit the use of some of these technologies in academic labs, and others are limited by the size and number of DNA fragments that can be assembled (Table 1).

The Omega-PCR (Ω-PCR) method (Chen et al., 2013) was developed as another promising megaprimer-based alternative to digestion-ligation methods and commercial cloning kits (see Supporting Information: Appendix). However, the Ω-PCR protocol failed in our laboratory with our particular gene cloning tasks. Thermodynamic calculations using NUPACK (http://www.nupack.org) (Zadeh et al., 2011) revealed a much greater free energy incentive for the megaprimers to re-anneal into a dsDNA product rather than anneal to our target plasmid. However, the Ω-PCR protocol inspired the design of λ-PCR and its variations.

The λ-PCR protocol ensures that the long ssDNA megaprimers are favored thermodynamically to bind to the DNA vector and facilitate assembly rather than re-anneal with themselves. There are different methods to produce ssDNA from dsDNA (Citartan et al., 2011, 2012) including (i) λ-exonuclease enzymatic digestion (Citartan et al., 2011; Higuchi & Ochman, 1989; Mitsis & Kwagh, 1999; Null et al., 2000), (ii) asymmetric PCR (Gyllensten & Erlich, 1988; Sanchez et al., 2004; Tang et al., 2006), (iii) streptavidin-biotin separation (Hultman et al., 1989), and (iv) denaturing-urea polyacrylamide gel electrophoresis separation following PCR amplification (Williams & Bartel, 1995). The λ-exonuclease digestion and asymmetric PCR were used to develop the three λ-PCR variations: complete digestion by λ-exonuclease (λ-PCR v1; Figure 1), asymmetric PCR (λ-PCR v2; Figure 2), and partial digestion by λ-exonuclease (λ-PCR v3; Figure 3). More is available about these variations in the Supporting Information: Appendix.

The advantages we have observed with λ-PCR are documented in Table 1. To demonstrate the different applications and capabilities of λ-PCR, four case studies are presented. Case Study 1 involves simplified cloning procedures involving *green fluorescent protein* (*gfp*) gene, *cyan fluorescent protein* (*amcyan*) gene, and the pUC19 plasmid. Three distinct genetic manipulations were performed using λ-PCR in this case study: (i) direct insertion of the *gfp* gene behind *lacZ α* in pUC19, (ii) simultaneous insertion of both *gfp* and *amcyan* genes in pUC19, and (iii) substitution of the *lacZ α* gene in pUC19 with *gfp*. λ-PCR variations v1 and v2 were both used in these applications. Case Study 2 involves the creation of plasmids for gene knockout by homologous recombination in the cyanobacterium *Synechocystis* PCC 6803. λ-PCR was used to construct plasmids containing a kanamycin resistance (*kan*^{r}) gene flanked by more than 100 bp sequences with chromosomal homology. The expansion of the homologous regions beyond 100 bp increased recombination efficiency, and λ-PCR enabled the simple construction of this plasmid in four steps. Case Study 3 demonstrates λ-PCR v3 to clone "hard to clone" genes used in metabolic and enzyme engineering research. Finally, Case Study 4 demonstrates the use of thermodynamic calculations using NUPACK (Zadeh et al., 2011) to troubleshoot λ-PCR, design effective primers, and simulate the annealing of the megaprimer.

TABLE 1 Comparison of molecular cloning techniques with λ-PCR

Methods	Throughput	Directional cloning	Sequence dependency	Need for dedicated vectors	Required steps for one gene	Cost/estimate fraction of λ-PCR cost
Restriction-ligation (Bertero et al., 2017; Chao et al., 2015)	Low to mid	Possible	Yes (restriction enzyme sites)	No	1. PCR to add restriction sites (optional if the insert is from a plasmid source) 2. Digestion with restriction enzymes (PCR and plasmid backbone) 3. Purification of digested products 4. Ligation	Low/~1.6×
Gibson assembly (Bertero et al., 2017; Gibson et al., 2009)	High	Yes	No	No	1. Generation of DNA fragments with overlapping ends (restriction digest or PCR) 2. Assembly	High (reagents)/~13×
Gateway (Bertero et al., 2017; Hartley et al., 2000)	High	Yes	No	Yes	1. Generation of entry clones: a. PCR of target gene to add recombination sites b. Reaction with donor vector to get entry clones 2. Creation of expression clones	High (reagents and vectors)/~19×
Ligation-independent cloning (Bertero et al., 2017; Stevenson et al., 2013)	Depends on fragment size (low for >1.5 kb)	Yes	No	No	1. Adding short sequences of DNA to the target fragment that are homologous to the destination vector 2. Generation of complementary cohesive ends between the vector and insert 3. Annealing	Medium (vectors)/~11×
λ-PCR	Works well for routine cloning and subcloning experiments	Yes	No	No	1. PCR of the target gene 2. Generation of ssDNA megaprimer (steps 1 and 2 can be combined if using λ–PCR v2) 3. Amplification of the target plasmid	Low

Abbreviation: λ-PCR, lambda-polymerase chain reaction.

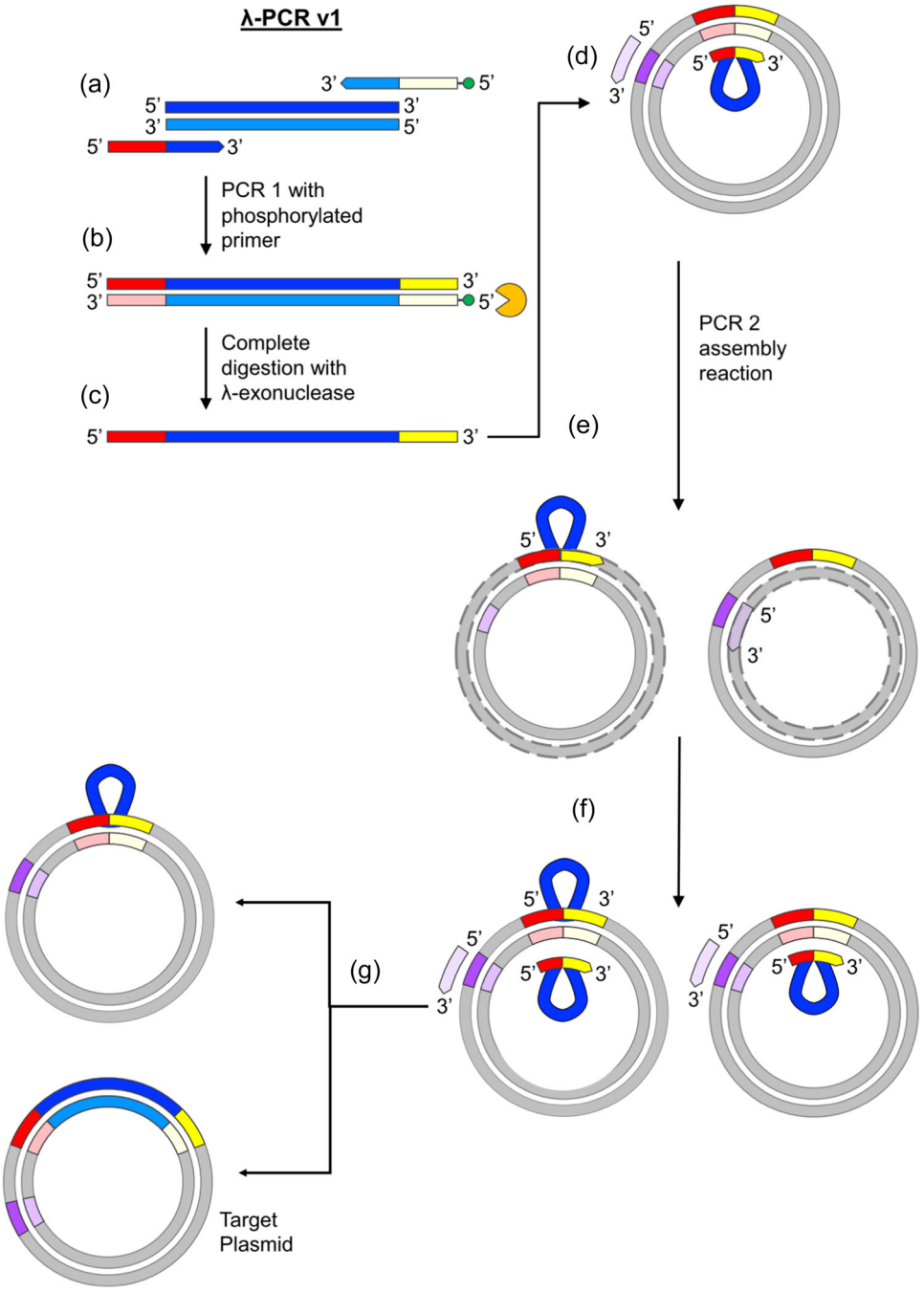

FIGURE 1 Schematic of the lambda-polymerase chain reaction (λ-PCR) v1 protocol, which uses complete digestion with λ-exonuclease to generate the ssDNA megaprimer. (a) PCR amplification of target gene with the phosphorylated reverse primer. (b) λ-exonuclease complete digestion of the reverse strand of the PCR fragment. (c) ssDNA megaprimer. (d) Annealing of megaprimer and plasmid reverse primer. (e) First PCR cycle and amplification of the plasmid. Dashed strands are newly amplified. (f) Second PCR cycle and plasmid amplification. (g) Third cycle of PCR and amplification of the target plasmid.

2 | MATERIALS AND METHODS

2.1 | Plasmids, genes, primers, and strains

The case studies made use of pUC19, pBAD24, pBAD/HisA, pACYC177, and pET28a plasmids as cloning vectors. All plasmids used and created are listed in Supporting Information: Table S1 and Appendix. The reporter genes for *gfp* and *amcyan* were obtained from plasmids pET-GFP and pAmCyan (Takara Bio USA). Chimeric primers for each DNA insertion product and one reverse primer complementary to the pUC19 backbone were designed and are listed in Supporting Information: Table S2. Chimeric primers contained two regions: (i) for amplifying the target DNA and (ii) homologous to the plasmid vector backbone to complete the cloning reaction, as shown in Figures 1–3. All primers were synthesized by Integrated DNA Technologies (Coralville). *Escherichia*

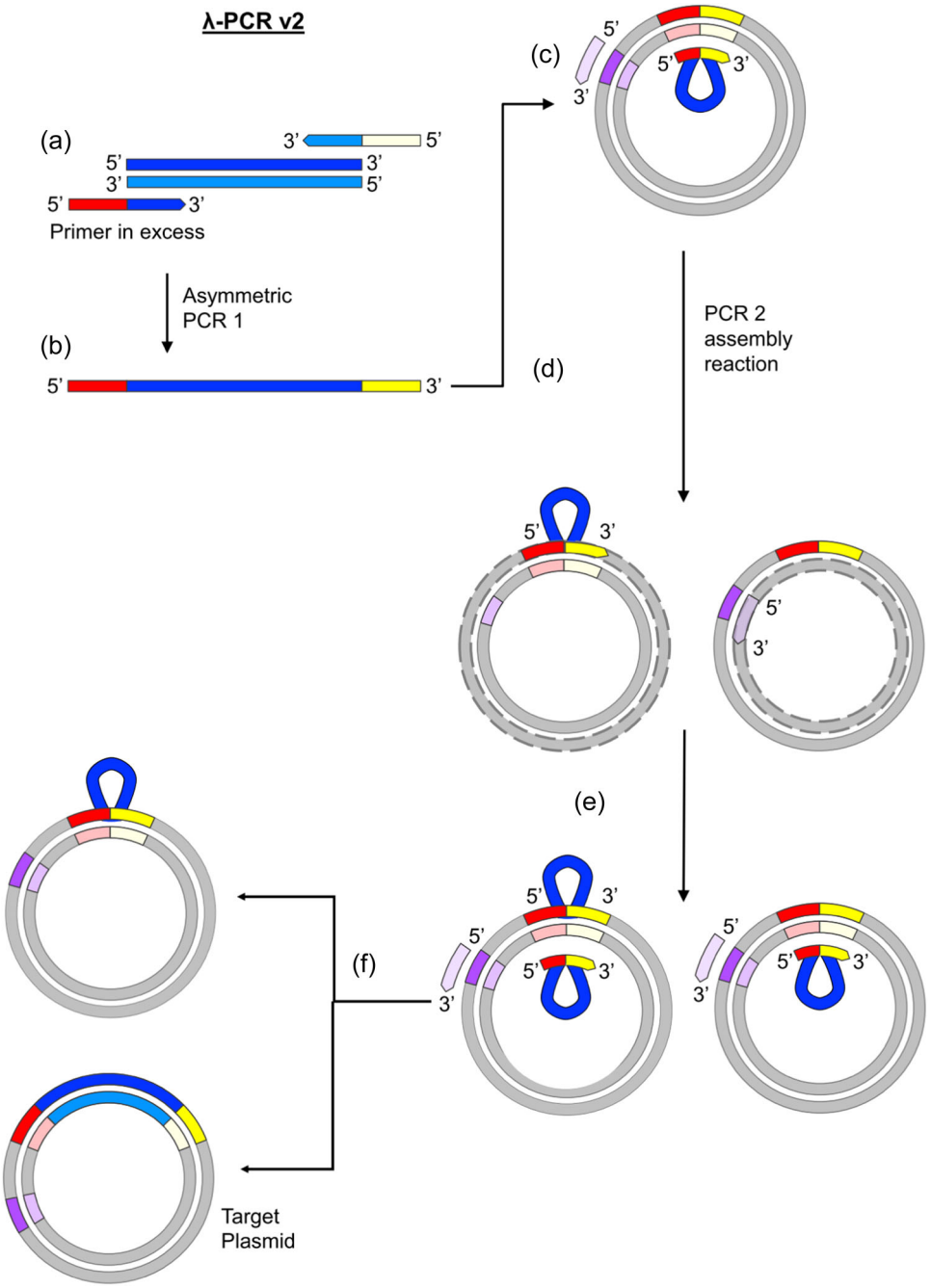

FIGURE 2 Schematic of the lambda-polymerase chain reaction (λ-PCR) v2 protocol, which uses asymmetric PCR to generate the ssDNA megaprimer. (a) PCR amplification of target gene with excess forward primer. (b) ssDNA megaprimer. (c) Annealing of megaprimer and plasmid reverse primer. Dashed strands are newly amplified. (d) First PCR cycle and amplification of the plasmid. (e) Second PCR cycle and plasmid amplification. (f) Third cycle of PCR and amplification of the target plasmid.

coli 10-beta, *E. coli* BL21DE3, and T7 Express® cells (New England Biolabs; Ipswitch) were used for transformations.

2.2 | Megaprimer generation by λ-exonuclease digestion

To generate the megaprimer for λ-PCR v1, the reverse primer in the first PCR was phosphorylated (Figure 1) as follows: (i) 0.5 µl of reverse primer (100 µM), (ii) 0.5 µl of 10× T4 DNA ligase buffer (New England Biolabs), (iii) 0.5 µl T4 polynucleotide kinase (New England Biolabs), and (iv) molecular biology grade water adjusted to 5.0 µl. The reaction was incubated at 37°C for 1 h. The phosphorylated reverse primer and unphosphorylated forward primer were used to amplify the target DNA sequence to be cloned. Final PCR volumes were 25 µl each and consisted of (i) 0.5 µl reverse primer, (ii) 0.5 µl forward primer, (iii) template DNA (~50 ng/µl), (iv) 12.5 µl Q5® High-Fidelity 2X Master Mix

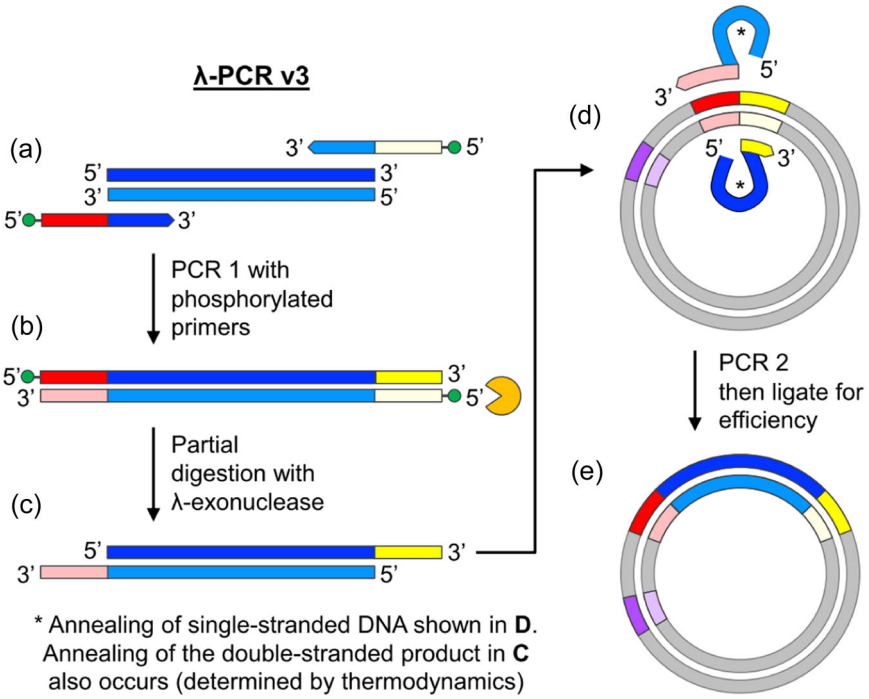

FIGURE 3 Schematic of the lambda-polymerase chain reaction (λ-PCR) v3 protocol, which uses partial digestion with λ-exonuclease to generate the dsDNA megaprimer with ssDNA overhangs. (a) PCR amplification of target gene with phosphorylated forward and reverse primer. (b) λ-exonuclease partial digestion of the PCR fragment. (c) dsDNA megaprimers. (d) Annealing of megaprimers and plasmid. PCR amplification to incorporate the megaprimers into a dsDNA fragment. (e) Ligation to circularize the target plasmid.

(New England Biolabs), and (v) molecular biology grade water adjusted to 25 µl. After 30 cycles of standard PCR, the amplified DNA was purified using the GeneJET™ PCR purification kit (ThermoFisher Scientific). The recovered PCR fragments were then digested with λ-exonuclease enzyme (New England Biolabs). The digestion reaction consisted of (i) 0.5 µl 10× reaction buffer, (ii) 5.0 µl of purified PCR product, (iii) 0.5 µl λ-exonuclease enzyme, and (iv) volume adjusted to 10 µl with molecular biology grade water. The reaction was incubated overnight, and then the λ-exonuclease was deactivated by incubation at 75°C for 20 min.

2.3 | Megaprimer generation by asymmetric PCR

A previously published asymmetric PCR protocol (Tang et al., 2006) was followed: (i) 1 pmol limit primer (reverse primer), (ii) 15 pmol excess primer (forward primer), (iii) template DNA (~50 ng/µl), (iv) 20.0 µl Q5® High-Fidelity 2X Master Mix (New England Biolabs), and (v) molecular biology grade water adjusted to 40 µl. The PCR cycling conditions included an initial denaturing at 98°C for 2 min followed by 30 cycles of (i) denaturing at 98°C for 30 s, (ii) annealing for 20 s, and (iii) elongating at 72°C (allowing 30 s per kb). A final extension step at 72°C for 3 min was then applied. PCR products were visualized by 1% agarose gel. To verify that the PCR yielded ssDNA, 5 µl of the mixture containing PCR products were treated with 20 U of S1 nuclease (ThermoFisher) to degrade ssDNA. The reaction was performed at room temperature for 1 h, and the DNA mixture was revisualized by gel.

2.4 | DNA cloning and assembly reactions

The ssDNA megaprimer, generated from a method described above, was used in a second PCR to complete the cloning reaction. The second PCR consisted of (i) 0.5 µl reverse primer (100 µM), (ii) 2.0 µl of the first PCR product containing the megaprimer, (ii) cloning plasmid (~50 ng/µl), (iv) 12.5 µl Q5® High-Fidelity 2X Master Mix (New England Biolabs), and (v) molecular biology grade water to 25 µl. A standard PCR program was run for 30 cycles. The newly amplified plasmid was recovered using a GeneJET™ PCR purification kit (ThermoFisher). The final product is a dsDNA plasmid with two nicks (one on each strand). It was then transformed into competent *E. coli*, which seal the nicks with DNA repair enzymes. In some instances, where only one of the chimeric primers has an overlap with the plasmid (Case Study 2), the final product is linear. An optional ligation step with T4 DNA ligase (New England Biolabs) was used here. DpnI treatment (New England Biolabs), according to the manufacturer's protocol, was used to digest original plasmid templates and improve cloning efficiency.

2.5 | Transformation, colony screening, and fluorescence detection

Competent *E. coli* 10-beta cells were transformed by heat-shock according to the manufacturer's protocol and incubated overnight in Luria-Bertani (LB) plates supplemented with 100 µg/ml ampicillin and 0.05 µM XGal/IPTG. Successful clones were identified by colony PCR using the One*Taq*® 2X master mix with standard buffer (New England Biolabs). PCR products were visualized by 1% agarose gel, and

positive colonies were grown in liquid LB at 30°C in 96-well microplates in an incubating Synergy H4 microplate reader (BioTek). GFP and AmCyan fluorescence intensity was monitored using excitation/emission wavelengths of 480/515 nm ($I_{480/515}$) and 458/489 nm ($I_{458/489}$), respectively. Culture growth was monitored by optical density at 600 nm (OD_{600}).

2.6 | Public availability

Commercially available vectors and fluorescent reporter genes were used as starting materials. All plasmids constructed are listed in Supporting Information: Table S1. Plasmids pUC19-psba2-kan, pBAD-B-MCLA, pBAD-MCRT, and pET-HBcAgEps are available through Addgene. All primers designed and used in this study are available in Supporting Information: Table S2, and all DNA gel pictures and analysis are available in the Supporting Information: Appendix.

3 | RESULTS

3.1 | Case Study 1: Fluorescent reporter gene insertions into pUC19

The λ-PCR protocol was demonstrated using the fluorescent reporter genes (*gfp* and *amcyan*) and the pUC19 plasmid. The following manipulations were performed: (i) insertion of *gfp* behind the native *lacZ α* of pUC19, (ii) simultaneous insertion of both *gfp* and *amcyan* genes behind *lacZ α*, and (iii) substitution of *lacZ α* by *gfp*. For this case study, two versions of λ-PCR were used: v1 (λ-exonuclease) and v2 (asymmetric PCR). All plasmids used and constructed are given in Supporting Information: Table S1, and all DNA primers used in this case study are given in Supporting Information: Table S2. A schematic of the λ-PCR cloning strategies is given in Supporting Information: Figure S1. The overall percentages (overall case studies) of positive colonies from PCR product transformants were: 45.2% for λ-PCR v1% and 40% for λ-PCR v2.

Successful clones were obtained for all three manipulations described above with all three variations of λ-PCR (DNA gel images provided as Supporting Information: Figure S2 and fluorescence data presented in Supporting Information: Figure S3). The λ-PCR v1 protocol led to the highest cloning efficiency (thousands of positive colonies per µg of DNA transformed). Transformation efficiencies are summarized in Table 2. These values were considered suitable for routine cloning and DNA assembly reactions.

3.2 | Case Study 2: Plasmids construction for Synechocystis PCC 6803 gene knockout

Gene knockout in Synechocystis PCC 6803 (Synechocystis) was performed by homologous recombination (Heidorn et al., 2011).

TABLE 2 Cloning efficiencies of the different versions of λ-PCR

λ-PCR version	Mode of λ-PCR	Plasmid name	Efficiency (c.f.u/ug DNA)
λ-PCR v1	Insertion	pUC19-lacZ α-GFP	1.86E + 04
	Substitution	pUC19-GFP	1.34E + 04
	Two insertions	pUC19-lacZ α-GFP-AmCyan	1.55E + 04
λ-PCR v2	Insertion	pUC19-lacZ α-GFP	8.57E + 03
	Substitution	pUC19-GFP	7.94E + 03
	Two insertions	pUC19-lacZ α-GFP-AmCyan	1.60E + 04
λ-PCR v1	Substitution	pUC19-KanR-PSBA2	1.22E + 04
	Insertion	pUC19-ME	1.79E + 04
	Insertion	pUC19-SDHB	1.67E + 04
	Substitution	pUC19-mdh-KanR	1.07E + 04
	Substitution	pUC19-ME-Kan	1.37E + 04
	Substitution	pUC19-SDHB-Kan	1.43E + 04
λ-PCR v2	Insertion	pUC19-mdh	1.60E + 04
λ-PCR v3	Insertion	pUC19-psba2	9.50E + 03

Note: Competent cell efficiency was tested in pUC19.
Abbreviation: λ-PCR, lambda-polymerase chain reaction.

In this case study, a *kan*^{r} gene was cloned into pUC19 and was flanked by regions (greater than 100 bp) with chromosomal homology. With conventional plasmid construction methods (i.e., restriction digestion and ligation), building this plasmid involves the ligation of three DNA fragments (two homologous region sequences flanking the antibiotic resistance gene) and insertion of this product into a multiple cloning site in the plasmid. In our experience, this has proven difficult, as the homologous region sequences can approach hundreds of base pairs for effective homologous recombination in Synechocystis. This has led to very low cloning efficiency and reliance on the availability of adequate restriction sites (Supporting Information: Figure S14).

With λ-PCR, this construction was simplified considerably and was done with high efficiency (overall percentage of positive colonies was 45.6%). The photosystem II (*psba2*) gene was chosen as the site of homologous recombination in Synechocystis because this cyanobacterium has two copies of this gene and knocking out of them does not result in lethality. Four steps were required for the final assembly of the plasmid. First, the target *psba2* gene (1083 bp) was amplified from the Synechocystis genome with phosphorylated forward and reverse primers. Next, it was partially digested with λ-exonuclease

for 30 min (λ-PCR v3). The resulting product, with ssDNA 3′ ends, was used as the megaprimer in the cloning reaction with pUC19 to replace the *lacZα* gene (DNA gel images and sequencing results provided in Supporting Information: Figure S4). Third, another PCR (with a phosphorylated reverse primer only) amplified the *kan*^{r} gene from the pACYC177 plasmid, and it was digested with λ-exonuclease overnight (λ-PCR v1). This served as the ssDNA megaprimer to insert the *kan*^{r} gene into the middle of the already cloned *psba2* gene (DNA gel images and sequencing results provided in Supporting Information: Figure S5). The resulting plasmid, pUC19-psba2-kan, was transformed into Synechocystis, and cultures were grown in BG11 medium (Rippka et al., 1979) under increasing levels of kanamycin, as described previously (Grigorieva & Shestakov, 1982; Schwarzkopf et al., 2014). After approximately 1 month, a culture capable of growing in 15 μg/ml of kanamycin was obtained, and genetic screening indicated the presence of the *kan*^{r} gene inserted into the genome.

Similarly, a series of plasmids were constructed for gene knockouts in Synechocystis. The target genes were malic enzyme (*me*), malate dehydrogenase (*mdh*), and succinate dehydrogenase (iron-sulfur subunit) (*sdhB*). The first step included the insertion of the target gene into the plasmid backbone using λ-PCR v1 for *me* and *sdhb* and λ-PCR v2 for *mdh* (details about gene amplification and sequencing are provided in Supporting Information: x Figures S6–7). The second step was performed to insert the *kan*^{r} gene into the middle of the already cloned target gene. Results for the construction of all plasmids were verified by sequencing (Supporting Information: Figures S6–8). We sequenced the junction regions in the vector constructs modified by the λ-PCR versions (we only picked three examples here). A diagram and the sequences of the two junction regions in the original plasmid pUC19 are shown in Supporting Information: Figure S8AB and these regions were sequenced for verification after modification (Supporting Information: Figure S8C–E). We found that all three resultant plasmids had been modified correctly. Transformation efficiencies are summarized in Table 2.

3.3 | Case Study 3: Cloning heterologous genes for enzyme engineering

Two genes were obtained for the construction of a de novo biosynthetic pathway. The first was malyl-CoA lyase (*mcla*), which was isolated from *Chloroflexus auriantiacus* (Herter et al., 2001, 2002). The second was malonyl-CoA reductase (*mcrt*), which was obtained from *Sulfolobus tokodaii* (Alber et al., 2006; Demmer et al., 2013). Here, we describe how to clone these genes using λ-PCR v3, as cloning these genes was unsuccessful (for reasons unknown) after months of using conventional methods and several commercial cloning kits. The pBAD/HisA plasmid of the pBAD/His kit (ThermoFisher) was selected as the cloning vector due to its histidine tag, reliable arabinose-inducible promoter, and ampicillin resistance (*amp*^{r}) gene. λ-PCR v3 was used to insert the 1047 bp *mcla* and 1071 bp *mcrt* genes into separate pBAD/HisA plasmids. This case study showed that λ-PCR v3 can accommodate full gene-sized inserts (~1 kb). With λ-PCR v3, *mcla* cloning was successful on the first transformation, after months of failures with other protocols. The *mcrt* gene required a few transformations to obtain positive colonies, but significantly improved cloning efficiency was observed for *mcrt* overall using λ-PCR v3, as opposed to our initial design using v1. This suggested that a full ssDNA megaprimer containing the *mcrt* gene in v1 may have been prone to forming secondary structures inhibitory to the cloning reaction. The improved efficiency using v3 (with a dsDNA megaprimer) illustrates the value of this variation for cases where the usually more efficient v1 fails. Additional details of this case study are also given in the Supporting Information: Figures S9 and S10. In addition, this occurrence of a failed v1 trial, led us to investigate the role(s) of inhibitory secondary structures and whether these could be predicted using thermodynamic calculations and engineered further.

3.4 | Case Study 4: Troubleshooting using thermodynamic calculations

After many successful trials with λ-PCR, we identified another case (in addition to *mcrt* with v1) where gene cloning was unsuccessful with both v1 and v3. This was cloning the Hepatitis b core antigen (*HBcAg*) gene (509 bp) into pET28a for expression and assembly into a virus-like particle. The purpose of this case study is to illustrate this potential problem, describe the solution, and demonstrate how to identify this problem a priori. The cloning of *HBcAg* was investigated by thermodynamic calculations using NUPACK (Zadeh et al., 2011), and a solution was found. To use the HBcAg protein as a backbone for vaccines to other viruses, foreign epitopes (128 bp) were to be inserted into the major immunodominant region (MIR) of the *HBcAg* gene using λ-PCR. The only change to the λ-PCR v1 protocol described previously is that plasmids were transformed into freshly thawed T7 Express® cells. Further details and figures related to this case study are also given in the Supporting Information: Appendix. Plasmids used are given in Supporting Information: Table S1 and all primers are available in Supporting Information: Table S2.

Following the attempted insertion of the foreign epitope by λ-PCR v1, the cloning products were visualized by gel electrophoresis. DNA fragment "streak," consisting of multiple indistinguishable bands of varying intensity was evident, as shown in Supporting Information: Figure S11A. This phenomenon is indicative of mispriming (i.e., binding of a primer or megaprimer in multiple or undesired locations) due to secondary structure formation in the binding region of a primer (or megaprimer). Risk of secondary structure formation increases in guanine-cytosine-rich regions. In this case study, the foreign epitope was designed to encode glycine-rich terminal ends by including three glycine residues upstream and downstream of the inserted peptides to improve the flexibility of the epitope peptide insert. The only codons available for glycine in *E. coli*

are $GG(X)_3$. Following initial λ-PCR v1 cloning, 112 colonies were screened to locate a positive clone, but none was found.

To solve this problem, thermodynamic calculations with NUPACK (Zadeh et al., 2011) were used to simulate the secondary structure formation of the ssDNA megaprimer during λ-PCR v1. The cloning reaction of λ-PCR was run with an annealing temperature of 64°C. The 3′ binding portion of the megaprimer must have affinity to the plasmid at this temperature, rather than a secondary structure within itself. NUPACK simulations of the megaprimer at 95°C, 80°C, 65°C, and 64°C are shown in Supporting Information: Figure S12A–D. Here, a large hairpin secondary structure was predicted to form near the 3′ end at 65°C, with an additional hairpin structure formation at the 5′ end at 64°C (although this should not impact gene insertion by λ-PCR). With much of the 3′ end of the megaprimer (with complementarity to the pET-28a plasmid) involved in a secondary structure, proper plasmid annealing in λ-PCR does not occur, and the gene cloning reaction failed. Alternatively, the presence of the secondary structure likely allowed other portions of the megaprimer to bind elsewhere to the plasmid, giving rise to several incomplete gene insertion products, as shown in Supporting Information: Figure S11A.

To implicate the $GG(X)_3$ nucleotide sequences in the failed cloning reaction, they were removed from the initial primers, and λ-PCR v1 was repeated. This time, the cloning reaction was successful (see Supporting Information: Figure S11B). NUPACK simulations (shown in Supporting Information: Figure S12E) indicated there were no longer inhibitory structures in the ssDNA megaprimer at the annealing temperature of the cloning reaction. Thus, the question remained as to how this cloning reaction can be performed if inhibitory secondary structure forms in the megaprimer? λ-PCR provides a unique solution to this "hard to clone" problem, which is common among all methods that involve ssDNA (even in shorter overhangs). New primers were designed that create a megaprimer to contain the foreign epitope DNA, but the 3′ end was extended further past the $GG(X)_3$ hairpin forming sequences, into the *HBcAg* gene itself. The goal was to create a megaprimer for the cloning reaction that did not form secondary structures at the 3′ annealing portion at the desired annealing temperature (64°C in this case). Because λ-PCR allows DNA cloning/assembly to occur anywhere (down to single nucleotide resolution, as shown in Supporting Information: Figure S8), the megaprimer can be extended as needed to minimize inhibitory secondary structures at the annealing temperature of the cloning/assembly reaction. This process is illustrated in Supporting Information: Figures S12 and 13 and demonstrates how thermodynamic calculations can be used to optimize λ-PCR. The NUPACK simulation of this final design is shown in Supporting Information: Figure S12F, and the successful cloning result is shown in Supporting Information: Figure S10C. In our experience, preventing the formation of secondary structures in the ssDNA binding portion of the megaprimer at the annealing temperature is key to success.

4 | DISCUSSION

λ-PCR is potentially a time- and resource-saving open-source method for DNA assembly and manipulation. It can be applied broadly and used for routine DNA cloning as well as for the "hard to clone" cases. DNA cloning technologies have moved away from restriction digest and ligation methods, and many versions of these methods are now available as commercial kits. However, their cost can be prohibitive to small and academic labs, especially when the cloning reaction efficiency is low for a particular DNA fragment. While commercial kits have a proven track-record of success, we offer λ-PCR as a complementary alternative, not as a replacement for proven methods. λ-PCR was developed in response to our failures to assemble particular "hard to clone" DNA genes/fragments using traditional methods and several commercial kits (see Supporting Information: Figure S14 and Appendix). With λ-PCR, we succeeded with the "hard to clone" DNA fragments and genes, and generally, higher efficiencies have been observed (Table 2). In addition to being restriction enzyme-free, the λ-PCR method also allows direct insertion of nearly any DNA fragment into nearly any vector at nearly any location (with single base pair resolution, Supporting Information: Figure S8), which is another advantage not available from traditional methods or commercial kits that rely on restriction digestion (Table 1).

The λ-PCR method (including the name) was inspired by the published Ω-PCR method (Chen et al., 2013) and resembles the overlap extension PCR technique (A. Bryksin & Matsumura, 2013; Gaugué et al., 2013; Heckman & Pease, 2007; Ho et al., 1989; Jiang et al., 2012; Quan & Tian, 2011). The major difference is that λ-PCR allows the incorporation of large DNA fragments without the custom synthesis of long primers, which is accomplished through the generation of a megaprimer by PCR. The three variations of λ-PCR deal with how to convert this dsDNA megaprimer product into a form with ssDNA ends that can then complete the overlap extension by PCR. In our experience, this added step in λ-PCR is crucial because secondary structures can inhibit ssDNA custom synthesis and cloning/assembly. The generation of the megaprimer may provide the flexibility to accommodate many more DNA fragments prone to secondary structure formation. While this scenario is unavoidable with some custom DNA projects, λ-PCR provides a way to mitigate this. Finally, as with overlap extension PCR, λ-PCR can be used to add DNA fragments serially to a plasmid or linear DNA strand. This makes DNA assembly limited by the molecular stability of the dsDNA strand itself, not by the assembly technique. In our practice, each new DNA fragment can be added in 1–2 days (PCR generation of the megaprimer, conversion of dsDNA to ssDNA, and cloning/assembly by PCR). This time can be reduced dramatically if the generation of multiple megaprimers and conversion to ssDNA is done in parallel. While the importance of thermodynamic calculations has been illustrated in Case Study 4, the adoption of λ-PCR more broadly will be aided by megaprimer design software that will automatically choose primers to minimize secondary structure formation in the

binding region. This software is in development and will be offered as a freely-available platform to design λ-PCR primers.

AUTHOR CONTRIBUTIONS

Imen Tanniche designed the study, carried out the experiments, analyzed the data, and wrote the manuscript with the input of all authors. Amanda K. Fisher and Frank Gillam conducted the experiments for Case Studies 3 and 4, respectively. Ryan S. Senger also designed the study, performed thermodynamics calculations, and reviewed and edited the manuscript. All authors have given approval to the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supporting Information of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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