PART I

CLONING AND GENE DELIVERY

CHAPTER 1

DNA Assembly Technologies Based on Homologous Recombination

BILLYANA TSVETANOVA,[1] LANSHA PENG,[1] XIQUAN LIANG,[1] KE LI,[1] JIAN-PING YANG,[1] TONY HO,[1,2] JOSH SHIRLEY,[1] LIEWEI XU,[1] JASON POTTER,[1] WIESLAW KUDLICKI,[1] TODD PETERSON,[1] and FEDERICO KATZEN[1]

[1]Life Technologies Corporation, Carlsbad, CA, USA
[2]AMGEN, Thousand Oaks, CA, USA

1.1 INTRODUCTION

The origins of the recombinant DNA technology can be traced back to the discovery of restriction enzymes and the generation of the first recombinant DNA molecule over 40 years ago (1–3). Since then, and with the emergence of PCR, scientists have generated a number of elegant cloning systems that enable the manipulation of DNA fragments in various ways (for recent reviews see Refs 4–7). Some remarkable examples include the polymerase cycling assembly of oligonucleotides (8), thermostable ligation (9), topoisomerase-based cloning (10), Gateway cloning (11,12), Golden Gate cloning (13), and Biobrick assembly (http://dspace.mit.edu/handle/1721.1/21168). Due to the value that these technologies have, many of them have turned into commercial products.

In the postgenomic era and with the advent of the emerging synthetic biology field, which uses complex combinations of genetic elements to design circuits with new properties, the manipulation and analyses of large set of genes becomes a crucial necessity. In this chapter we offer a review of DNA assembly strategies based on homologous recombination, which present important advantages over other methods.

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1.2 BACKGROUND

1.2.1 Homologous Recombination *In Vitro*

Homologous recombination is the exchange of genetic information between two similar or identical molecules of DNA. This exchange occurs in a precise, specific, and faithful manner, and thus presents an excellent tool for genetic engineering for seamless gene fusion. The mechanism requires the presence of homologous regions, DNA sequence stretches shared by the recombining fragments.

The term "homologous recombination *in vitro*" is commonly referred to the joining of two or more DNA fragments that share end-terminal homology. The reaction is driven by purified enzymes involved in the double-strand DNA break-repair mechanisms such as DNA polymerases, DNA ligases, exonucleases, and single-strand DNA binding proteins. *In vitro* recombination protocols take advantage of improved PCR techniques to introduce sequence identity at the ends of adjacent DNA fragments that otherwise do not share significant homology. A common element in the *in vitro* recombination methods is the generation of complementary single-strand overhangs in the DNA fragments to be joined. Different techniques employ different approaches to generating complementary overhangs.

1.2.2 Existing Cloning Methods Based on Homologous Recombination

A number of *in vitro* methods use digestion with exonucleases to generate long complementary overhangs, thereby allowing efficient base pairing between adjacent fragments. For example "ligation-independent cloning" (LIC), uses PCR primers that are designed in such a way that the first 12 bases at the 5′-end must lack one particular nucleotide. The ($3' \rightarrow 5'$) exonuclease activity of T4 DNA polymerase is used in combination with the corresponding dNTP to specifically remove 12 nucleotides from each 3′-end of the PCR fragments. Because of the complementarity of the ends that are generated, circularization can occur between vector and insert (14,15). The technology is being commercialized by EMD under the name of Radiance. Disadvantages of this method include sequence constraints imposed on the overhangs.

Another approach is the uracil-excision-based cloning. It uses PCR amplification with primers containing deoxyuridines (dUs) in place of deoxythymidines (dTs) within the homology region, a stretch of 10–12 bases from the 5′-end of the amplicon. The resulting PCR products are then treated with uracil DNA glycosylase (UDG) for selective degradation of

the deoxyuracil residues in one strand thus promoting strand separation and the generation of 10–12 nt long complementary 3′ single-stranded overhangs (16). The method was further refined by the incorporation of DNA glycosylase-lyase Endonuclease VIII, which breaks the phosphodiester backbone at the 3′ and 5′ sides of the abasic site (17). The enzymatic mix is commercialized by New England Biolabs as Uracil-Specific Excision Reagent (USER) enzyme. The shortcoming of this method is its sequence dependency, as multiple dUs must be incorporated into the 5′-end of the primer and compatible dU tails must be introduced on the plasmid.

A related technology requires PCR amplification with primers containing three consecutive ribonucleotides or a single 2′-*O*-methylribonucleotide (18). Here the polymerase stops processing when it encounters the modified nucleotides in the template thus generating PCR products with 3′ recessed ends, whose length is defined by the position of the modified nucleotide.

Oligonucleotides containing one or more phosphorothioate bonds have been also used to generate single-stranded overhangs of specific length (19,20). 5′–3′ exonuncleases are ordinarily unable hydrolyze phosphorothioate bonds, thereby leaving behind a 5′ recessed end.

A flexible and versatile technique is the so-called sequence-and-ligation-independent cloning (SLIC) (21). The method relies on ssDNA overhangs generated by T4 DNA polymerase. It does not require a specific sequence or the use of modified nucleotides. The annealed recombination intermediates may still have gaps and overhangs, which are efficiently repaired inside the cell upon transformation. A likely similar principle is applied in the commercially available In-Fusion PCR cloning system (Clontech), which uses a proprietary enzyme mix to join together any PCR product with a linearized vector. Up to three inserts can be simultaneously assembled into a vector using this system (22).

The above described methods are based on the generation of complementary single-stranded overhangs between adjacent fragments that can anneal and be directly transformed without being ligated. Though these methods are applicable for most common cloning experiments, they are rather inefficient in the assembly of constructs larger than 15 kbp. An *in vitro* method for genome-scale assemblies has been recently published (23). The reaction takes place at 50°C and uses a heat labile 5′–3′ exonuclease and heat stable polymerase and ligase enzymes. This enzymatic formulation limits the exonuclease activity, and at the same time it allows filling-in the gaps and sealing the nicks. The authors have used this strategy to assemble a quarter of the *Mycoplasma genitalium* genome (144 kb) (24). The drawback of the approach is that it requires significantly larger overlap regions (80–360 bp) than other strategies. The method was further adapted for the assembly of DNA molecules directly from overlapping oligonucleotides, which combined

with PCR amplification allowed the synthesis of the mouse mitochondrial genome (25).

We have recently developed a novel DNA assembly system based on *in vitro* homologous recombination, GENEART® Seamless Cloning and Assembly (26), which combines some of the advantages of the methods above.

1.3 PROTOCOL

1.3.1 *In Vitro* Recombination Protocol

The reliability of our method is only limited by the fidelity of the PCR reaction and the quality of the primers used. The assembly reaction takes 30 min at room temperature followed by a transformation into chemically competent *Escherichia coli*. The strongest advantage of this cloning system is the ability to assemble multiple fragments bearing a small overlap (15 bp) with very high efficiency (Figure 1.1a). This system also enables internal recombination up to 200 bp from the end of the fragments (Figure 1.1b). In addition, we have developed a free online software, the Oligo Designer Webtool (www.invitrogen.com/DesignDNAassembly), which utilizes input fragment sequences to design the oligonucleotides with the required overlap tails providing a GenBank® annotated sequence of the assembled construct.

1.3.1.1 Assembly Reaction Add the following components in a microcentrifuge tube in the order they are listed below:

Insert(s)[a]	20–100 ng each
Linearized vector	100 ng
Sterile water	to 14 µL
5× Reaction buffer	4 µL
10× Enzyme mix	2 µL

[a]We recommend a 2:1 molar ratio of insert:vector for optimum result.

Incubate the reaction at room temperature for 30 min. Then place the tube on ice and proceed immediately to transformation.

1.3.1.2 Transformation Transform One Shot® TOP10 chemically competent *E. coli* (Life Technologies, Carlsbad, CA) or similar following standard protocols. *Note*: Do not use electrocompetent cells. Plate the cells on LB agar plates supplemented with the corresponding antibiotics.

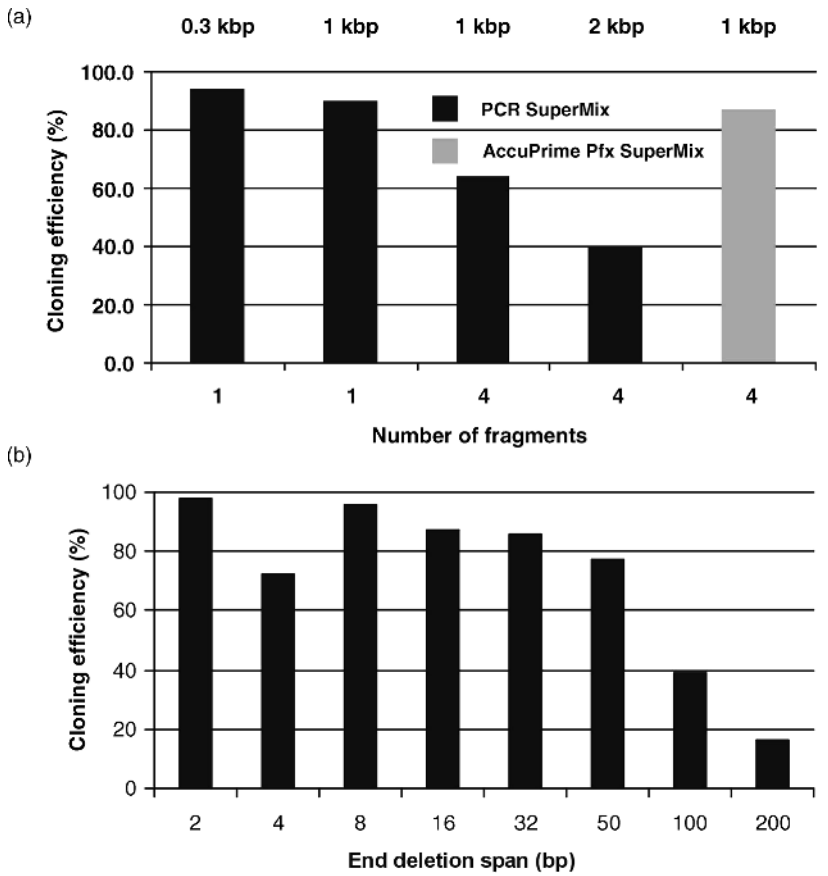

FIGURE 1.1 *In vitro* recombination assemblies. PCR-amplified inserts of different sizes were assembled into a restriction enzyme-linearized pUC19 vector. (a) At least four fragments can be joined simultaneously in a predetermined order into a vector with high cloning efficiencies. Fragments amplified with a proofreading polymerase (AccuPrime Pfx Supermix, Life Technologies, Carslbad, CA) resulted in significantly higher cloning efficiencies than those amplified with a standard polymerase (PCR supermix, Life Technologies, Carlsbad, CA). (b) The approach allows for recombination to occur within the fragments. Two fragments were assembled into linearized pUC19. The first fragments had 15 bp homology to an internal region located at 2–200 bp away from the end of the second fragment. This attribute is useful for generating cloning variants using a single linearized vector.

1.3.2 Homologous Recombination *In Vivo*

A general limitation of *in vitro* cloning methods is that they are particularly inefficient with extremely large constructs. The larger the assembly, the more refractory to transformation the cells are and the more susceptible to physical distress the DNA molecules would be. A way to overcome these constraints is

to let the host organism perform the assembly process, limiting the *ex vivo* manipulation of DNA to the smaller component fragments.

Although *E. coli* is the preferred host for conventional cloning, its natural homologous recombination system is very inefficient. Homologous recombination in *E. coli* can be RecA dependent or RecA independent. RecA-mediated recombination requires relatively long homology regions between the fragments (>1 kb) and occurs at very low frequency (27). The RecA-independent mechanism occurs in *recA* strains, requires 15–40 bp overlap between two linear fragments, and it works at a very low efficiency (28). Recombination efficiency can be boosted by overexpressing the *E. coli* endogenous *recE* and *recT* genes or their counterparts from other organisms such as the red system from the phage λ (Murphy, 1998; Datsenko et al., 2000; Yu et al., 2000). Limitations of the method include the dependence on a host expressing the *red/recET* genes and the relatively low efficiency for the assembly of multiple fragments. The λ red system has been successfully used in combination with *in vivo* expression of homing endonucleases and conjugal mating, resulting in mating-assisted genetically integrated cloning (MAGIC) (29). The system circumvents problems associated with the *in vitro* manipulation of DNA, but it relies on the use of specific vectors and engineered hosts.

In vivo recombination has also been reported using *Bacillus subtilis* as the host organism. Mitochondrion and chloroplast genomes have been successfully assembled in this bacterium (30). The method is based on nonreplicating episomes with different antibiotic resistance markers containing sequences with end-homology that are subsequently transformed into the cells. Assembly takes place step by step in the *Bacillus* genome and can be later recovered by homologous recombination with a retrieval plasmid or a bacterial artificial chromosome (BAC).

The most broadly used *in vivo* DNA assembly platform has been the one based on *Saccharomices cerevisiae* cells. The natural ability of yeast to recombine DNA fragments with homologous sequences during transformation has been observed over 30 years ago (31,32). Since then this property has been applied to the cloning of large DNA fragments into artificial yeast chromosomes (transformation-associated recombination or TAR) (33,34). More recently, the TAR approach has been used to assemble the *Mycoplasma* genome (24,35,36). Furthermore, it has been shown that the yeast system can join DNA fragments that share no homology by using short oligonucleotides that overlap the ends of the adjacent fragments (stitching oligonucleotides), thus guiding the homologous recombination (37,38). Finally, it has been demonstrated that yeast can efficiently assemble DNA fragments starting from oligonucleotide building blocks (39). The TAR cloning method eliminates the need for enzymatic treatment of the DNA and greatly reduces the *in vitro* manipulation of large DNA fragments.

We have recently developed a simplified yeast-based DNA assembly system (GENEART High Order Genetic Assembly) (26). It can efficiently assemble constructs of up to 110 kb using multiple fragments with only 30–50 bp overlap in one single transformation event. Our approach is compatible with fragments that do not share end-homology, provided that stitching oligonucleotides are added to the transformation mix. This feature allows reusing fragments and editing the junctions by removal or addition of end sequences such as restriction sites, tags, or small watermarks. We have also developed an *E. coli*–yeast shuttle vector that permits a rapid and efficient transfer of the assembled construct back to *E. coli* for subsequent manipulations and an adaptation cassette that permits the conversion of any *E. coli* vector into a yeast–*E. coli* shuttle vector (Figure 1.2).

Our Oligo Designer Webtool (www.invitrogen.com/DesignDNAassembly) facilitates the oligonucleotide design and also warns when unwanted homology is shared among the fragments.

1.3.3 Yeast-Based DNA Assembly Protocol

1.3.3.1 Transformation of Competent Yeast Cells Mix the following components into a microcentrifuge tube:

pYES1L (Figure 1.2), or your own linearized vector	100 ng
DNA inserts	100 ng each (if final construct is $\leq$25 kb) 200 ng each (if final construct is >25 kb)
Stitching oligonucleotides (if necessary)	500 ng each

If the total volume of the DNA mix is larger than 10 µL, reduce total volume to 5–10 µL using a SpeedVac® or a centrifugal filter device.

Add 100 µL of *S. cerevisiae* MaV203 yeast competent cells thawed at 30°C into the DNA mix (the volume of the DNA mix should be $\leq$10 µL). Mix well by tapping the tube. Then add 600 µL of the PEG/LiAc solution to the DNA/competent cell mixture. Mix by inverting the tube five to eight times until the mixture becomes homogeneous. Incubate in 30°C water bath for 30 min. Invert the tube occasionally (every 10 min) to resuspend the components. Add 35.5 µL of DMSO to the tube. Mix by inverting the tube. Heat-shock the cells in 42°C water bath for 20 min. Invert the tube occasionally to resuspend the components. Centrifuge at 1800 rpm (200–400 $\times$ *g*) for 5 min. Carefully

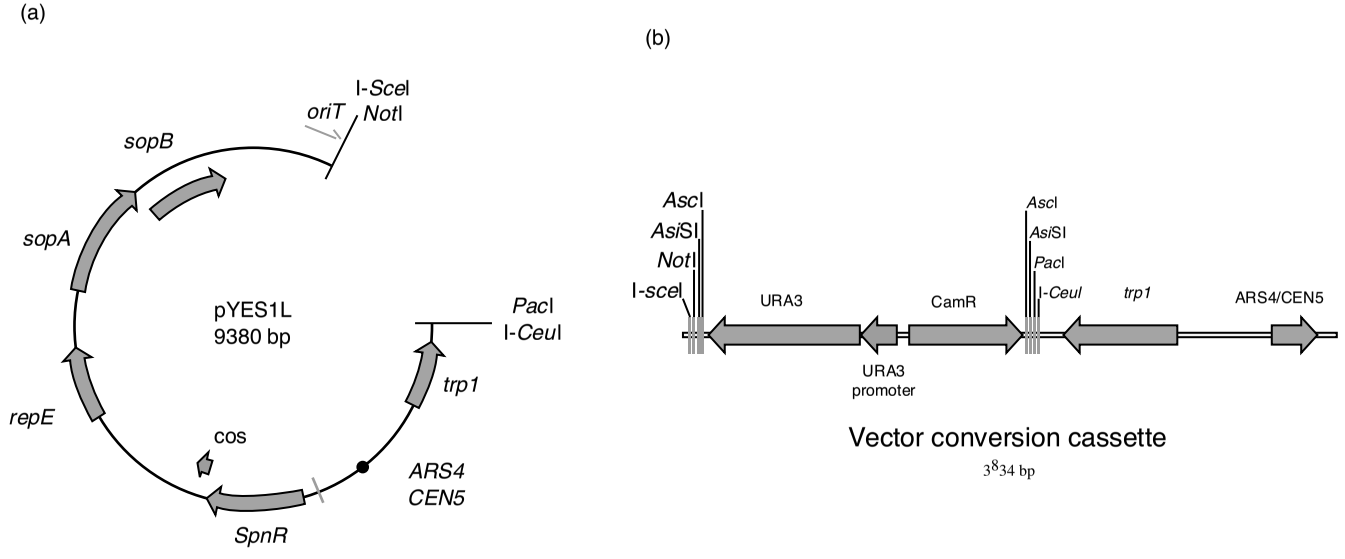

FIGURE 1.2 Yeast-*E. coli* shuttle vector and plasmid conversion cassette. (a) The *in vivo* approach uses the pYES1L shuttle vector that carries replication and selection elements for *S. cerevisiae* and *E. coli*. An autonomously replicating sequence (ARS) and a centromere (CEN) are responsible for replication and partitioning in yeast, whereas *trp1* is used as selection marker that complements yeast strains deficient in tryptophan biosynthesis. To maximize DNA capacity when replicating in *E. coli*, the F′ replication origin and accessory genes from the mini-F plasmid (41) were incorporated. Other elements such as the spectinomycin resistance gene (SpnR), an origin of transfer (*oriT*) for plasmid mobilization, and the cos site from bacteriophage λ were also included. (b) Scheme of the yeast adaptation cassette.

TABLE 1.1 Assemblies Using Fragments with End-Homology

No. of Fragments[a]	No. × Length of Precloned Fragments (kbp)[b]	No. × Length of PCR-Amplified Fragments (kbp)	Total Size (kbp)[c]	Overlap (bp)	Insert (ng)	Cloning Efficiency
3	3 × 10	0 × 0	30	80	100	100
5	5 × 10	0 × 0	50	80	100	100
10	10 × 10	0 × 0	100	80	100	50
20	8 × 10	12 × (0.5–2.5)	100	80	100	58
20	8 × 10	12 × (0.5–2.5)	100	80	200	83
1	0 × 0	1 × 0.7	0.7	30	100	100
1	0 × 0	1 × 10	10	80	200	100
1	0 × 0	1 × 10	10	30	200	100
10	0 × 0	10 × 5	50	30	200	92

[a]Vector not counted as a fragment.
[b]Fragments were initially cloned into pACYC184 and excised by *Not*1 digestion.
[c]Vector size not considered.

remove the supernatant from the tube and resuspend the cell pellet in 1 mL of sterile 0.9% NaCl by gentle pipetting. Plate 100 µL of the transformation onto CSM-Trp agar plates. For final constructs of >60 kb, we recommend that you centrifuge the remaining 900 µL of the transformation mixture, remove ~750 µL of the supernatant, resuspend the cell pellet in the remaining 100–150 µL supernatant, and plate all cells onto another CSM-Trp agar plate. Incubate the cells at 30°C for 3 days and proceed to screening for correct clones (see below). Typical results are presented in Table 1.1 and Figure 1.3.

1.3.3.2 Screening for Positive Clones

Diagnostic Primers We recommend that you first screen the yeast colonies by colony PCR assays using a pair of diagnostic primers that amplify each expected junction. For example, for a 5-fragment (plus vector) assembly, six pairs of diagnostic primers should be used. Oligonucleotide pairs (forward and reverse) should be designed at a distance of 100–250 bp from the ends of each DNA fragment (including the cloning vector) so that the colony PCR products would be 200–500 bp in size and span the junctions between the fragments.

Yeast Colony PCR Pick individual yeast colonies using a sterile pipette tip and resuspend the cells by pipetting up and down in 15 µL of yeast lysis buffer (Life Technologies, Carlsbad, CA) or equivalent. Transfer 5 µL of each cell suspension into fresh PCR tubes and store at 4°C until verification

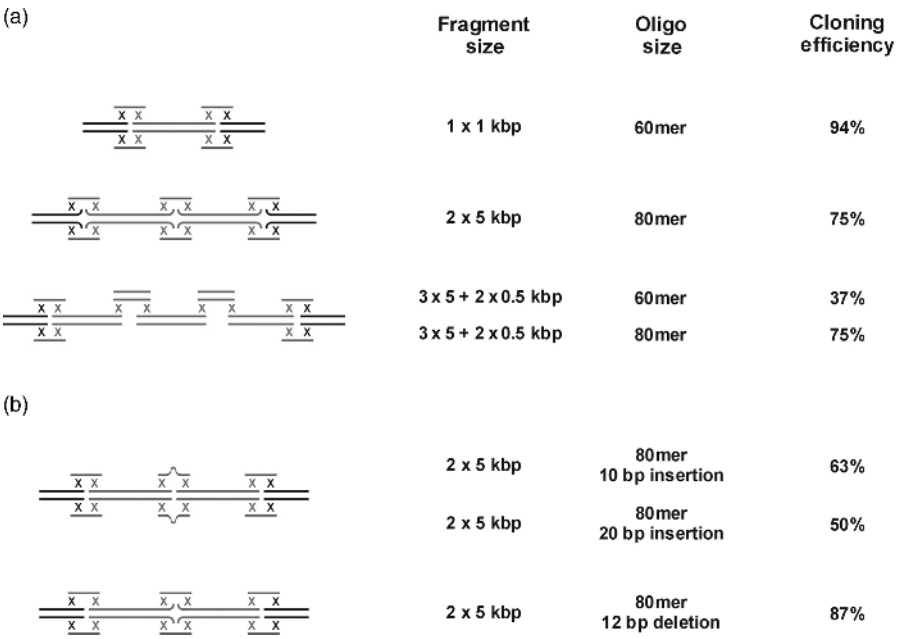

FIGURE 1.3 *In vivo* yeast recombination assemblies of fragments without end-homology. (a) One, two, and five fragments of different sizes were simultaneously assembled into the yeast–*E. coli* shuttle vector pYES1L assisted with stitching oligonucleotides. The use of longer oligonucleotides with larger size overlap to the adjacent fragments results in higher cloning efficiency especially for the more complex 5-fragment assembly. (b) Junction editing by using stitching oligonucleotides. Insertions of up to 20 bp and deletions of up to 12 bp can be readily obtained using the system.

(see below). Heat the remaining cell suspension (10 µL) at 95°C for 5 min in a thermocycler and place the tube on ice. Briefly centrifuge the PCR tubes to bring down condensed water. Add 40 µL of nuclease-free water to each lysate and pipet up and down three to five times to mix. Set up a PCR master mix for each junction and aliquot 49.5 µL of it into fresh PCR tubes or plates. Add 0.5 µL of the diluted yeast lysate into each PCR tube or well. Do not exceed 0.5 µL of lysed yeast cells for 50 µL of PCR volume. Vortex to mix the contents and briefly centrifuge to bring down all liquid. Perform PCR cycling in a thermocycler. Load 10 µL onto an agarose gel to visualize the PCR products. We recommend sequencing of the PCR products.

Yeast–E. coli Transfer Plasmid DNA preparation from yeast cells usually results in very low yield and poor DNA quality. Therefore, it is a common laboratory practice to retrieve the shuttle plasmids out of yeast and transfer back into *E. coli* for additional analysis or manipulation. We have developed a

highly efficient protocol with a few modifications to an earlier strategy (40) that streamline the process.

Aliquot four to five glass beads (Life Technologies, Carlsbad, CA or similar) into a fresh PCR tube and add 10 µL of yeast lysis buffer. Add 5 µL of the cell suspension that was stored at 4°C (see above) into the lysis buffer/glass beads mix and pipet up and down three to five times to mix. Vortex the cells at room temperature for 5 min. Do not heat the lysed cells. Add 1 µL of the lysed cells into a vial of electrocompetent cells and mix gently. Do not add more than 1 µL of the lysed cells to avoid arcing during electroporation. We recommend using One Shot TOP10 electrocompetent *E. coli* cells (Life Technologies, Carlsbad, CA) or similar. Transfer the cells into a chilled electroporation cuvette on ice. Electroporate the cells following the manufacturer's recommended protocol. Add 250 µL of prewarmed SOC medium to each vial. Transfer the solution to a 15 mL snap-cap tube and incubate for at least 1 h at 37°C with shaking to allow the expression of the antibiotic resistance gene. Spread 10–50 µL from each transformation on a prewarmed LB plate supplemented with the appropriate antibiotic. Invert the selective plate(s) and incubate at 37°C overnight.

1.3.3.3 Guidelines for Designing Stitching Oligonucleotides

- Each pair of adjacent fragments requires two stitching oligonucleotides (sense and antisense) to link them together.
- Stitching oligonucleotides can help assemble up to five fragments plus vector, provided that not more than three junctions are formed by the stitching oligonucleotides and the remaining junctions are produced by shared end-terminal homology.
- To assemble up to three nonhomologous fragments of <5 kb the stitching oligonucleotides must be 80 mers (i.e., they must have a 40 bp overlap with each adjacent fragment).
- The stitching oligonucleotide stocks are prepared at a final concentration of 100 µM in 1× TE buffer, pH 8 (10 mM Tris-HCl, 1 mM EDTA, pH 8).
- For making insertions the stitching oligonucleotides must have a 30-nucleotide overlap with each adjacent fragment in addition to the inserted bases (up to 20 bases).
- Stitching oligonucleotides used for deletions must have a 40-nucleotide overlap with each adjacent fragment. They must anneal to the fragment ends at a distance of not more than six nucleotides from the junction.
- The stitching oligonucleotides are added to the inserts and vector mix at a concentration of 50 ng/µL each (2 pmol/µL each).

1.4 PERSPECTIVES

Over the last 10 years significant advances on DNA assembly have been reported. New and improved assembly techniques continue to be developed with an emphasis on addressing the new challenges from the emerging field of synthetic biology. Different methods address different scales of assembly. Currently there is not a single "ideal" technique that would allow the rapid simultaneous assembly of multiple preexisting or synthesized, small or large fragments in a seamless predetermined order without sequence-specific requirements. Here we show that a combination of *in vitro* and *in vivo* homologous recombination-based methods can provide a solution for a wide range of assemblies. For example, the seamless assembly of multiple small fragments into larger constructs can be accomplished by the *in vitro* approach whereas the large fragments can then be efficiently assembled by the *in vivo* yeast-based method. A good illustration of this strategy is the two-step assembly of the *Mycoplasma* genome (24), where small and intermediate-size fragments were assembled *in vitro* and used subsequently to generate genome sized sequences using the yeast approach.

In the future, the small scale assemblies of a single gene or a group of genes might be gradually replaced by DNA synthesis. However, the demand for larger scale assemblies at the level of complete pathways up to whole genomes will continue to grow and it will require reliable high throughput-adapted DNA assembly methods. These approaches should allow the rapid combinatorial construction of customized pathways and genomes from any number of modules at any number of defined positions.

DISCLOSURE

Life Technologies products are for research use only; not intended for any animal or human therapeutic or diagnostic use.

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