Enzymatic Assembly of Overlapping DNA Fragments

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Contents

Abstract

Three methods for assembling multiple, overlapping DNA molecules are described. Each method shares the same basic approach: (i) an exonuclease removes nucleotides from the ends of double-stranded (ds) DNA molecules, exposing complementary single-stranded (ss) DNA overhangs that are specifically annealed; (ii) the ssDNA gaps of the joined molecules are filled in by DNA polymerase, and the nicks are covalently sealed by DNA ligase. The first method employs the 3′-exonuclease activity of T4 DNA polymerase (T4 pol), *Taq* DNA polymerase (*Taq* pol), and *Taq* DNA ligase (*Taq* lig) in a two-step thermocycled reaction. The second method uses 3′-exonuclease III (ExoIII), antibody-bound *Taq* pol, and *Taq* lig in a one-step thermocycled reaction. The third method employs 5′-T5 exonuclease, Phusion® DNA polymerase, and *Taq* lig in a one-step isothermal reaction and can be used to assemble both ssDNA and dsDNA. These assembly methods can be used to seamlessly construct synthetic and natural genes, genetic pathways, and entire genomes and could be very useful for molecular engineering tools.

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1. INTRODUCTION

For nearly 40 years, scientists have had the ability to join DNA sequences to produce combinations that are not present in nature. "Recombinant DNA technology" was initiated soon after the discovery of DNA ligase (Gellert, 1967; Weiss and Richardson, 1967) and restriction endonucleases (Smith and Wilcox, 1970). In 1972, Berg and colleagues constructed the first recombinant DNA fragment, an SV40 hybrid DNA molecule (Jackson et al., 1972). In the following year, Cohen et al. (1973) combined two antibiotic resistance markers in a single plasmid and showed that when bacterial cells take up the recombined plasmid, they become resistant to both antibiotics. By then, a 77-nucleotide gene encoding a yeast alanine transfer RNA had been synthesized from 17 overlapping oligonucleotides (oligos) (Agarwal et al., 1970). It was not long before it was announced that the human genes for somatostatin (Itakura et al., 1977) and insulin (Crea et al., 1978; Goeddel et al., 1979) were synthesized and inserted into a vector for expression in Escherichia coli.

Polymerase chain reaction (PCR) allows specific genes to be amplified from a complex mixture of genomic DNA and cloned into a vector. Restriction sites can be added to PCR primers to allow amplified products to be inserted into vector cloning sites. Overlapping DNA fragments can be assembled during PCR (Horton et al., 1990) using primers specific to the ends of the assembly; however, the size of the product cannot exceed the length that can be readily amplified by PCR. Complementary vector sequence is added to PCR primers in a technique known as ligation–independent cloning (LIC) (Aslanidis and de Jong, 1990). An improvement on this method was made and is known as sequence and ligation–independent cloning (SLIC) (Li and Elledge, 2007), which removes the sequence constraints of LIC. Both methods utilize exonuclease activity to expose complementary ssDNA sequences that can be specifically annealed. In these two systems, ligation of the DNA molecules is not performed in vitro but instead performed within a host organism following transformation of the annealed molecules.

Type II restriction enzymes have traditionally been used to clone DNA fragments by inserting them into a vector. However, this approach is of limited value for assembling multiple pieces at once and for producing large DNA fragments that do not contain a restriction site "scar." On the other hand, type IIS restriction enzymes, which cleave adjacent to but outside their recognition sites to produce sticky ends specific for the sequences to be joined, have become widely used for constructing seamless DNA molecules. For example, this scheme was used to assemble a 31.5-kb full-length infectious cDNA of the group II coronavirus mouse hepatitis virus strain

A59 (Yount *et al.*, 2002) and a completely synthetic 32-kb polyketide synthase gene cluster (Kodumal *et al.*, 2004). However, as the recombined DNA molecules get larger, it becomes increasingly difficult to find a type IIS restriction enzyme that does not cut within the assembly.

Extensive reengineering of genetic elements relies on technology that enables the assembly of small synthetic oligos. A number of *in vitro* enzymatic strategies are available for the assembly of single-stranded (ss) oligos into larger double-stranded (ds) DNA constructs (Czar *et al.*, 2008; Xiong *et al.*, 2008a,b). For example, oligos can be joined by polymerase cycling assembly and subsequently amplified by PCR (Smith *et al.*, 2003; Stemmer *et al.*, 1995). These dsDNA fragments can then be assembled into a vector by any of the methods described earlier and cloned in *E. coli*.

Overlapping DNA molecules can also be joined by the action of three enzymes: (i) an exonuclease, which chews back the ends of the fragments and exposes ssDNA overhangs that are specifically annealed; (ii) a polymerase, which fills the gaps in the annealed products; and (iii) a ligase, which covalently seals the resulting nicks. A two-step thermocycled *in vitro* recombination method was used to join 101 overlapping DNA cassettes into four quarter molecules of the *Mycoplasma genitalium* genome, each between 136 and 166 kb in size (Gibson *et al.*, 2008). Since then, two additional *in vitro* recombination methods were described that can join and clone DNA molecules larger than 300 kb, and can be carried out in a single step (Gibson *et al.*, 2009). The simplest approach is a one-step isothermal (ISO) reaction that can be used to join both ssDNA and dsDNA (Gibson *et al.*, 2009, 2010). All three assembly systems utilize the three enzymes above to produce DNA molecules that are covalently joined, and polyethylene glycol (PEG), a macromolecular crowding agent, to stimulate recombination. Because the DNA products are covalently joined, PCR or rolling-circle amplification (RCA) can be performed directly from the reactions (Gibson *et al.*, 2009, 2010).

Although three recombination methods are described later, the one-step ISO system is typically used due to its simplicity. We found that all components of this assembly system can be premixed and kept frozen until needed. Thus, all that is required for DNA assembly is for input ssDNA or dsDNA to be added to this mixture, and the mixture to be briefly incubated at 50 °C. These approaches could be very useful for cloning multiple inserts into a vector without relying on the availability of restriction sites and for rapidly constructing large DNA molecules. For example, regions of DNA too large to be amplified by a single PCR event can be divided into multiple overlapping PCR amplicons and then assembled into one piece. The ISO system is advantageous for assembling circular products, which accumulate because they are not substrates for any of the three enzymes. The one-step thermocycled method, however, can be used to generate linear assemblies because the exonuclease is inactivated during the reaction. Protocols for these recombination methods are provided later.

2. Design and Preparation of the dsDNA for *In Vitro* Recombination

DNA molecules are designed such that neighboring fragments contain at least 40 bp of overlapping sequence. However, as much as 500 bp can be used if the procedures are slightly modified as indicated below. If the DNA fragments will originate from PCR products, 40 bp overlapping sequences are introduced at the 5′ends of the primers used in the amplification reactions. DNA molecules can also be assembled from overlapping restriction fragments. The noncomplementary partial restriction sites will be removed during recombination and form a contiguous piece of DNA without intervening sequence. DNA fragments are often assembled with a vector to form a circular product. PCR amplification can be used to produce a unique vector containing terminal overlaps to the ends of the DNA fragments being joined. To produce these cloning vectors, each PCR primer includes an overlap with one end of the vector, a restriction site (e.g., *Not*I) not present within the insert to allow its release from the vector, and an overlap with one end of the DNA fragment assembly. An example of how to assemble a linear fragment into PCR–amplified pUC19 is shown in Fig. 15.1.

1. *E. coli* strains carrying overlapping restriction fragments, contained within a plasmid, are propagated in Luria Broth (LB) containing the appropriate antibiotic and incubated at 30 or 37 °C for 16 h. The cultures are harvested, and the DNA molecules are isolated using a commercially available kit (e.g., Qiagen's HiSpeed Plasmid Maxi Kit) or by following a standard alkaline–lysis procedure. Plasmid DNA is

A

5′-GAAAATGAAGATTATGATGACTTTCTTGAAATCCCTTTACAAGCAGCTAACAAAATAAACAGTTCATT
GCAATTAGGTGATGTGTTGCGAAAACCAATCCCCTTAAAAAA..
....ACCACCTATTGTTACTATCATGGGTCATGTTGACCATGGT
AAAACTTCGCTTTTAGACACAATTAGAAAAACTAATGTAACTGCTAAGGAGTTTGGCGGAATTACCC-3′

B
Oligo 1 =
5′-GTAAAGGGATTTCAAGAAAGTCATCATAATCTTCATTTTC***GCGGCCGC***gatcctctagagtcgacctg-3′

Oligo 2 =
5′AAAACTAATGTAACTGCTAAGGAGTTTGGCGGAATTACCC***GCGGCCGC***cgggtaccgagctcgaattc-3′

Figure 15.1 *Assembly vector primer design*. (A) A linear DNA sequence that is to be assembled into a vector. The first and last 40 bp of DNA sequence is underlined. (B) Two primers that could be used to PCR-amplify pUC19 to produce a vector containing overlaps to the sequence shown in (A), thus producing a circle. The primer sequences include regions that can anneal to pUC19 (nonbolded, lowercase), *Not*I restriction sites (bolded and italicized, uppercase) to release the insert from the vector, and 40-bp overlaps (underlined, uppercase) to the ends of the DNA sequence shown in (A).

eluted or resuspended in Tris–EDTA buffer, pH 8.0 (TE buffer). Overlapping DNA fragments are then released from the vector by restriction digestion. These reactions are terminated by heat inactivation or phenol–chloroform–isoamyl alcohol (PCI) extraction and ethanol precipitation. DNA is dissolved in TE buffer and then quantified by gel electrophoresis with standards.

2. Overlapping PCR fragments are produced with a high-fidelity (HF) DNA polymerase such as the hot-start Phusion® polymerase (New England Biolabs, NEB) with the HF buffer. Results may be improved by gel purifying the PCR products prior to DNA assembly. However, this is not necessary and instead, reactions may be column purified (e.g., QIAquick PCR purification Kit, Qiagen). For the one-step thermocycled and ISO systems, which are not inhibited by the presence of dNTPs, PCR products may be directly used in assembly reactions without additional purification. PCR products are quantified by gel electrophoresis with standards.

3. TWO-STEP THERMOCYCLED ASSEMBLY OF OVERLAPPING dsDNA

This two-step *in vitro* recombination method for assembling overlapping DNA molecules makes use of the 3′-exonuclease activity of T4 DNA polymerase (T4 pol) to produce ssDNA overhangs, and a combination of *Taq* DNA polymerase (*Taq* pol) and *Taq* DNA ligase (*Taq* lig) to repair the annealed joints (Gibson *et al.*, 2008, 2009; Fig. 15.2). The reaction is carried out in a thermocycler, in two steps. In the first step, the 3′-ends of the DNA fragments are digested to expose the overlap regions using T4 pol in the absence of dNTPs. The T4 pol is then inactivated by incubation at 75 °C, followed by slow cooling to anneal the complementary overlap regions. In the second step, the annealed joints are repaired using *Taq* pol and *Taq* lig at 45 °C in the presence of all four dNTPs. *Taq* pol is generally used as the gap-filling enzyme in this system because it does not strand-displace, which would lead to disassembly of the joined DNA fragments. It also has inherent 5′-exonuclease activity (or nick translation activity) (Chien *et al.*, 1976), which eliminates the need to phosphorylate the input DNA (a requirement for DNA ligation). This is because 5′-phosphorylated ends are created following nick translation. Further, this activity removes any noncomplementary sequences (e.g., partial restriction sites at the ends of overlapping restriction fragments), which would otherwise end up in the final joined product.

1. A 4× chew-back and anneal (CBA) reaction buffer is prepared. This buffer consists of 20% PEG-8000 (United States Biochemical), 800 m*M*
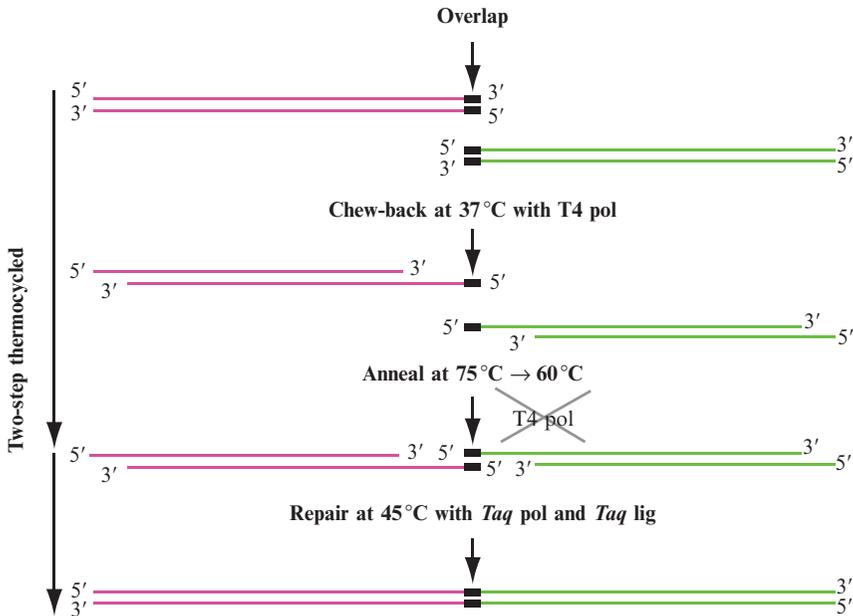
Figure 15.2 *Two-step thermocycled in vitro recombination*. **Two adjacent DNA fragments (magenta and green) sharing terminal sequence overlaps (thickened black line) are joined into one covalently sealed molecule by a two–step thermocycled reaction.**

Tris–HCl, pH 7.5, 40 mM MgCl$_2$, and 4 mM DTT and can be aliquoted and stored at -20 °C for several years.

2. The DNA fragments to be assembled are mixed in a volume not exceeding 10 μl. Approximately 10–100 ng of each DNA segment is used in equimolar amounts. For 5–8 kb DNA fragments, 25 ng substrate DNA is ideal. For larger assemblies, the amount of DNA is increased accordingly (e.g., for 20–32 kb DNA fragments, 100 ng DNA substrate is used). It is best to use ≤ 1 kb DNA fragments in 5- to 10-fold excess.

3. In a 0.2-ml PCR tube on ice, a 20-μl reaction is prepared and consists of 5 μl 4× CBA reaction buffer, 0.2 μl of 10 mg/ml BSA (NEB), 0.4 μl of 3 U/μl T4 pol (NEB), and the DNA prepared in step 2.

4. The tube is added to a thermocycler and cycled as follows: 37 °C for 5 ($\leq$80-bp overlaps) or 15 min ($\geq$80-bp overlaps), 75 °C for 20 min, 0.1 °C/s to 60 °C, 60 °C for 30 min, and then 0.1 °C/s to 4 °C.

5. *Taq* repair buffer (TRB) is prepared. This buffer consists of 5.83% PEG–8000, 11.7 mM MgCl$_2$, 15.1 mM DTT, 311 μM each of the four dNTPs, and 1.55 mM β-nicotinamide adenine dinucleotide (NAD) and can be aliquoted and stored at -20 °C for up to 1 year.

6. Ten microliters of the CBA reaction, following step 4, is added to 25.75 µl of TRB in a tube on ice. In all, 4 µl of 40 U/µl *Taq* lig (NEB) and 0.25 µl of 5 U/µl *Taq* pol (NEB) are then added.
7. The reaction is incubated at 45 °C for 15 min.
8. Samples may be used in downstream applications such as PCR or transformed into *E. coli*. For transformation, 3 µl of the undiluted TRB reaction can be directly electroporated into 30 µl Epi300 cells (Epicentre) in a 1-mm cuvette (BioRad) at 1200 V, 25 µF, and 200 Ω using a Gene Pulser Xcell Electroporation System (BioRad). Cells are allowed to recover in 1 ml Super Optimal broth with Catabolite repression (SOC) medium and then plated onto LB medium containing the appropriate antibiotic.
9. Assembly reactions are analyzed by agarose gel electrophoresis for product formation.

4. ONE-STEP THERMOCYCLED ASSEMBLY OF OVERLAPPING dsDNA

A DNA assembly method that requires the absence of dNTPs to achieve exonuclease activity, such as the T4 pol–based system described earlier, cannot be completed in one step. This is because dNTPs are required at a later point to fill in the gapped DNA molecules. ExoIII, which removes nucleotides from the 3′-ends of dsDNA, is fully functional even in the presence of dNTPs, so it is a candidate for a one-step reaction. However, it will compete with polymerase for binding to the 3′-ends. To eliminate this competition, and allow for one-step DNA assembly, antibody-bound *Taq* pol (Ab–*Taq* pol) is used in combination with ExoIII (Fig. 15.3). In this assembly method, overlapping DNA fragments and all components necessary to covalently join the DNA molecules (i.e., ExoIII, Ab–*Taq* pol, dNTPs, *Taq* lig, etc.) are added in a single tube and placed in a thermocycler (Gibson *et al.*, 2009). At 37 °C, ExoIII is active (but Ab–*Taq* pol remains inactive) and recesses the 3′-ends of the dsDNA molecules. The reaction is then shifted to 75 °C, which inactivates ExoIII. Annealing of the DNA molecules commences and the antibody dissociates from *Taq* pol, thus activating this enzyme. Further annealing, extension, and ligation are then carried out at 60 °C.

1. A 4× chew-back, anneal, and repair (CBAR) reaction buffer is prepared. This buffer consists of 20% PEG-8000, 600 mM Tris–HCl, pH 7.5, 40 mM MgCl$_2$, 40 mM DTT, 800 µM each of the four dNTPs, and 4 mM NAD and can be aliquoted and stored at −20 °C for up to 1 year.
2. ExoIII (NEB) is diluted 1:25 from 100 to 4 U/µl in its storage buffer (50% glycerol, 5 mM KPO$_4$, 200 mM KCl, 5 mM β–mercaptoethanol,
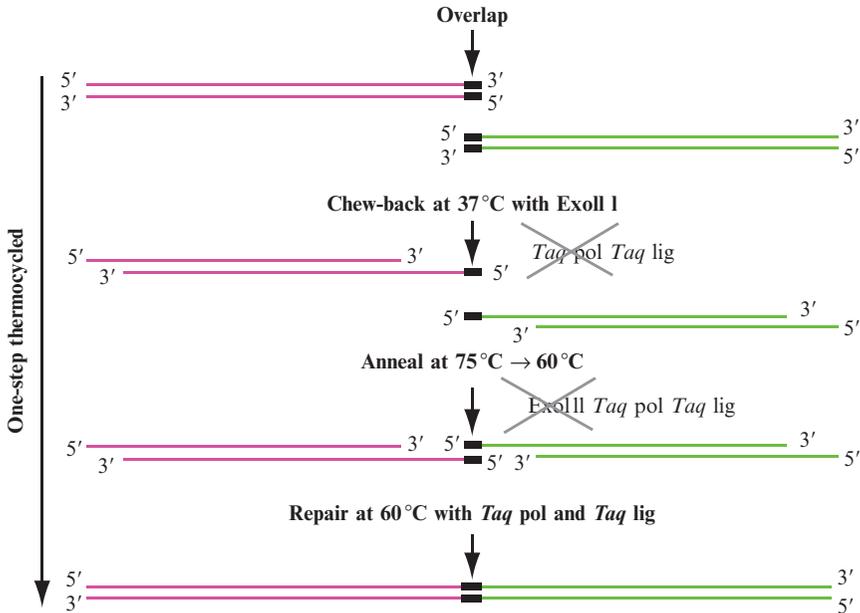
Figure 15.3 *One-step thermocycled assembly of overlapping DNA segments.* **Two adjacent DNA fragments sharing terminal sequence overlaps are joined into one covalently sealed molecule by a one-step thermocycled reaction.**

0.05 mM EDTA, and 200 µg/ml BSA, pH 6.5). This enzyme dilution can be stored at -20 °C for up to 1 year.

3. The DNA fragments to be assembled are mixed in a volume not exceed-ing 10 µl. Approximately 10–100 ng of each DNA segment is used in equimolar amounts. For 5–8 kb DNA fragments, 25 ng substrate DNA is ideal. For larger assemblies, the amount of DNA is increased accordingly. It is best to use ≤ 1 kb DNA fragments in 5- to 10-fold excess.

4. In a 0.2-ml PCR tube on ice, a 40-µl reaction is prepared and consists of 10 µl 4× CBAR buffer, 0.35 µl of 4 U/µl ExoIII, 4 µl of 40 U/µl *Taq* lig, and 0.25 µl of 5 U/µl Ab-*Taq* pol (Applied Biosystems).

5. The tube is added to a thermocycler and cycled as follows: 37 °C for 5 ($\leq$80-bp overlaps) or 15 min ($\geq$80-bp overlaps), 75 °C for 30 min, 0.1 °C/s to 60 °C, and then 60 °C for 1 h.

6. Samples are diluted 1:5 with sterile water and used in downstream applications such as PCR or *E. coli* transformation as described earlier.

7. Assembly reactions are analyzed by agarose gel electrophoresis for product formation.

8. ExoIII is less active on $3'$ protruding termini, which can result from digestion with certain restriction enzymes. This can be overcome by removing the ssDNA overhangs to form blunt ends, prior to assembly, with the addition of T4 pol and dNTPs, as described in the previous method.

5. ONE-STEP ISO ASSEMBLY OF OVERLAPPING DSDNA

Exonucleases that recess dsDNA from 5′-ends, and are not inhibited by the presence of dNTPs, are also candidates for a one-step DNA assembly reaction. Further, these exonucleases will not compete with polymerase activity. Thus, all activities required for DNA assembly can be simultaneously active in a single ISO reaction. A 50 °C ISO assembly system has been optimized using the activities of the 5′-T5 exonuclease (T5 exo), Phusion® DNA polymerase (Phusion® pol), and *Taq* lig (Gibson *et al.*, 2009; Fig. 15.4). *Taq* pol can be used in place of Phusion® pol; however, Phusion® pol is preferred, as it has inherent proofreading activity for removing noncomplementary sequences from assembled molecules. In addition, Phusion® pol incorporates the incorrect nucleotide at a significantly lower rate.

1. A 5× ISO reaction buffer is prepared. This buffer consists of 25% PEG-8000, 500 mM Tris–HCl, pH 7.5, 50 mM MgCl$_2$, 50 mM DTT, 1 mM each of the four dNTPs, and 5 mM NAD and can be aliquoted and stored at −20 °C for up to 1 year. This mix can be prepared by combining 3 ml of 1 M Tris–HCl, pH 7.5, 150 μl of 2 M MgCl$_2$,
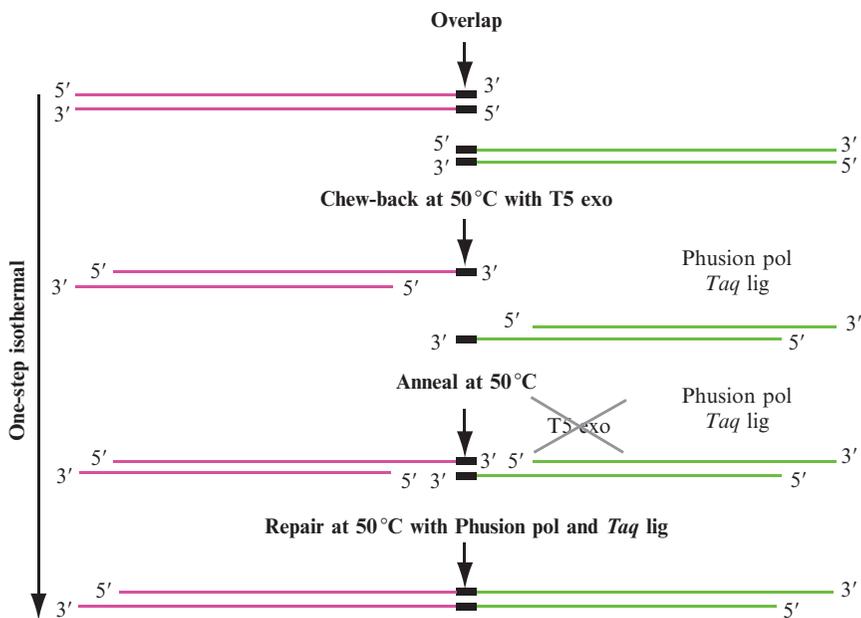

Figure 15.4 *One-step isothermal assembly of overlapping DNA fragments.* Two adjacent DNA fragments sharing terminal sequence overlaps are joined into one covalently sealed molecule by a one-step isothermal reaction.

60 μl of 100 mM dGTP, 60 μl of 100 mM dATP, 60 μl of 100 mM dTTP, 60 μl of 100 mM dCTP, 300 μl of 1 M DTT, 1.5 g PEG–8000, and 300 μl of 100 mM NAD. This will produce 6 ml of 5× ISO buffer, which can be aliquoted and stored at −20 °C for up to 1 year.

2. An enzyme–reagent master mixture is prepared by combining 320 μl of 5× ISO reaction buffer, 0.64 μl of 10 U/μl T5 exo (Epicentre), 20 μl of 2 U/μl Phusion® pol, 160 μl of 40 U/μl *Taq* lig (NEB), and water up to a final volume of 1.2 ml. Fifteen microliters of this enzyme-reagent mix can be aliquoted and stored at −20 °C for up to 2 years. This exonuclease amount is ideal for overlaps that are ≤80 bp. For overlaps that are ≥80 bp, 3.2 μl exonuclease is used in the mixture.

3. The DNA fragments to be assembled are mixed in a volume not exceeding 5 μl. Approximately 10–100 ng of each DNA segment is used in equimolar amounts. For 5–8 kb DNA fragments, 25 ng substrate DNA is ideal. For larger assemblies, the amount of DNA is increased accordingly. It is best to use ≤1 kb DNA fragments in 5- to 10-fold excess.

4. In a tube on ice, a 20-μl reaction consisting of 5 μl DNA and 15 μl enzyme–reagent master mixture is prepared, and the reaction is mixed by pipetting.

5. The reaction is incubated at 50 °C for 1 h.

6. Samples are diluted 1:5 with sterile water and used in downstream applications such as PCR or *E. coli* transformation as described earlier.

7. Assembly reactions are analyzed by agarose gel electrophoresis for product formation.

6. ONE-STEP ISO DNA ASSEMBLY OF OVERLAPPING ssDNA

The one-step ISO reaction described earlier can also be used to directly assemble oligos into the pUC19 cloning vector (Gibson *et al.*, 2010; Fig. 15.5). The assembled products are then cloned in *E. coli*, and
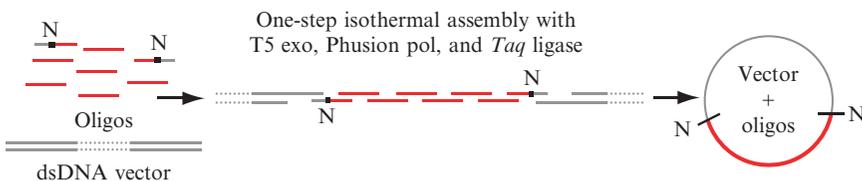

Figure 15.5 *Isothermal assembly of overlapping oligonucleotides into pUC19.* Eight 60–base oligos (red lines) are directly assembled into pUC19 (gray lines), *in vitro*, to produce a dsDNA fragment. N indicates the *Not*I restriction site (black line), which is added to release the fragment from the pUC19 vector.

the errors originating from the chemical synthesis of the oligos are weeded out by DNA sequencing. To ensure that error-free molecules are obtained at a reasonable efficiency, only eight to twelve 60-base oligos are assembled at one time. The resulting dsDNA molecules can then be assembled by any of the methods described earlier.

1. The pUC19 assembly vector is prepared. To reduce the background of undesired vector-only clones following assembly and transformation, pUC19 plasmid DNA can be linearized by restriction digestion with *Bam*HI then extracted from an agarose gel following electrophoresis. This linearized vector can then be diluted to 2 ng/μl and used as template in a PCR with a forward primer having the sequence 5′-gatcctctagagtcgacctg-3′ and a reverse primer having the sequence 5′-cgggtaccgagctcgaattc-3′. Following a standard PCI extraction and ethanol precipitation, the DNA pellets are suspended in TE buffer and then diluted to 200 ng/μl.
2. An enzyme–reagent mixture is prepared as in step 2 in the above protocol, but 20 μl of PCR-amplified pUC19 (200 ng/μl) is included in the 1.2-ml mix.
3. The oligos to be assembled are designed. Adjacent oligos overlap by 20 bp. The oligos at each end of the assembly contain a 20-bp overlap to the termini of PCR-amplified pUC19 and restriction sites not present in the assembled insert (e.g., *Not*I sites) to allow the release of the synthesized product from pUC19. An example of how this method can be used to synthesize a 284-bp fragment from eight overlapping 60-base oligos is shown in Fig. 15.6. If larger constructs are to be made from a series of

A
5′-*caggtcgactctagaggatc* **_gcggccgc_**GAAAAAAAGAACCTTTCGGCTATATAGGAATAGTATGAGCAATAATGT
CTATTGGCTTTCTAGGCTTTATTGTATGAGCCCACCACATATTCACAGTAGGATTAGATGTAGACACACGA
GCTTACTTTACATCAGCCACTATAATTATCGCAATTCCTACCGGTGTCAAAGTATTTAGCTGACTTGCAAC
CCTACACGGAGGTAATATTAAATGATCTCCAGCTATACTATGAGCCTTAGGCTTTATTTTCTTATTTACAGT
TGGTGGTCTAACCGGAATTGTT **_gcggccgc_***cgggtaccgagctcgaattc* -3′

B
Oligo 1 = 5′-*caggtcgactctagaggatc* **_gcggccgc_**GAAAAAAAGAACCTTTCGGCTATATAGGAATA-3′
Oligo 2 = 5′-CAATAAAGCCTAGAAAGCCAATAGACATTATTGCTCATACTATTCCTATATAGCCGAAAG-3′
Oligo 3 = 5′-TGGCTTTCTAGGCTTTATTGTATGAGCCCACCACATATTCACAGTAGGATTAGATGTAGA-3′
Oligo 4 = 5′-TGCGATAATTATAGTGGCTGATGTAAAGTAAGCTCGTGTGTCTACATCTAATCCTACTGT-3′
Oligo 5 = 5′-CAGCCACTATAATTATCGCAATTCCTACCGGTGTCAAAGTATTTAGCTGACTTGCAACCC-3′
Oligo 6 = 5′-CATAGTATAGCTGGAGATCATTTAATATTACCTCCGTGTAGGGTTGCAAGTCAGCTAAAT-3′
Oligo 7 = 5′-TGATCTCCAGCTATACTATGAGCCTTAGGCTTTATTTTCTTATTTACAGTTGGTGGTCTA-3′
Oligo 8 = 5′-*gaattcgagctcggtacccg* **_gcggccgc_**AACAATTCCGGTTAGACCACCAACTGTAAATA-3′

Figure 15.6 *Overlapping oligonucleotide design for assembly into pUC19.* (A) A 340-bp sequence, which includes 20 bp overlapping sequence to PCR-amplified pUC19 (nonbolded lowercase) and *Not*I restriction sites (bolded and underlined). Because 56 bp is used for assembly into and release from pUC19, only 284 bp of unique sequence (uppercase) is synthesized. (B) The sequence in (A) can be synthesized from the eight 60-mer oligos shown, which contain 20 bp overlaps.

synthetic cassettes, 40-bp overlapping sequences can also be designed into these oligos.

4. The oligos to be assembled are pooled. Oligos are prepared without modification or additional purification and suspended to 50 μM in TE buffer. Equal volumes of each oligo are then pooled in groups of 8 or 12 and diluted in TE buffer to a per-oligo concentration of 180 or 75 nM, respectively.

5. In a tube on ice, a 20-μl reaction consisting of 5 μl DNA and 15 μl enzyme–reagent master mixture containing the pUC19 vector is prepared. The reaction is mixed by pipetting up and down.

6. The reaction is incubated at 50 °C for 1 h.

7. Samples are diluted 1:5 with sterile water and used in downstream applications such as PCR or *E. coli* transformation as described earlier.

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REFERENCES

Agarwal, K. L., Buchi, H., Caruthers, M. H., Gupta, N., Khorana, H. G., Kleppe, K., Kumar, A., Ohtsuka, E., Rajbhandary, U. L., Van de Sande, J. H., Sgaramella, V., Weber, H., *et al.* (1970). Total synthesis of the gene for an alanine transfer ribonucleic acid from yeast. *Nature* **227,** 27–34.

Aslanidis, C., and de Jong, P. J. (1990). Ligation-independent cloning of PCR products (LIC-PCR). *Nucleic Acids Res.* **18,** 6069–6074.

Chien, A., Edgar, D. B., and Trela, J. M. (1976). Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. *J. Bacteriol.* **127,** 1550–1557.

Cohen, S. N., Chang, A. C., Boyer, H. W., and Helling, R. B. (1973). Construction of biologically functional bacterial plasmids in vitro. *Proc. Natl. Acad. Sci. USA* **70,** 3240–3244.

Crea, R., Kraszewski, A., Hirose, T., and Itakura, K. (1978). Chemical synthesis of genes for human insulin. *Proc. Natl. Acad. Sci. USA* **75,** 5765–5769.

Czar, M. J., Anderson, J. C., Bader, J. S., and Peccoud, J. (2008). Gene synthesis demystified. *Trends Biotechnol.* **27,** 63–72.

Gellert, M. (1967). Formation of covalent circles of lambda DNA by E. coli extracts. *Proc. Natl. Acad. Sci. USA* **57,** 148–155.

Gibson, D. G., Benders, G. A., Andrews-Pfannkoch, C., Denisova, E. A., Baden-Tillson, H., Zaveri, J., Stockwell, T. B., Brownley, A., Thomas, D. W., Algire, M. A., Merryman, C., Young, L., *et al.* (2008). Complete chemical synthesis, assembly, and cloning of a Mycoplasma genitalium genome. *Science* **319,** 1215–1220.

Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., 3rd, and Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **6,** 343–345.

Gibson, D. G., Smith, H. O., Hutchison, C. A., 3rd, Venter, J. C., and Merryman, C. (2010). Chemical synthesis of the mouse mitochondrial genome. *Nat. Methods* **7,** 901–903.

Goeddel, D. V., Kleid, D. G., Bolivar, F., Heyneker, H. L., Yansura, D. G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K., and Riggs, A. D. (1979). Expression in Escherichia coli of chemically synthesized genes for human insulin. *Proc. Natl. Acad. Sci. USA* **76,** 106–110.

Horton, R. M., Cai, Z. L., Ho, S. N., and Pease, L. R. (1990). Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques* **8,** 528–535.

Itakura, K., Hirose, T., Crea, R., Riggs, A. D., Heyneker, H. L., Bolivar, F., and Boyer, H. W. (1977). Expression in Escherichia coli of a chemically synthesized gene for the hormone somatostatin. *Science* **198,** 1056–1063.

Jackson, D. A., Symons, R. H., and Berg, P. (1972). Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. *Proc. Natl. Acad. Sci. USA* **69,** 2904–2909.

Kodumal, S. J., Patel, K. G., Reid, R., Menzella, H. G., Welch, M., and Santi, D. V. (2004). Total synthesis of long DNA sequences: synthesis of a contiguous 32-kb polyketide synthase gene cluster. *Proc. Natl. Acad. Sci. USA* **101,** 15573–15578.

Li, M. Z., and Elledge, S. J. (2007). Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nat. Methods* **4,** 251–256.

Smith, H. O., and Wilcox, K. W. (1970). A restriction enzyme from Hemophilus influenzae. I. Purification and general properties. *J. Mol. Biol.* **51,** 379–391.

Smith, H. O., Hutchison, C. A., 3rd, Pfannkoch, C., and Venter, J. C. (2003). Generating a synthetic genome by whole genome assembly: phiX174 bacteriophage from synthetic oligonucleotides. *Proc. Natl. Acad. Sci. USA* **100,** 15440–15445.

Stemmer, W. P., Crameri, A., Ha, K. D., Brennan, T. M., and Heyneker, H. L. (1995). *Gene* **164,** 49–53.

Weiss, B., and Richardson, C. C. (1967). Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Proc. Natl. Acad. Sci. USA* **57,** 1021–1028.

Xiong, A. S., Peng, R. H., Zhuang, J., Gao, F., Li, Y., Cheng, Z. M., and Yao, Q. H. (2008a). Chemical gene synthesis: strategies, softwares, error corrections, and applications. *FEMS Microbiol. Rev.* **32,** 522–540.

Xiong, A. S., Peng, R. H., Zhuang, J., Liu, J. G., Gao, F., Chen, J. M., Cheng, Z. M., and Yao, Q. H. (2008b). Non-polymerase-cycling-assembly-based chemical gene synthesis: strategies, methods, and progress. *Biotechnol. Adv.* **26,** 121–134.

Yount, B., Denison, M. R., Weiss, S. R., and Baric, R. S. (2002). Systematic Assembly of a Full-Length Infectious cDNA of Mouse Hepatitis Virus Strain A59. *J. Virol.* **76,** 11065–11078.