Harnessing homologous recombination *in vitro* to generate recombinant DNA via SLIC

Mamie Z Li & Stephen J Elledge

We describe a new cloning method, sequence and ligation–independent cloning (SLIC), which allows the assembly of multiple DNA fragments in a single reaction using *in vitro* homologous recombination and single-strand annealing. SLIC mimics *in vivo* homologous recombination by relying on exonuclease-generated ssDNA overhangs in insert and vector fragments, and the assembly of these fragments by recombination *in vitro*. SLIC inserts can also be prepared by incomplete PCR (iPCR) or mixed PCR. SLIC allows efficient and reproducible assembly of recombinant DNA with as many as 5 and 10 fragments simultaneously. SLIC circumvents the sequence requirements of traditional methods and functions much more efficiently at very low DNA concentrations when combined with RecA to catalyze homologous recombination. This flexibility allows much greater versatility in the generation of recombinant DNA for the purposes of synthetic biology.

The assembly of recombinant DNA by restriction enzyme cutting and re-ligation was a crowning achievement of biology in the 20th century[1–4]. Many variations on this theme have emerged that allow greater precision to be achieved with respect to sequence alterations and sites of junctions of recombinant molecules. Two methods that made critical improvements are site-directed mutagenesis[5] and PCR[6,7]. Site-directed mutagenesis allows alteration of specific sequences to allow structure-function studies of molecules. PCR has made several contributions including the ability to select a precise sequence from low concentrations of DNA and to place specific sequences at fragment ends to allow conventional assembly with other fragments. PCR has also been used to introduce changes into gene sequences[8].

Presently the DNA sequence and coding capacities of entire organisms are being determined. This presents the opportunity to manipulate and analyze large sets of genes for genetic and biochemical properties. Furthermore, a new field, synthetic biology, is emerging, which uses complex combinations of genetic elements to design circuits with new properties. These endeavors require the development of new cloning technologies. Three recombination-based cloning methods have emerged for accomplishing parallel processing of large gene sets. Two use *in vitro* site-specific recombination, the Univector Plasmid-fusion System and Gateway[9–14]. The third, MAGIC, is an *in vivo* method that relies upon homologous recombination and bacterial mating[15]. These methods offer a uniform and seamless transfer of genes from one expression context to another, thereby allowing different clones to be treated identically. These methods, however, lack important features. First, they do not generally facilitate initial assembly of the gene of interest into the origin plasmid. The exception, Gateway, requires the use of expensive enzymes for initial cloning and requires specific long sequences on each primer that contain recombination sites. Second, these methods are useful only for cloning into specific vectors containing defined sequences. If a cloning reaction requires a specialty assembly, that is, replacing a fragment in an existing plasmid, perhaps within a gene, these methods cannot be used. Finally, these methods generally allow only the combination of two fragments in a single experiment.

Homologous recombination has important advantages over site-specific recombination in that it does not require specific sequences. Two types of homologous recombination exist in *Escherichia coli*[16,17], RecA-mediated recombination and a RecA-independent pathway called single-strand annealing. We have addressed limitations of present systems by the development of a new *in vitro* homologous recombination method called SLIC. We show that homologous recombination intermediates, such as large gapped molecules assembled *in vitro* by RecA or single-strand annealing, efficiently transform *E. coli,* removing the sequence constraints inherent in other methods. This system circumvents many problems associated with conventional cloning methods, providing a multifaceted approach for the efficient generation of recombinant DNA.

RESULTS

In vitro homologous recombination with and without RecA

Homologous recombination *in vivo* depends upon a double-stranded break, generation of ssDNA by exonucleases, homology searching by recombinases, annealing of homologous stretches, and repair of overhangs and gaps by enzymes that include resolvases, nucleases and polymerases. We reasoned it might be possible to generate recombination intermediates *in vitro* and introduce these into cells to allow the cell endogenous repair machinery to finish the repair to generate recombinant DNA (**Fig. 1a**). To generate

Howard Hughes Medical Institute, Department of Genetics, Harvard Partners Center for Genetics and Genomics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA. Correspondence should be addressed to. S.J.E. (selledge@genetics.med.harvard.edu).

RECEIVED 12 SEPTEMBER 2006; ACCEPTED 4 JANUARY 2007; PUBLISHED ONLINE 11 FEBRUARY 2007; DOI:10.1038/NMETH1010

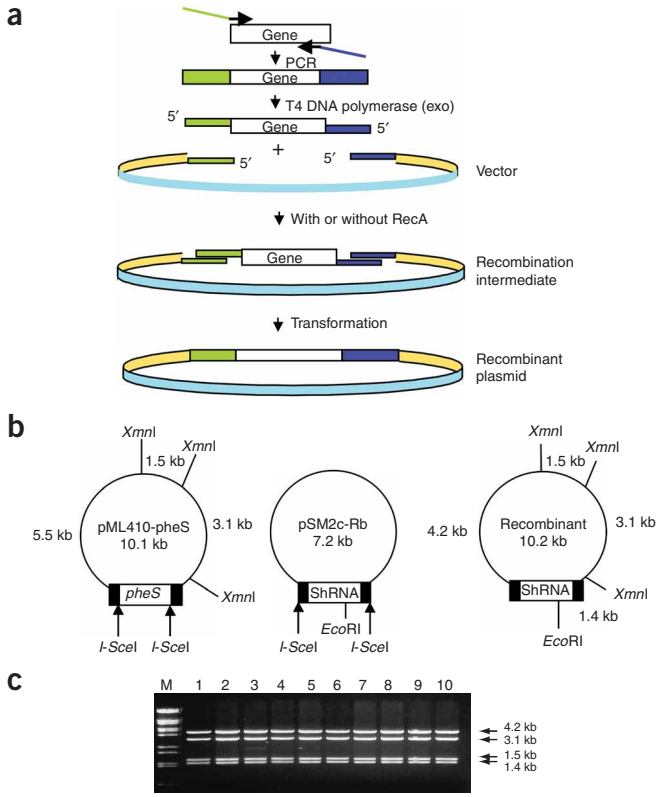

Figure 1 | *In vitro* recombination of MAGIC vectors mediated by RecA. (**a**) A schematic for production of recombinant DNA through *in vitro* homologous recombination and single-strand annealing. (**b**) *Xmn*I and *Eco*RI restriction maps of MAGIC plasmids pML410-pheS, pSM2c-Rb and recombinant plasmid. (**c**) An *Xmn*I-*Eco*RI restriction analysis of the recombinants derived from SLIC reaction. Plasmid DNA from ten independent carbenicillin-resistant colonies (lanes 1–10) were digested with *Xmn*I-*Eco*RI. The digestion products were separated on a 0.8% agarose gel and visualized after ethidium bromide staining. Recombinants contain one *Eco*RI site from the insert and three *Xmn*I sites from the vector (4.2, 3.1, 1.5 and 1.4 kb).

overhangs for homology searching, we used exonucleases to chew back one strand to reveal ssDNA overhangs. We tested both 3′ and 5′ exonucleases including T7 exonuclease, lambda exonuclease and T4 DNA polymerase[18]. We chose T4 DNA polymerase, which produces 5′ overhangs, because it gave the best and most reproducible results (**Supplementary Table 1** online), and had the ability to terminate excision by addition of a single dNTP.

For the cloning reaction we generated the vector by cleavage with a restriction enzyme and generated the insert by PCR. We treated both the vector and the insert with T4 DNA polymerase in the absence of dNTPs to generate overhangs, then incubated vector and insert with and without RecA protein and ATP to promote recombination, and transformed the products into *E. coli*. Transformation with vector alone gave some background, which we traced to a small amount of uncleaved vector. We reduced the background in these experiments by placing the negative selection marker *pheS* A294G between the restriction sites in the vector and growing transformants on plates containing chlorophenylalanine. In most cases however, chlorophenylalanine selection gave only a twofold reduction in background. Transformation with vector alone yielded very few transformants, whereas incubation of the vector with equimolar insert fragments and RecA produced 400–600-fold increase over background. All 20 clones analyzed had the correct restriction map (data not shown). We observed an increase in formation of recombinants over background with addition of an insert in the absence of RecA, indicating that single-strand annealing could produce recombinants under these conditions. By varying the length of overlap, we found that 30-bp homology gave the greatest stimulation of recombination (**Table 1**).

To examine how this method might apply to different vector and insert systems, we chose a vector-insert combination previously shown to efficiently recombine *in vivo* via MAGIC cloning. MAGIC donor vectors have greater than 60 bp homology with recipient vectors on each end and generate inserts by cleavage with *I-Sce*I of both donor and recipient plasmids. The recipient used was a lentivector and the donor fragment was a short hairpin RNA cassette from a short hairpin RNA library. We incubated prepared fragments with and without RecA, electroporated into *E. coli* and selected for carbenicillin resistance. Without RecA, vector alone gave 1.3 transformants per nanogram of vector, whereas vector plus insert gave 51. With RecA, vector alone gave 8 transformants per nanogram of vector whereas vector plus insert gave 3,900 transformants, a 500-fold stimulation of recombination, similar to what we observed *in vivo*. Ten of ten clones yielded restriction fragments consistent with the predicted restriction map (**Fig. 1b,c**). Additionally, the isolation of 3,900 transformants per nanogram of vector means libraries can be transferred by this method *in vitro* without losing complexity.

The requirement of RecA for efficient recombination suggested that at the DNA concentrations used, efficient homology searching required enzymatic facilitation. We wondered whether the less efficient single-strand annealing pathway could be made more

Table 1 | Stimulation of recombination by RecA

	20 bp	30 bp	40 bp	50 bp
Vector[a] only	<2.7	8.1	2.7	<2.7
Vector[a] and Skp1	210	440	89	120
Vector[a] and RecA	2.7	<2.7	<2.7	<2.7
Vector[a] and Skp1 and RecA	1,700	4,900	1,200	1,300

Cloning efficiencies are given as colony forming units per nanogram of vector for the indicated regions of homology. [a]The recipient vector pML385 was linearized with *NcoI-BamHI* and the Skp1 fragment was prepared by PCR. Both were treated with T4 DNA polymerase to generate 5′ overhangs. This experiment was performed at low DNA concentration (0.075 ng/μl).

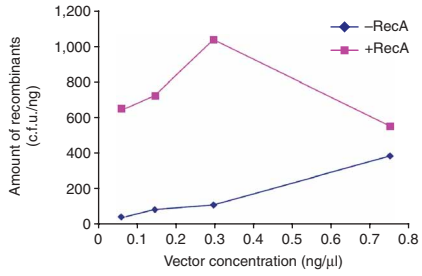

Figure 2 | The dependency on RecA can be overcome by increasing DNA concentration. One microgram of linear vector pML385 and 1 μg of 40-bp-homology *Skp1* insert fragment were treated with 0.5 U of T4 DNA polymerase for 1 h. The vector and inserts were then diluted and annealed with and without RecA in a 1:1 molar ratio at different concentrations.

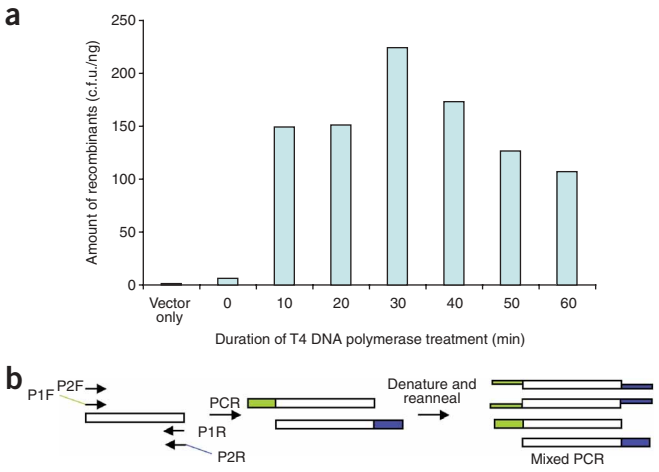

Figure 3 | iPCR and mixed PCR can be used to prepare inserts for SLIC cloning without nuclease treatment. (**a**) Effect of the duration of T4 DNA polymerase treatment on recombination. A 20-bp-homology *Skp1* fragment was treated with T4 DNA polymerase for the indicated times and annealed at a 1:1 molar ratio with linearized vector pML385 treated with T4 DNA polymerase for 30 min, and transformed into cells. (**b**) A schematic illustrating the production of mixed PCR products. Two PCR reactions were prepared using primer pairs P1F-P1R and P2F-P2R. Primers P1F and P2R are longer than P1R and P2F and produce 5′ and 3′ overhangs respectively. The two PCR products were mixed and heated to 95 °C for 5 min to denature, and then cooled slowly to 22 °C to reanneal.

efficient by increasing the concentration of input DNA to lessen dependency on RecA. Increasing the amount of both vector and insert by tenfold greatly increased the efficiency of RecA-independent recombination (**Fig. 2**).

Homologous recombination using iPCR or mixed PCR inserts

While optimizing the amount of T4 DNA polymerase for recombination, we observed that inserts prepared by PCR displayed a low level of recombination without T4 treatment (**Fig. 3a**). One potential explanation is that incomplete synthesis of DNA during later cycles of PCR occurred such that some insert fragments have 5′ overhangs. To test this, we used a fragment prepared by PCR and the identical fragment excised from a plasmid using restriction enzymes. Before T4 DNA polymerase treatment, the PCR-generated material gave 16-fold stimulation of transformation, whereas the restriction fragment did not, confirming the incomplete-synthesis-by-PCR hypothesis (**Table 2**). We call this iPCR, and although inserts prepared by iPCR stimulate recombination with a lower efficiency, it is a quick method for subcloning. We find that recombinant generation using iPCR-generated inserts is more robust with higher insert concentration, likely because only a subset of iPCR molecules contain clonable overhangs. It should be noted that if iPCR is used, one round of denaturation and renaturation without primer extension at the end should be performed before use of the products in subcloning so molecules with overhangs on both ends will be present.

Mixing two PCR products, each of which has one homology region, upon denaturation and renaturation yields 25% of resulting fragments with correct overhangs[19] (**Fig. 3b**), which should be sufficient for SLIC. We tested this hypothesis using a large recipient plasmid vector, ptmGIPZ-pheS (12 kb). At the higher insert-to-vector ratio of 6:1, mixed PCR inserts gave a 38-fold stimulation of transformation over background, whereas a T4 DNA polymerase–treated insert gave 70-fold stimulation (**Table 2**).

The ability to generate recombinant DNA by traditional methods varies depending upon the insert size and molar ratio with vector. The optimal insert to vector ratio varied between 2:1 and 4:1 (**Supplementary Fig. 1** online). By varying insert sizes we found that inserts up to 3.2 kb still showed robust homologous recombination *in vitro* (**Supplementary Fig. 2** online). Plasmids in which one fragment was as large as 7 kb assembled with good efficiency and one plasmid of 12 kb assembled at a reduced but sufficient efficiency, demonstrating that recombination with larger fragments is feasible.

In vitro homologous recombination with multiple fragments

The efficiency of SLIC suggested it might be possible to generate recombinant DNA with multiple inserts. We first attempted a three-way cloning consisting of a 0.6-kb insert and an approximately 75-bp *lacO* fragment generated by annealing two oligonucleotides that left 5′ overhangs on each side, one homologous to the 5′ end of the insert and the other to the vector (**Fig. 4a**). We treated only the insert and vector with T4 DNA polymerase. We chose *lacO* because we had previously developed a genetic selection for subcloning *lac* operators in high copy, which titrates out the endogenous *lac* repressor and induces a *lac* promoter driving *bla*[9,15]. We reasoned that if three-way cloning was inefficient, rare recombinants could be selected. We plated recombinants on plates containing kanamycin to select for the vector, plus chlorophenylalanine to select against uncut vector. To determine the effects of selecting for *lacO*, we plated cells on these plates with and without carbenicillin. In the presence of carbenicillin, the background of vector and *lacO* alone upon transformation was extremely low and the presence of the third fragment stimulated recombinant formation by 20,000-fold (**Table 3**). To our surprise, in the absence of

Table 2 | Comparison of cloning efficiencies with iPCR, mixed PCR and restriction enzyme–generated inserts

	No treatment		T4 treatment	
	Cloning efficiency (c.f.u./ng[a])	Fold induction	Cloning efficiency (c.f.u./ng[a])	Fold induction
Vector 1[b] only (3.1 kb)	0.8	1	0.8	1
Vector 1[b] and iPCR fragment	13	16	220	280
Vector 1[b] and restriction fragment	1	1.3	650	810
Vector 2[c] only (12 kb)	0.2	1	0.2	1
Vector 2[c] and mixed PCR fragment	7.6	38	–	–
Vector 2[c] and T4 treated PCR fragment	–	–	14	70

[a]Colony forming units per ng of vector. [b]The linear vector 1 (pML385, 3.1 kb) was treated with T4 DNA polymerase. The 20 bp homology Skp1 insert generated by iPCR or the identical insert generated by *Sma*I digestion were heated to 95 °C for 5 min to denature, and then cooled slowly to 22 °C to reanneal. T4 treatment, inserts (iPCR and restriction fragment) were treated with T4 DNA polymerase. The vector and the appropriate amount of inserts (1:1 molar ratio) were then annealed and transformed into cells. [c]The linear vector 2 (ptmGIPZ-pheS, 12 kb) and insert (using primer pair P1F-P2R) were treated with T4 DNA polymerase. The vector and the insert generated by T4 DNA polymerase were annealed at 1:2 molar ratio of vector to insert and transformed. The vector and the insert generated by mixed PCR were annealed at 1:6 molar ratio of vector to insert and transformed into cells. –, not tested.

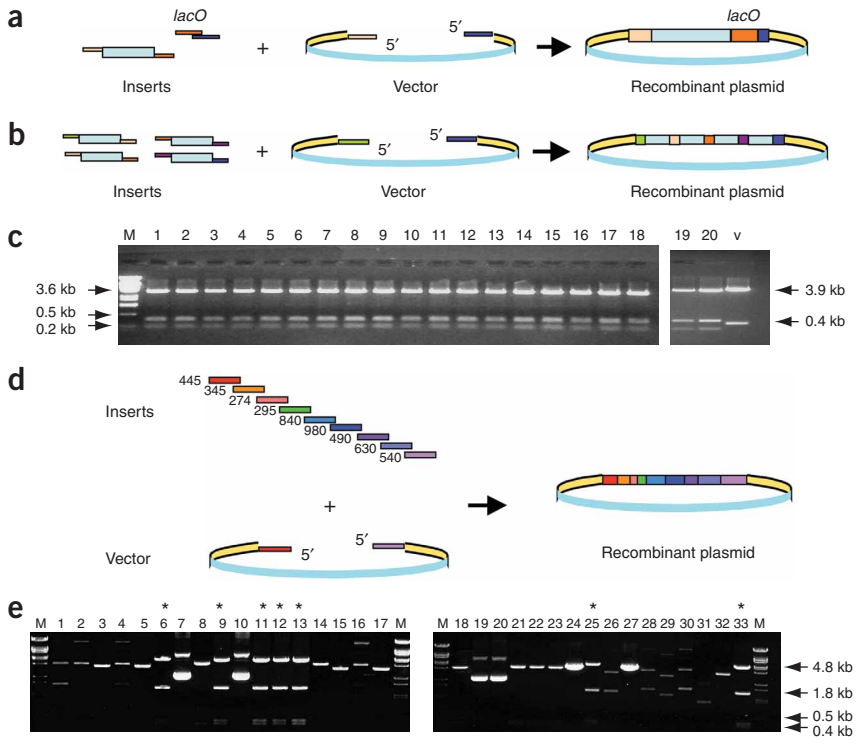

Figure 4 | Multi-fragment assembly using SLIC. (**a**) A schematic illustrating the three-way SLIC reaction with *lacO* oligos. (**b**) A schematic illustrating the five-way SLIC reaction in which T4 DNA polymerase–treated linear vector, pML385, and inserts with different amounts of homology were annealed in an equimolar ratio and transformed into cells. (**c**) An *Afe*I restriction analysis of the recombinants derived from the five-way SLIC reaction (40 bp homology) in **b**. Plasmid DNAs from 20 independent kanamycin-resistant colonies (lanes 1–20) and vector control (lane "v") were digested with *Afe*I. The recombinants contain three bands (3.6, 0.5 and 0.2 kb). The vector contains two bands (3.9 and 0.4 kb). (**d**) A schematic of the ten-way SLIC reaction in which T4 DNA polymerase–treated linear vector, pML385, and inserts with 40-bp homology were annealed in equimolar ratios. Numbers on the left refer to length of each fragment in nucleotides. (**e**) *Nco*I restriction analysis of the recombinants derived from ten-way SLIC. Plasmid DNAs from 33 independent kanamycin-resistant colonies (lanes 1–33) were digested with *Nco*I. The correct recombinants should have 4.8, 1.8, 0.5 and 0.4 kb bands. Asterisks indicate recombinants with the correct restriction map. Thirty-three of 42 total plasmids are shown, including all of the correct clones.

lacO selection, background increased to only 150 colonies/μg, but we observed nearly 42,000 transformants and 280-fold stimulation when all three fragments were present. Thus, three-way assembly is robust.

To attempt a five-way reaction, we generated four inserts of sizes from 275 bp to 400 bp by PCR (**Fig. 4b**). The amount of overlapping homology varied from 20 to 40 bp overlaps. There is an inherent problem with multiple fragment assembly because if assemblies occur on both ends of a vector, they may inhibit circularization if more than four fragments anneal to the vector ends. To minimize this potential problem, we did not use the excess of inserts that is optimal for two-way assemblies. Inserts gave stimulation to each overhang class (**Table 4**). For 20-bp overhangs, we observed a 14-fold stimulation, which increased to 20-fold with 30-bp overlap and 60-fold with 40-bp overlaps. Restriction analysis of the 40-bp recombinants revealed that 20 of 20 had the correct restriction digest pattern (**Fig. 4c**). We selected ten recombinants for complete sequence analysis. Eight had completely wild-type sequence. One had a mutation in sequence complementary to one of the primers and another had a PCR-induced mutation in one of the fragments.

We next attempted a ten-fragment assembly with nine PCR-generated fragments of sizes ranging between 275 and 980 bp with 40 bp overlaps inserted into a 3.1 kb vector (**Fig. 4d**). Unlike other assemblies, there was no stimulation of transformation upon addition of inserts. Restriction analysis revealed that 7 of 42 transformants had the correct restriction pattern (**Fig. 4e**). Unlike five-way reactions, in this case we observed clones that had a subset of the insert fragments present as a result of faulty recombination. Nevertheless, nearly 20% of the ten-way assemblies were correct, which is sufficient for complex component assembly *in vitro*. We obtained similar results when reactions were performed with RecA (data not shown). These data together indicate that the assembly of complex recombinant DNA assemblies can be achieved using *in vitro* homologous recombination.

DISCUSSION

Unlike conventional methods that use restriction enzymes or site-specific recombinases, recombinant DNA assembled by SLIC achieves a seamless transfer of genetic elements *in vitro* without the need for specific sequences required for ligation or site-specific recombination. This is accomplished by harnessing the power of homologous recombination *in vitro* to assemble recombinant DNA that resembles recombination intermediates. These intermediates are then introduced into bacteria where they are repaired to regenerate double-stranded, covalently closed plasmids.

Using bacterial recombinases such as RecA, recombinant DNA can be assembled efficiently with very small amounts of DNA.

Table 3 | Three-way SLIC with *lacO* selection

	Cl⁻Phe Kan Cb	Cl⁻Phe Kan
Vector[a] and *lacO*	1.6	150
Vector[a] and insert and *lacO*	36,000	42,000

Cloning efficiencies are given as colony forming units per microgram of vector under the indicated selection conditions. [a]T4 DNA polymerase–treated pML403 and a 20-bp homology *Skp1* insert were annealed with a pair of *lacO* oligos (1:1:1 molar ratio) and transformed. Cl⁻Phe, chlorophenylalanine; Kan, kanamycin; Cb, carbenicillin.

Table 4 | Five-way SLIC

	20 bp	30 bp	40 bp
Vector[a] only	390	410	360
Vector[a] and inserts	5,300	8,700	22,000

Cloning efficiencies are given as colony forming units per microgram of vector for the indicated regions of homology. [a]T4 DNA polymerase–treated pML385 and inserts with different amounts of homology were annealed in equimolar ratio and transformed.

Homologous recombination events that occur *in vivo* such as those carried out by MAGIC cloning can be efficiently recapitulated *in vitro* using SLIC. This method can be used to assemble DNA made by PCR or restriction fragments. The only requirement is that the fragments to be assembled contain on their ends sequences of 20 bp or longer to allow stable annealing. Excision by the proofreading exonuclease of T4 DNA polymerase has proven to be the most reproducible and easiest to manipulate method for generating 5′ overhangs. Although much less efficient, iPCR also gives substantial stimulation of transformation. This might be sufficient for routine subcloning purposes, although there is likely to be more variability depending on the completeness of the PCR synthesis.

The SLIC reactions described here that do not use RecA bear a resemblance to ligation-independent cloning[20–22]. There are important differences, however. For ligation-independent cloning, PCR primers for inserts are designed to contain appropriate 5′ extension sequences lacking a particular dNTP that, after treatment with T4 DNA polymerase in the presence of the particular dNTP, generate specific 12-nt ssDNA overhangs that are complementary to overhangs engineered into the vector. Notably, these overhangs have sequence constraints as they must be devoid of a common dNTP, which limits their use to specialized vectors bearing that sequence. The realization that alternative recombination intermediates with imprecise junctions such as large gaps and overhangs can be efficiently repaired *in vivo* completely liberates SLIC from the sequence constraints from which the ligation-independent cloning method suffers. Having the ability to generate overlaps of greater lengths of unrestrained sequence provides much greater utility for SLIC, and its combination with RecA makes it able to function at much lower DNA concentrations.

An important advantage of the SLIC method is its flexibility with respect to sequence junctions. We have also shown that fragments with considerable nonhomologies of up to 20 nt at the ends can be assembled as long as the homologous regions are made single-stranded (data not shown). Presumably these branched molecules are efficiently trimmed *in vivo* to generate recombinant plasmids. Unlike the site-specific recombination or restriction-enzyme methods, SLIC allows alterations of fragments internal to a gene in a plasmid. For example, it would be simple to introduce a PCR fragment into a restriction site *in vitro* even if that fragment contained multiple sites for the enzyme. Also, as the homologous junctions of fragments can be controlled, SLIC offers a new approach to the generation of site-directed mutations.

Among the strongest advantages offered by homologous recombination *in vitro* is the ability to assemble multimeric fragments. In conventional cloning experiments usually two and sometimes three fragments are assembled in one reaction assuming proper restriction sites are available. Our data indicate that five fragments can be easily assembled with high efficiency using SLIC, and 10 fragments can be joined with reduced efficiency. The fidelity of the assembled molecules is limited only by the fidelity of PCR and the oligonucleotide primers used to generate the insert fragments. SLIC compares favorably with other multifragment cloning strategies such as multisite Gateway because SLIC allows complete control over junction fragments unlike Gateway, which requires a defined site-specific recombination site between each fragment[23]. Furthermore, SLIC works with PCR fragments, whereas multisite Gateway has only been demonstrated with cloned fragments on donor plasmids[23]. The ability to assemble complex combinations of DNA sequence elements in defined orders will be particularly important in the field of synthetic biology. We made no attempts to optimize the ten-way assemblies, and it is likely one could considerably improve the yield in future experiments. Thus, it is likely that in the future it will be possible to assemble molecules with greater than 10 fragments.

The utility of the SLIC system is not limited to gene assembly. Genetic elements of any kind can be assembled using this system. One can now envision vectors being assembled in a combinatorial fashion from component parts. For example, using the highly efficient five-way assembly one could combine an open reading frame together with a particular epitope tag, a tissue-specific promoter, a retroviral vector together with a selectable marker of choice to generate a custom expression assembly. Thus, in the future vectors might exist in virtual form and be assembled in final form as needed. The advent of SLIC now brings the ability to manipulate DNA sequences with much greater ease than previously possible. Other complex assemblies such as homologous recombination targeting vectors could be assembled in one step by SLIC. These advances should save investigators substantial amounts of time, effort and expense.

METHODS

SLIC. We digested the vector pML385 with *Nco*I-*Bam*HI and gel purified it using a QIAEX II gel extraction kit (Qiagen; **Supplementary Protocols 1** and **2** online). We amplified inserts using *Taq* DNA polymerase. After PCR amplification, we added 20 U of *Dpn*I to the reaction and incubated it at 37 °C for 1 h to digest the template. We purified the inserts by QIAquick PCR purification column (Qiagen). We treated 1 μg of the vector and 1 μg of the inserts separately with 0.5 U of T4 DNA polymerase in 20-μl reactions at 22 °C for various amounts of time depending on the length of the homologous region. The optimal treatment for a 20-bp overlap was 30 min and for a 40-bp overlap, 60 min. We stopped the reactions by adding 1/10 volume of 10 mM dCTP. We routinely use 150 ng of the vector and appropriate amount of inserts in a 1:1 or 2:1 insert to vector molar ratio in a 10-μl annealing reaction with 1× ligation buffer and incubation at 37 °C for 30 min. We transformed 5 μl of the annealing mix into 150 μl of chemically competent cells and plated them on chlorophenylalanine plates. In cases when we were transforming DNA in low concentrations, we used electrocompetent cells.

SLIC with iPCR or mixed PCR products. We generated iPCR products under the same conditions as regular PCR products and purified them by a QIAquick PCR purification column (**Supplementary Protocol 3** online). We denatured the purified iPCR products at 95 °C for 5 min and renatured them slowly to 22 °C in 1 h. We annealed the vector and inserts at a 1:1 molar ratio and transformed them into cells.

We generated the two mixed PCR products separately using primer pairs P1F-P1R and P2F-P2R (see **Supplementary Methods** online for primer sequences). Primers P1F and P2F are overlapping on the 3′ end, but P1F is longer and contains a 5′ 30-bp homology region to the vector, ptmGIPZ-pheS. Primers P1R and P2R are overlapping on the 3′ end, but P2R is longer and contains the second 30-bp homology region to the vector. After PCR, we purified the two PCR products using QIAquick PCR purification columns, mixed them in equal amounts, denatured at 95 °C for

5 min and renatured slowly to 22 °C in 1 h. We annealed the vector and inserts at 1:6 molar ratio and transformed these reactions into cells.

Additional methods. Information about bacterial strains, media, plasmid constructions and primer sequences are available in **Supplementary Methods**.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

We thank B. Wanner, G. Hannon and T. Moore for providing plasmids, bacterial strains and advice concerning their use. We thank M. Schlabach for comments on the manuscript. This work was supported by a grant from US National Institutes of Health. S.J.E. is an investigator with the Howard Hughes Medical Institute.

AUTHOR CONTRIBUTIONS

M.Z.L. performed all experiments. S.J.E. helped in experimental design. M.Z.L. and S.J.E. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at http://www.nature.com/naturemethods/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Smith, H.O. & Wilcox, K.W. A restriction enzyme from Hemophilus influenzae. I. Purification and general properties. *J. Mol. Biol.* **51**, 379–391 (1970).
2. Danna, K. & Nathans, D. Specific cleavage of simian virus 40 DNA by restriction endonuclease of Hemophilus influenzae. *Proc. Natl. Acad. Sci. USA* **68**, 2913–2917 (1971).
3. Cohen, S.N., Chang, A.C., Boyer, H.W. & Helling, R.B. Construction of biologically functional bacterial plasmids *in vitro*. *Proc. Natl. Acad. Sci. USA* **70**, 3240–3244 (1973).
4. Backman, K. & Ptashne, M. Maximizing gene expression on a plasmid using recombination *in vitro*. *Cell* **13**, 65–71 (1978).
5. Hutchison, C.A. *et al.* Mutagenesis at a specific position in a DNA sequence. *J. Biol. Chem.* **253**, 6551–6560 (1978).
6. Rumsby, G. An introduction to PCR techniques. *PCR Methods Mol. Biol.* **324**, 75–89 (2006).
7. Saiki, R.K. *et al.* Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**, 1350–1354 (1985).
8. Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. & Pease, L.R. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59 (1989).
9. Liu, Q., Li, M.Z., Liebham, D., Cortez, D. & Elledge, S.J. The univector plasmid fusion system, a method for rapid construction of recombinant DNA without restriction enzymes. *Curr. Biol.* **8**, 1300–1309 (1998).
10. Hartley, J.L., Temple, G.F. & Brasch, M.A. DNA cloning using *in vitro* site-specific recombination. *Genome Res.* **10**, 1788–1795 (2000).
11. Walhout, A.J. *et al.* GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. *Methods Enzymol.* **328**, 575–592 (2000).
12. Bethke, B. & Sauer, B. Segmental genomic replacement by Cre-mediated recombination: Genotoxic stress activation of the p53 promoter in single-copy transformants. *Nucleic Acids Res.* **25**, 2828–2834 (1997).
13. Nebert, D.W., Dalton, T.P., Stuart, G.W. & Carvan, M.J. Gene-swap knock-in cassette in mice to study allelic differences in human genes. *Ann. NY Acad. Sci.* **919**, 148–170 (2000).
14. Siegel, R.W. *et al.* Recombinatorial cloning using heterologous lox sites. *Genome Res.* **14**, 1119–1129 (2004).
15. Li, M.Z. & Elledge, S.J. MAGIC: An *in vivo* genetic method for the rapid construction of recombinant DNA molecules. *Nat. Genet.* **37**, 311–319 (2005).
16. Amundsen, S.K. & Smith, G.R. Interchangeable parts of the *Escherichia coli* recombination machinery. *Cell* **112**, 741–744 (2003).
17. Kuzminov, A. Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage lambda. *Microbiol. Mol. Biol. Rev.* **63**, 751–813 (1999).
18. Konforti, B.B. & Davis, R.W. 3′ homologous free ends are required for stable joint molecule formation by the RecA and single-stranded binding proteins of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **84**, 690–694 (1987).
19. Tillett, D. & Neilan, B.A. Enzyme-free cloning: A rapid method to clone PCR products independent of vector restriction enzyme sites. *Nucleic Acids Res.* **27**, e26 (1999).
20. Aslanidis, C. & de Jong, P.J. Ligation-independent cloning of PCR products (LIC-PCR). *Nucleic Acids Res.* **18**, 6069–6074 (1990).
21. Haun, R.S., Servanti, I.M. & Moss, J. Rapid, reliable ligation-independent cloning of PCR products using modified plasmid vectors. *Biotechniques* **13**, 515–518 (1992).
22. Aslanidis, C., de Jong, P.J. & Schmitz, G. Minimal length requirement of the single-stranded tails for ligation-independent cloning (LIC) of PCR products. *PCR Methods Appl.* **4**, 172–177 (1994).
23. Cheo, D.L. *et al.* Concerted assembly and cloning of multiple DNA fragments using *in vitro* site-specific recombination: Functional analysis of multi-site expression clones. *Gen. Res.* **14**, 2111–2120 (2004).