

# GENETIC ASSEMBLY TOOLS FOR SYNTHETIC BIOLOGY

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## Abstract

With the completion of myriad genome sequencing projects, genetic bioengineering has expanded into many applications including the integrated analysis of complex pathways, the construction of new biological parts and the redesign of existing, natural biological systems. All these areas require the precise and

concerted assembly of multiple DNA fragments of various sizes, including chromosomes, and the fine-tuning of gene expression levels and protein activity. Current commercial cloning products are not robust enough to support the assembly of very large or very small genetic elements or a combination of both. In addition, current strategies are not flexible enough to allow further modifications to the original design without having to undergo complicated cloning strategies. Here, we present a set of protocols that allow the seamless, simultaneous, flexible, and highly efficient assembly of genetic material, designed for a wide size dynamic range (10s to 100,000s base pairs). The assembly can be performed either *in vitro* or within the living cells and the DNA fragments may or may not share homology at their ends. A novel site-directed mutagenesis approach enhanced by *in vitro* recombineering is also presented.

## 1. OVERVIEW

The consensual definition of the “synthetic biology” term refers to the design and construction of new biological parts, devices, and systems, and the redesign of existing, natural biological systems for useful purposes ([www.syntheticbiology.org](http://www.syntheticbiology.org)).

Challenges of these aspirations include the standardization of parts and devices, the modeling of intricate metabolic circuits, and the seamless assembly of fairly large pieces of DNA in a predetermined order to program the circuits above. The ultimate goal would be to convert a digitized sequence of a chromosome into its biochemical counterpart in a single step.

This latter ambition implies that the classical recombinant DNA technology, based on restriction enzymes, ligase, and PCR, needs to evolve to cover a wide range of DNA sizes and number of fragments.

Significant progress has been made during the past few years. Advanced systems for assembling multiple DNA fragments have been developed. Some of them such as RecA-independent recombination, Red/ET recombination, Gateway, or *loxP* based are practical and fast but they work with a limited number of fragments. (Bubeck *et al.*, 1993; Cheo *et al.*, 2004; Datsenko and Wanner, 2000; Hartley *et al.*, 2000; Liu *et al.*, 1998; Zhang *et al.*, 1998). Other approaches, such as ligation-independent cloning, overlap PCR, SLIC, or the use of Type IIS restriction enzymes, require considerable design and/or preparation time (Aslanidis and de Jong, 1990; Gao *et al.*, 2003; Lebedenko *et al.*, 1991; Li and Elledge, 2007).

In our experience, the most viable strategies are those that make use of single step either yeast-based or *in vitro* homologous recombination.

In this chapter, we provide a set of protocols based on homologous recombination useful for successfully assembling and editing small, intermediate, and large DNA fragments.



## 2. YEAST-BASED HOMOLOGOUS RECOMBINATION

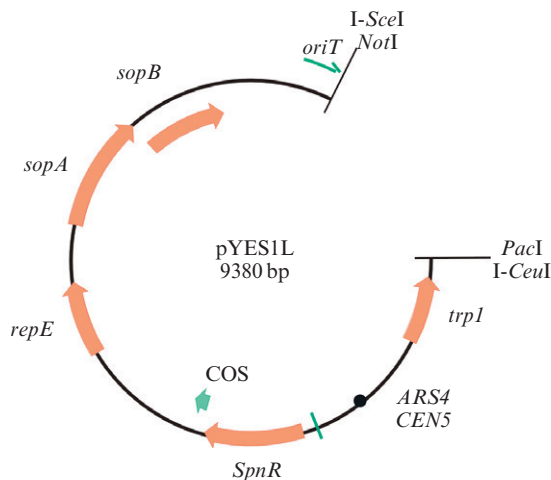
The approach described in this section relies on the yeast's powerful ability to take up and recombine DNA fragments. This property has been first described over 30 years ago (Hinnen *et al.*, 1978; Orr-Weaver *et al.*, 1981), and since then it has been applied to the generation of plasmids and yeast artificial chromosomes under the name of transformation-associated recombination (TAR; Ebersole *et al.*, 2005; Larionov *et al.*, 1994, 1996; Ma *et al.*, 1987; Marykwas and Passmore, 1995). At the same time, it has been shown that any given DNA sequence can be joined to a vector using short synthetic linkers that bridge the ends (Raymond *et al.*, 1999, 2002). More recently, the technology has been used to build an artificial bacterial chromosome and to assemble genes from oligonucleotides (Gibson, 2009; Gibson *et al.*, 2008a,b, 2010; Lartigue *et al.*, 2009). All these approaches used linearized yeast vectors and fragments with overlapping ends, and thereby can undergo recombination that restores the circular topology and functionality of the plasmid as linearized plasmids lacking telomeres fail to replicate (Szostak and Blackburn, 1982).

### 2.1. Yeast–*Escherichia coli* shuttle vector

A fundamental requirement for the TAR approach is a *Saccharomyces cerevisiae* replicating vector with a selectable marker. Replication elements routinely used in yeast plasmids consist of either an autonomously replicating sequence (ARS) and a centromere (CEN) or replication and partitioning sequences from the endogenous 2- $\mu$ m plasmid (Clarke and Carbon, 1980; Murray, 1987; Struhl *et al.*, 1979). The most common selectable markers are those that restore prototrophy for an essential metabolite in auxotrophic cells.

We designed a yeast plasmid vector that has ARS4 and CEN5 as replication elements plus the *trp1* gene encoding the yeast phosphoribosylanthranilate isomerase as a selectable marker that complements strains deficient in the synthesis of tryptophan such as those harboring homozygous *trp1-901* alleles (Fig. 14.1).

As genetics in yeast is hampered by genetic instability and low yields of purified DNA products, we decided to clone into the plasmid a bacterial replication origin, thus effectively converting it into a yeast–*E. coli* shuttle vector. In order to maximize DNA capacity, the F' replication origin and accessory genes from the mini-F plasmid (reviewed by Kline, 1985) were cloned. Other elements such as a spectinomycin resistance gene (*SpnR*), an origin of transfer (*oriT*) for plasmid mobilization, and the *cos* site from bacteriophage  $\lambda$  were also included (Fig. 14.1).



**Figure 14.1** Map of the linearized BAC/YAC vector *pYES1L*. Circularization of the plasmid results in a functional *E. coli*–*S. cerevisiae* shuttle episome. Unique rare restriction sites such as *PacI*, *I-CeuI*, *I-SceI*, and *NotI* were added for mapping or further linearization or cloning. For additional details and abbreviations see text.

## 2.2. DNA assembly using fragments with identical ends

The method presented here is similar to that one described earlier (Raymond *et al.*, 1999) with the following modifications: (i) we use the yeast strain MaV203 (MAT $\alpha$  *leu2-3,112 trp1-901*; *his3 $\Delta$ 200*; *ade2-101*; *cyh2R*; *can1R*; *gal4 $\Delta$* ; *gal80 $\Delta$* ; *GAL1::lacZ SPAL10::URA3 HIS3<sub>UASGAL1</sub>::HIS3@LYS2*), a derivative from a cross between two nonisogenic strains, PCY2 and MaV99 (Chevray and Nathans, 1992; Vidal *et al.*, 1996); (ii) we use chemically competent yeast cells; and (iii) we recommend 30 bp of fragment overlap for constructs < 60 kbp, and 50 bp of fragment overlap for constructs > 60 kbp.

End homology can be added manually to the fragments by PCR-amplifying the elements with oligonucleotides bearing additional sequences at their 5' end, or can be exposed by excising the fragments with restriction endonucleases from larger DNA entities. Residual nucleotides derived from the original restriction site do not interfere and are readily eliminated during the recombination process.

The recombinogenic properties of *S. cerevisiae* strongly promote DNA recombination between any pair of highly homologous sequences 50 bp or larger. Therefore, we recommend verifying that the fragment sequences do not share patches of DNA identity other than those at the end of adjacent fragments.

To avoid using fragments with unwanted similarity, we internally blast our sequences using the Oligo Designer Web tool ([www.invitrogen.com/DesignDNAAssembly](http://www.invitrogen.com/DesignDNAAssembly)), a free online software that detects potential patches of internal homology, designs oligonucleotides for TAR recombination, and provides a GenBank annotated sequence of the assembled construct.

### 2.2.1. Materials needed

- DNA fragments to assemble
- Stitching oligonucleotides (only if necessary; see above and below)
- pYES1L linear cloning vector or your own yeast-adapted cloning vector
- MaV203 competent yeast cells (Life Technologies) or equivalent
- PEG/LiAc solution (Life Technologies) or any other DNA condensing reagent
- 0.9% NaCl solution (sterile)
- DNase-, RNase-free water
- CSM-Trp agar plates (Life Technologies) or equivalent
- 30 and 42 °C water baths
- 30 °C incubator
- Microcentrifuge
- SpeedVac<sup>®</sup> (Thermo Scientific, Waltham, MA; optional)

In addition, the following materials are needed for screening for the positive clone:

- Pair of diagnostic primers for each DNA junction including the cloning vector
- Yeast lysis buffer (Life Technologies) or equivalent
- Thermocycler and sterile PCR tubes or plates
- Platinum<sup>®</sup> PCR SuperMix (Life Technologies) or similar

Finally, required materials for DNA transfer from yeast to *E. coli* are

- Plates with yeast colonies containing the plasmid of interest
- Yeast lysis buffer (Life Technologies) or similar
- Glass beads (Life Technologies) or similar
- SOC medium at room temperature
- One Shot<sup>®</sup> TOP10 electrocompetent *E. coli* cells (Life Technologies) or similar
- Electroporation cuvettes, chilled on ice
- Electroporator
- LB plates with the appropriate selection antibiotics, prewarmed to 37 °C

### 2.2.2. Performing the DNA assembly

1. Add the following components to a microcentrifuge tube and mix:

Linearized vector	100 ng
DNA fragments	100 ng each (if final construct is $\leq 25$ kbp)
	200 ng each (if final construct is $> 25$ kbp)

If the total volume of the DNA mix is smaller than 10  $\mu\text{L}$ , proceed to Step 2. If the total volume of the DNA mix is larger than 10  $\mu\text{L}$ , reduce total volume down to 5–10  $\mu\text{L}$  using a SpeedVac<sup>®</sup> or a centrifugal filter device. Do not let the liquid dry out completely.

2. Add 100  $\mu\text{L}$  of 30 °C thawed MaV203 cells into the DNA mix (the volume of the DNA mix should be  $\leq 10$   $\mu\text{L}$ ). Mix well by tapping the tube.
3. Add 600  $\mu\text{L}$  of the PEG/LiAc solution to the DNA/competent cell mixture. Mix by inverting the tube five to eight times until the mix is homogeneous.
4. Incubate the mixture in the 30 °C water bath for 30 min. Invert the tube occasionally (every 10 min) to resuspend the components.
5. Add 35.5  $\mu\text{L}$  of DMSO to the tube. Mix by inverting the tube five to eight times.
6. Heat-shock the cells by incubating the tube in the 42 °C water bath for 20 min. Invert the tube occasionally to resuspend the components.
7. Centrifuge the tube at 1800 rpm ( $200\text{--}400 \times g$ ) for 5 min.
8. Carefully discard the supernatant from the tube and resuspend the cell pellet in 1 mL of sterile 0.9% NaCl by gentle pipetting.
9. Plate 100  $\mu\text{L}$  of the transformed cells onto CSM-Trp agar plates. For final constructs  $> 60$  kbp, we recommend centrifuging the remaining 900  $\mu\text{L}$  of the transformation mixture, removing approximately 750  $\mu\text{L}$  of the supernatant, resuspending the cell pellet in the remaining 100–150  $\mu\text{L}$  of supernatant, and plate all cells onto another CSM-Trp agar plate to ensure that you have sufficient number of colonies to screen.
10. Incubate the cells at 30 °C for 3 days and proceed to screening for the correct clone (see below).

### 2.3. DNA assembly using fragments without identical ends

The TAR approach has been applied to recombine adjacent fragments that do not share end homology (DeMarini *et al.*, 2001; Raymond *et al.*, 2002). In this case, the necessary homology is provided in *trans* by complementary oligonucleotides that overlap both fragments, thereby serving as recombination linkers (stitching oligonucleotides). The method is particularly well

suited for reusing fragments in a new sequence context, or for cloning DNA targets that cannot be readily amplified by PCR.

The linker-mediated recombinational feature (oligonucleotide stitching) allows editing the fragment junctions, thus generating required imperfections. This is particularly useful when the removal of end sequences such as restriction sites or primer tails is required. It also permits the opposite type of alterations such as the addition of foreign sequences to insert restriction sites, small tag coding regions, or small watermarks. We successfully inserted up to 20 bp and effectively deleted up to 12 nucleotides from up to three fragment junctions.

### 2.3.1. Guidelines for designing the stitching oligonucleotides

- Each junction between adjacent fragments requires two oligonucleotides for oligonucleotide stitching, a sense and an antisense oligonucleotides.
- Up to five fragments plus a vector can be assembled using stitching oligonucleotides, provided that not more than three junctions are formed by the stitching oligonucleotides and the remaining junctions are produced by shared end-terminal homology.
- Oligonucleotides used for oligonucleotide stitching of up to three non-homologous fragments of < 5 kbp must be 80-mers (i.e., they must have a 40-bp overlap with each adjacent fragment).
- Prepare stitching oligonucleotide stocks at a final concentration of 100  $\mu\text{M}$  in  $1\times$  TE buffer, pH 8 (10 mM Tris-HCl, 1 mM EDTA, pH 8).
- Stitching oligonucleotides used for insertion editing must have a 30-nucleotide overlap with each adjacent fragment in addition to the insertion bases (for a total length of up to 80-mer, including up to 20 insertion bases).
- Stitching oligonucleotides used for deletion editing must have a 40-nucleotide overlap with each adjacent fragment, annealing up to six nucleotides from the junction into each fragment, thus leaving up to 6 bp at the end of each fragment to be deleted during TAR.

### 2.3.2. Materials needed

Required materials are the same as those depicted in [Section 2.2.1](#) plus the corresponding double-stranded stitching oligonucleotides (up to three pairs per assembly reaction) as described in [Section 2.3.1](#).

### 2.3.3. Performing the DNA assembly

1. Add the following components to a microcentrifuge tube and mix:

---

pYES1L or your own linearized vector	100 ng
DNA fragments	100 ng each (if final construct is $\leq 25$ kbp) 200 ng each (if final construct is $> 25$ kbp)
Stitching oligonucleotides	500 ng each (20 pmol each)

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If the total volume of the DNA mix is smaller than 10  $\mu\text{L}$ , proceed to Step 2 in [Section 2.2.2](#). If the total volume of the DNA mix is larger<sup>®</sup> than 10  $\mu\text{L}$ , reduce total volume down to 5–10  $\mu\text{L}$  using a SpeedVac or a centrifugal filter device. Do not let the liquid dry up completely.

2. Proceed to Step 2 of [Section 2.2.2](#).

## 2.4. Screening for the positive clone

The fastest way to screen for yeast colonies containing the right assembled construct is by performing colony-PCR assays using pair of diagnostic primers that amplify each single expected junction. For example, for a five-fragment (plus vector) assembly configuration, six pair of diagnostic primers should be used.

We recommend designing oligonucleotide pairs (forward and reverse) 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.

1. Aliquot 15  $\mu\text{L}$  of lysis buffer into PCR tubes or plates.
2. Pick individual yeast colonies one at a time using a sterile 20  $\mu\text{L}$  pipette tip. Leave the tip in the PCR tube or the well until all the colonies have been picked.
3. Resuspend the cells by pipetting up and down three times.
4. Transfer 5  $\mu\text{L}$  of each cell suspension into fresh PCR tubes and store at 4 °C until verified that the colony is positive (see below).
5. Heat the remaining cells (10  $\mu\text{L}$ ) at 95 °C for 5 min in a thermocycler and cool them down at 4 °C or on ice. Briefly centrifuge the PCR tubes or plates to bring down condensed water.
6. Add 40  $\mu\text{L}$  of nuclease-free water into each lysate and pipette up and down three to five times to mix.
7. Set up a PCR master mix for each junction and aliquot 49.5  $\mu\text{L}$  of it into fresh PCR tubes or plates.



8. Add 0.5  $\mu\text{L}$  of each diluted yeast lysate (from Step 4) into each PCR tube or well. Do not exceed 0.5  $\mu\text{L}$  of lysed yeast cells for 50  $\mu\text{L}$  of PCR volume.
9. Vortex to mix the contents and briefly centrifuge to bring down all liquid.
10. Perform PCR cycling in a thermocycler.
11. Load 10  $\mu\text{L}$  onto an agarose gel to visualize the PCR products. Sequencing these PCR products is recommended.

## 2.5. Yeast-*E. coli* transfer

Plasmid DNA preparations from yeast cells usually result in very low yield and poor DNA quality. Therefore, it is a common laboratory practice to retrieve these shuttle plasmids out of yeast and transfer back into *E. coli* for additional analysis or manipulation. Established protocols for the retrieval procedure, however, are sometimes more cumbersome and time consuming than the initial plasmid transfer into yeast and in some cases, the transfer efficiency is not high enough to actually obtain *E. coli* colonies (Gunn and Nickoloff, 1995; Marcil and Higgins, 1992). We have developed a highly efficient protocol with a few modifications to an earlier strategy (Summers and Withers, 1990) that streamline the process.

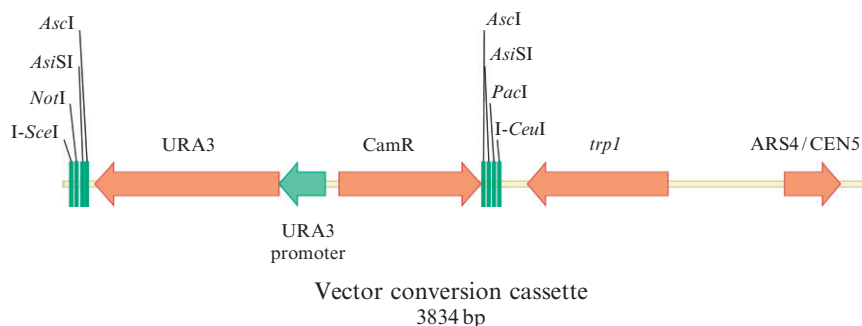
1. Aliquot four to five glass beads into a fresh PCR tube and add 10  $\mu\text{L}$  of lysis buffer.
2. Add 5  $\mu\text{L}$  of the cell suspension that was stored into the lysis buffer/glass beads mix (Section 2.4). Pipette up and down three to five times to mix.
3. Vortex the cells at room temperature for 5 min. Do not heat the lysed cells.
4. Add 1  $\mu\text{L}$  of the lysed cells (from Step 3, above) into a vial of electrocompetent cells and mix gently. Do not add more than 1  $\mu\text{L}$  of the lysed cells to avoid arcing during electroporation.
5. Transfer the cells to the chilled electroporation cuvette on ice.
6. Electroporate the cells following the manufacturer's recommended protocol.
7. Add 250  $\mu\text{L}$  of prewarmed SOC medium to each vial.
8. Transfer the solution to a 15-mL snap-cap tube and shake for at least 1 h at 37 °C to allow expression of the antibiotic resistance gene.
9. Spread 10–50  $\mu\text{L}$  from each transformation on a prewarmed LB plate supplemented with the appropriate selection antibiotic.
10. Invert the selective plate(s) and incubate at 37 °C overnight.

## 2.6. Yeast plasmid conversion cassette

In order to extend the usability of this system to any *E. coli* vector, we designed a linear adaptation cassette with all the features necessary for yeast cloning and replication (Fig. 14.2). The fragment contains all the yeast-related features described for the plasmid pYES1L (see Section 2.1 and Fig. 14.1) plus the *ura3* gene encoding yeast's orotidine 5-phosphate decarboxylase (Boeke *et al.*, 1984). This counter-selectable marker becomes lethal when 5-fluoroorotic acid (5-FOA) is added to the media, as it converts it into the toxic compound 5-fluorouracil. During development, we learned that the use of this feature is not really necessary, as the frequency of plasmid recircularization is negligible. The cassette contains also the bacterial chloramphenicol acetyl transferase (or chloramphenicol resistance gene, *CamR*), which facilitates plasmid adaptation by selecting recombinants in agar plates containing chloramphenicol and the corresponding antibiotic specific to the vector backbone. Convenient rare restriction sites were added in order to linearize the final adapted molecule for cloning in yeast.

## 2.7. Typical results

Standard assemblies were performed using different fragment numbers and sizes. Here, we used (i) preexisting fragments that had been previously cloned into pACYC184, (ii) PCR-amplified fragments, or (iii) a combination of both. The recipient plasmid was pYES1L. Additional variables tested were (i) fragment overlap (80 or 30 bp) and amount of each insert (100 or 200 ng per reaction). For large or complicated assemblies, 200 ng and 80 bp overlap are recommended. However, for simple assemblies (e.g., one fragment), 100 ng of DNA and 30 bp overlap are sufficient for attaining virtually 100% cloning efficiency (Table 14.1).



**Figure 14.2** Scheme of the yeast adaptation cassette. For details and abbreviation see text.

**Table 14.1** Assemblies using fragments with end homology

Number of fragments <sup>a</sup>	Number × length of precloned fragments (kbp) <sup>b</sup>	Number × length of PCR-amplified fragments (kbp)	Total size (kbp) <sup>c</sup>	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




<sup>a</sup> Vector not counted as a fragment.  
<sup>b</sup> Fragments were initially cloned into pACYC184 and excised by *NotI* digestion.  
<sup>c</sup> Vector size not considered.

For assemblies using stitching oligonucleotides, we have tried several conditions, which included (a) the use single- or double-stranded oligonucleotides, (b) the use of oligonucleotides with varying lengths, and (c) the use of different oligonucleotide:fragment molar ratios. Best results were obtained using 80-mer double-stranded oligonucleotides. However for simple assemblies (one vector + one fragment), 60-mers exhibited satisfactory performance (Fig. 14.3A). Single-stranded oligonucleotides are not recommended. Up to five fragments plus vector plus three pairs of stitching oligonucleotides can be successfully assembled.

We also performed assemblies with imperfect junctions using a combination of two 5 kbp fragments plus a vector (Fig. 14.3B). Recombination efficiency of imperfect junctions should be significantly higher if only one fragment plus vector are joined by stitching oligonucleotides. Oligonucleotides shorter than 60-mers did not perform satisfactorily (not shown).

### 3. IN VITRO RECOMBINEERING—DNA ASSEMBLY

Most of the *in vitro* methods for joining two or more DNA fragments require specific sequences that leave watermarks or scars at the end of the process. In addition, the number of fragments that can be simultaneously assembled is limited due to either poor cloning efficiency or limited number of available recognition elements. In the case of restriction–ligation

A	Fragment size	Oligo size	Cloning efficiency (%)
	1 × 1 kbp	60 mer	94
	2 × 5 kbp	80 mer	75
	3 × 5 + 2 × 0.5 kbp	60 mer	37
	3 × 5 + 2 × 0.5 kbp	80 mer	75
B	2 × 5 kbp	80 mer 10 bp insertion	63
	2 × 5 kbp	80 mer 20 bp insertion	50
	2 × 5 kbp	80 mer 12 bp deletion	87

**Figure 14.3** Bridging DNA fragments with stitching oligonucleotides. (A) One or more DNA fragments were bridged with the vector or adjacent fragment using double-stranded oligonucleotides perfectly complementary to the bridged molecules’ ends. Hundred to two hundred nanograms of each DNA fragment plus 40 pmol of double-stranded oligonucleotides were transformed into MaV203 yeast competent cells and processed as indicated in the text. (B) Adjacent DNA fragments were bridged with imperfect double-stranded 80 bp oligonucleotides that generate insertions or deletions. DNA fragments are represented by green lines, vector (ends only) by black lines, and oligonucleotides by red lines.

approaches, further constraints are imposed by the availability of unique sites in the vector and fragments.

Homologous recombination strategies have proven to overcome the issues above. Two methods stand out, which are based on chewing back and repairing the DNA ends. The first one likely uses vaccinia polymerase (Hamilton *et al.*, 2007; Willer *et al.*, 2000; Zhu *et al.*, 2007). The enzyme has both exonuclease and polymerase activities. It works quite efficiently for the assembly of one or two fragments, but requires two incubation steps at different temperatures, and cloning efficiencies for assembling more than three fragments are quite low (not shown). The second strategy uses a combination of thermostable polymerase, and ligase plus a heat-labile exonuclease and works in an isothermal context (Gibson *et al.*, 2009). This approach efficiently assembles large number of fragments of considerable size; however, adjacent fragments must share at least 40 bp of end homology, which is sometimes difficult to accommodate in standard PCR

oligonucleotides. It also requires electroporating the cells, which precludes the use of this method in a high-throughput context.

Our method combines the advantages of the strategies above, namely it requires only 15 bp of end homology, it efficiently assembles more than three fragments, and readily works with standard transformation protocols. In addition, the experimental design of our approach is greatly facilitated by using of the Oligo Designer Web tool ([www.invitrogen.com/DesignDNAassembly](http://www.invitrogen.com/DesignDNAassembly)), a free online software that designs the oligonucleotides with the required overlap tails and provides a GenBank annotated sequence of the assembled construct.

### 3.1. Materials needed

- DNA fragments for DNA assembly
- Linearized *E. coli* vector
- *In vitro* recombination buffer (Life Technologies or equivalent)
- *In vitro* recombination enzyme mix (Life Technologies or equivalent)
- Deionized, sterile water
- One Shot<sup>®</sup> TOP10 chemically competent *E. coli* (Life Technologies or similar)
- SOC medium at room temperature
- LB plates with the appropriate selection antibiotics, pre-warmed to 37 °C

### 3.2. Assembly reaction

1. In a microcentrifuge tube, add the components below in the order they are listed:

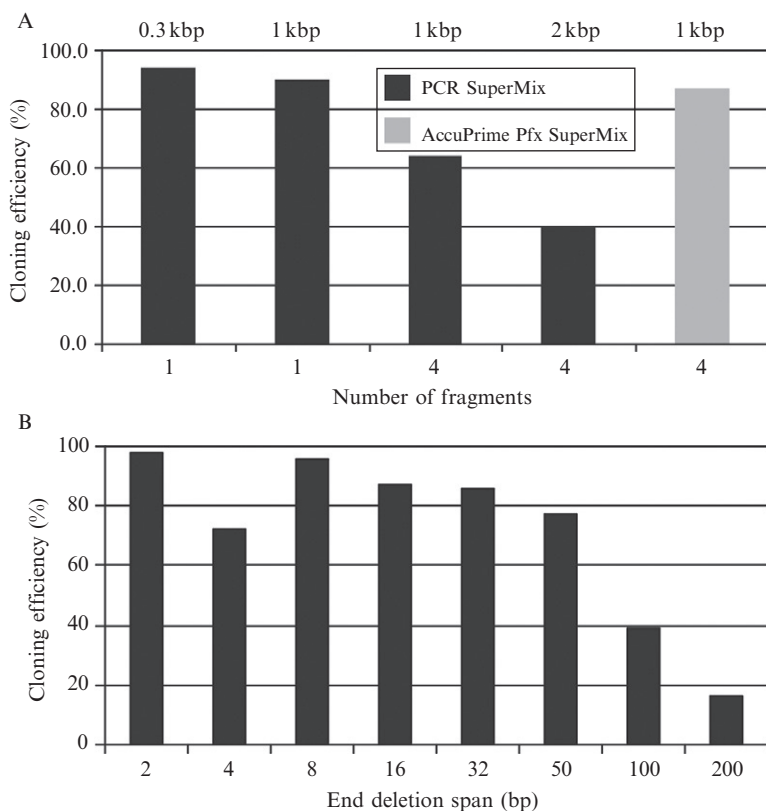
Insert(s)	20–200 ng each
Linearized vector	100 ng
5× reaction buffer <sup>a</sup>	4 μL
Deionized water	18 μL
10× Enzyme mix <sup>a</sup>	2 μL

<sup>a</sup>As an example, we list the *in vitro* recombination buffer and enzyme mix in the GENEART<sup>®</sup> Seamless Cloning and Assembly Kit (Life Technologies). Other kits are available from different commercial sources.

- For optimum results, use a 2:1 molar ratio of insert:vector.
2. Incubate at room temperature for 30 min.
  3. Immediately transform competent *E. coli* cells, following standard protocols. *Important:* do not use electrocompetent cells.
  4. Plate the cells on LB agar plates with the corresponding antibiotics.

### 3.3. Typical results

Fragments of different sizes were amplified and recombined into a linearized vector (Fig. 14.4A). Results showed that at least four fragments can be joined in a predetermined order into a vector with high cloning efficiencies. Results also indicated that fragments amplified with a proofreading polymerase produce significantly higher cloning efficiencies than those amplified with a standard polymerase (Fig. 14.4A).



**Figure 14.4** *In vitro recombineering: DNA assembly.* (A) Fragments of the indicated size were amplified using a standard PCR polymerase (PCR SuperMix, Life Technologies) or a proofreading thermostable DNA polymerase (AccuPrime Pfx SuperMix, Life Technologies) and recombined into *Pst*I–*Kpn*I linearized pUC19 plasmid. Identity between the fragments and the vectors was generated by the addition of 15 nucleotides to the 5′-end of the oligonucleotides, whereas identity between adjacent fragments was created with 8 bp primer extensions. (B) Junction editing. Two DNA fragments were simultaneously recombined into a vector. One of the fragments shared 15 bp of homology with the vector at the indicated distances from the vector’s end, generating constructs bearing up to 200 bp deletions.

**Table 14.2** Effect of the topology of the end of the vector

Number of fragments <sup>a</sup>	Observed cloning efficiency (%) <sup>b</sup>				
	3' protruding	5' protruding	Blunt ended	PCR amplified	Average
1	99.4	92.4	99.6	96.0	96.9
2	74.5	65.0	65.5	71.0	69.0
3	95.2	88.5	99.0	96.0	94.7
4	87.0	51.8	79.0	83.0	75.2

<sup>a</sup> Fragments were PCR amplified with AccuPrime Pfx SuperMix (Life Technologies) using standard oligonucleotides.

<sup>b</sup> Cloning efficiency determined by colony PCR and sequencing.

In addition, 1-, 2-, 3-, and 4-fragment assembly reactions were performed using a plasmid linearized with restriction enzymes that leave behind 3' protruding, 5' protruding, and blunt ends. Also, a PCR-amplified vector was used instead of a digested one. Results showed that the cloning efficiency does not depend on the topological ends of the vector (Table 14.2).

An interesting feature of our approach is that recombination can occur not only at the end of the fragments, but it also works at least up to 200 bp away from their ends (Fig. 14.4B). This attribute is useful for generating cloning variants using a single linearized vector.

## 4. IN VITRO RECOMBINEERING—SITE-DIRECTED MUTAGENESIS

During the past three decades, site-directed mutagenesis has become one of the most powerful tools in genetics. Its power lies in its ability, by chemical and/or enzymatic manipulation, to change a specific DNA target in a definable and predetermined way. With the advent of synthetic biology and rational design, the manipulation of genes to produce enzymes with subtle differences with respect to the natural ones and the modification of promoters to finely tune metabolic flows depend even more on reliable site-directed mutagenesis approaches.

Site-directed mutagenesis kits commercially available use, at least, one of the following approaches: (i) the isolation of single strand template DNA and the generation of the mutation with one complementary primer (Hutchison *et al.*, 1978); (ii) the design of two sets of PCR primers that overlap the mutation site, amplify the template by two PCR reactions, and then the cloning of the two PCR fragments and the vector by three-piece

ligation (Stemmer and Morris, 1992); (iii) the PCR amplification of a plasmid using complementary oligonucleotides and the subsequent elimination of the template molecule (Hemsley *et al.*, 1989; Kunkel, 1985).

We applied our homologous recombination approach (Section 3) to join the ends of a single DNA molecule, thereby enabling a highly efficient site-directed mutagenesis strategy. The system relies on the inherent properties of a CpG methyltransferase, a high fidelity thermostable DNA polymerase, recombination enzymes, and the *E. coli* McrBC restriction–modification system. The DNA methylation and amplification steps are combined into a single reaction followed by a 10-min recombination step. This short *in vitro* recombination reaction of PCR products increases the colony output by 3- to 10-fold. Finally, the products are transformed into a host strain that degrades the methylated DNA template (Fig. 14.5).

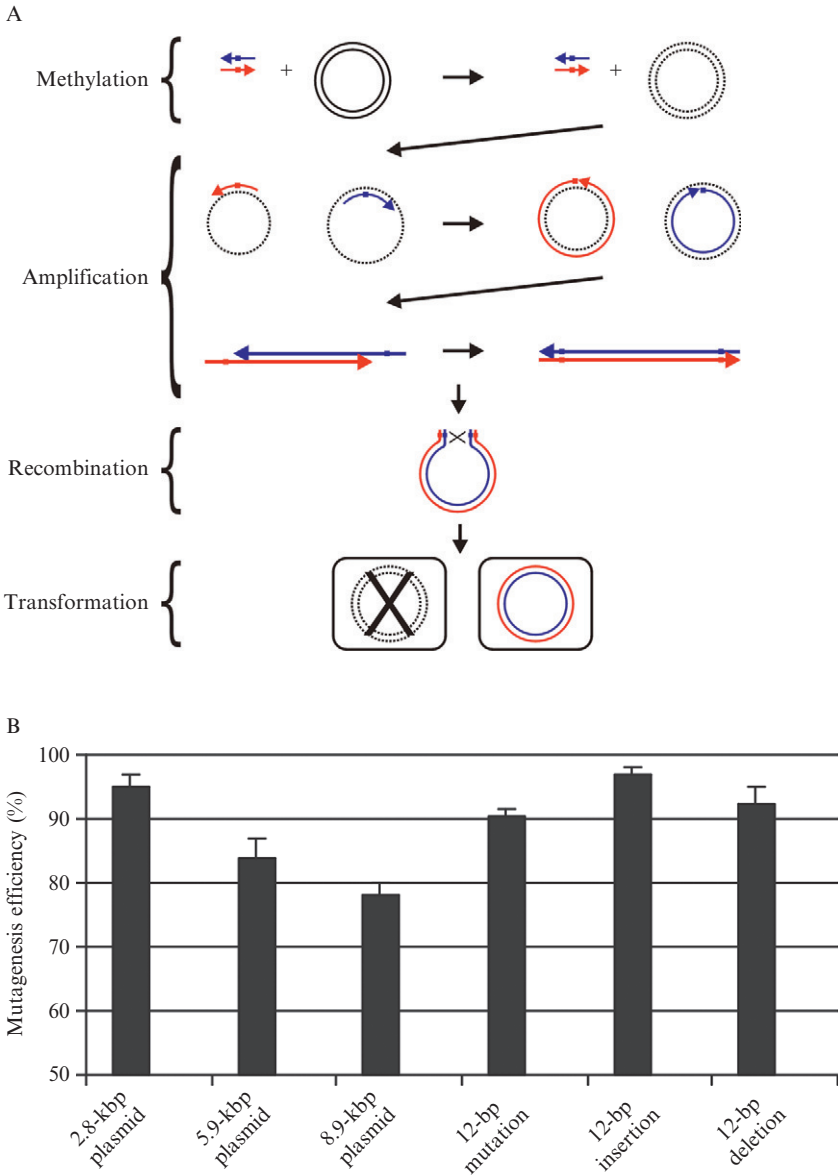
#### 4.1. Primer specifications

- Both primers (forward and reverse) should contain the desired mutation.
- The mutation site should be centrally located on both primers and can be up to 12 bases (deletions, insertions, and/or any substitutions).
- Both primers (forward and reverse) should be approximately 30–45 nucleotides in length, not including the mutation site. Primers longer than 45 nucleotides increase the likelihood of secondary structure formation, which may affect the efficiency of PCR amplification.
- Primers should have an overlapping region at the 5' ends of 15–20 nucleotides, for efficient end-joining of mutagenesis product.
- For most applications, DNA oligonucleotides purified by desalting are generally sufficient, although oligonucleotides purified by HPLC or PAGE may increase the mutagenesis efficiency.

#### 4.2. Materials needed

- Target plasmid DNA
- Custom mutagenic oligonucleotide pair
- AccuPrime<sup>TM</sup> Pfx DNA polymerase (Life Technologies) or similar
- CpG methyltransferase
- S-adenosyl methionine
- PCR enhancer (Life Technologies or similar)
- Thermocycler
- *In vitro* recombination buffer (Life Technologies) or equivalent
- *In vitro* recombination enzyme mix (Life Technologies) or equivalent
- Deionized, sterile water
- EDTA





**Figure 14.5** *In vitro* recombineering: site-directed mutagenesis. (A) Strategy's workflow. Template strands are shown in black. Methylated strands are shown as dotted lines. Oligonucleotides and new strands are shown in blue and red. For details see main text. (B) Plasmids with frameshift mutations in the *lacZ* $\alpha$  gene were subjected to the mutagenesis protocol described in the text, transformed into DH5 $\alpha$ -T1R cells, and then plated onto LB agar ampicillin X-gal plates. The mutagenesis efficiency was calculated by the ratio blue/total colonies.

- One Shot<sup>®</sup> MAX Efficiency<sup>®</sup> DH5 $\alpha$ -T1R competent cells (Life Technologies) or similar.
- SOC medium at room temperature
- LB plates with the appropriate selection antibiotics, prewarmed to 37 °C

### 4.3. Mutagenesis protocol

1. In a PCR tube, add the components below:

10 $\times$ AccuPrime <sup>TM</sup> Pfx Reaction mix	1 $\times$
10 $\times$ PCR enhancer	1 $\times$
Oligonucleotide pair	0.3 $\mu$ M each
Plasmid DNA	20 ng
CpG DNA methyl transferase	4 units
S-adenosyl methionine	160 $\mu$ M
AccuPrime <sup>TM</sup> Pfx	1 unit
Deionized, sterile water	50 $\mu$ L

2. Perform PCR using the following parameters:

37 °C	12–20 min <sup>a</sup>
94 °C	2 min
(a) 94 °C	20 s
(b) 57 °C	30 s
(c) 68 °C	30 s/kbp of plasmid
12–18 cycles of (a), (b) and (c) <sup>b</sup>	
68 °C	5 min
4 °C	As needed

<sup>a</sup>Perform methylation of the plasmid at 37 °C for 12–20 min. We recommend 12 min for 2.8–4 kbp plasmids and 20 min for 4–14 kbp plasmids.

<sup>b</sup>The cycling parameters specify a 30-s extension for each 1 kbp of DNA. For optimal mutagenesis efficiency, we recommend 12–15 cycles for 2.8–4 kbp plasmids and 18 cycles for 4–14 kbp plasmids.

3. Analyze 5  $\mu$ L of the product on a 0.8% agarose gel.

4. Set up the recombination reaction as follows:

10 $\times$ reaction buffer	4 $\mu$ L
Deionized, sterile water	10 $\mu$ L
PCR sample	4 $\mu$ L
10 $\times$ enzyme mix	2 $\mu$ L

5. Mix well and incubate at room temperature for 10 min.

6. Stop the reaction by adding 1  $\mu$ L 0.5 M EDTA. Mix well and place the tubes on ice.

7. Immediately transform competent *E. coli* cells, following standard protocols. *Important:* do not use electrocompetent cells.
8. Plate the cells on LB agar plates with the corresponding antibiotics

#### 4.4. Typical results

In our experiments, we used pUC19-derivative plasmid templates of sizes ranging from 2.8 to 8.9 kbp (Fig. 14.5B). All these plasmids encoded a *lacZ $\alpha$*  gene derivative with a frameshift mutation that enabled us to readily calculate the mutagenesis efficiency by simply plating the cells onto LB agar plates supplemented with X-gal (the mutagenic primers were designed to revert the mutation to a wild-type *lacZ $\alpha$*  gene). We also designed derivatives with insertions, deletions, and substitutions spanning multiple base pairs. Cognate primer pairs were designed to revert those mutants to a wild-type *lacZ $\alpha$*  allele. Results revealed a remarkable high mutagenesis efficiency, with a negative trend that followed larger plasmid sizes (Fig. 14.5B). In addition, we generated a 14 kbp plasmid, which was used as a template for a 1-bp transversion using an arbitrary mutagenic primer pair. Ten out of 10 independent clones sequenced, revealed the presence of the expected mutation.

### 5. CONCLUDING REMARKS

The emergence of the synthetic biology field clearly calls for reconsideration of the current paradigms in different life science and engineering areas. As this field continues to expand, an agile back and forth conversion process between digital information and “analog” biological systems will become a necessity.

Our ultimate goal is to attain a comprehensive solution to generate any DNA molecule from small assemblies up to high-level genetic systems starting from digital sequences stored in a computer. The approaches presented here are relevant not only for the area of synthetic biology, but they also have remarkable implications on the current cloning standards.

### 6. DISCLOSURE

Products are for research use only; not intended for any animal or human therapeutic or diagnostic use.

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