



# Reiterative Recombination for the in vivo assembly of libraries of multigene pathways

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The increasing sophistication of synthetic biology is creating a demand for robust, broadly accessible methodology for constructing multigene pathways inside of the cell. Due to the difficulty of rationally designing pathways that function as desired in vivo, there is a further need to assemble libraries of pathways in parallel, in order to facilitate the combinatorial optimization of performance. While some in vitro DNA assembly methods can theoretically make libraries of pathways, these techniques are resource intensive and inherently require additional techniques to move the DNA back into cells. All previously reported in vivo assembly techniques have been low yielding, generating only tens to hundreds of constructs at a time. Here, we develop "Reiterative Recombination," a robust method for building multigene pathways directly in the yeast chromosome. Due to its use of endonuclease-induced homologous recombination in conjunction with recyclable markers, Reiterative Recombination provides a highly efficient, technically simple strategy for sequentially assembling an indefinite number of DNA constructs at a defined locus. In this work, we describe the design and construction of the first Reiterative Recombination system in *Saccharomyces cerevisiae*, and we show that it can be used to assemble multigene constructs. We further demonstrate that Reiterative Recombination can construct large mock libraries of at least  $10^4$  biosynthetic pathways. We anticipate that our system's simplicity and high efficiency will make it a broadly accessible technology for pathway construction and render it a valuable tool for optimizing pathways in vivo.

in vivo DNA assembly | homing endonuclease | cell engineering | metabolic engineering | combinatorial libraries

A key bottleneck to reengineering cells for diverse synthetic biology applications is the technical difficulty of constructing optimized, multigene pathways in vivo. The advent of synthetic biology has raised the tantalizing prospect of reprogramming cells at will for purposes ranging from the biosynthesis of high-value feedstocks and natural product analogs to the development of cell-based sensors and therapeutics (1). Engineering cells for such tasks requires the introduction of numerous exogenous genes into the genome to create customized "pathways." However, standard molecular biology and genetic techniques, developed for the manipulation of single genes, become unwieldy or ineffective when applied to much larger multigene constructs. A new generation of robust, accessible tools for building pathways inside the cell is needed.

The difficulty of rationally designing complex systems that operate as desired in the cellular milieu (2) further argues that the ability to construct not only individual pathways but also libraries of pathways in vivo will be essential. Precedent has indicated that multicomponent systems introduced into the cell typically require refinement to function optimally (3, 4). By analogy to the directed evolution approaches that empowered the routine discovery of proteins and nucleic acids with prescribed functions, generating large numbers of variant pathways in parallel and screening for those that exhibit the required behavior could streamline optimization efforts (5). Library-based approaches could thus circumvent the gaps in our knowledge, immediately yielding functional systems. One invaluable tool

for implementing such strategies will be DNA assembly methods that can reliably generate sizable collections of pathways ( $>10^3$ ) inside of the cell, an especially high standard of efficiency.

While several creative strategies for the in vitro and in vivo assembly of multigene constructs have been developed, building such pathways has not become routine outside of expert laboratories. One class of techniques is comprised of in vitro methods (6–11) such as in vitro recombination (12–14) that stretch the limits of standard molecular biology tools for large-scale DNA assembly. These methods can sometimes meet the standard of being high yielding, but they are resource intensive and do not intrinsically address the issue of moving the DNA into the cell. Though certain of these techniques can theoretically generate large numbers of pathways simultaneously, only a very few examples of constructing libraries that were relatively small ( $\leq 10^2$  variants) have been reported (15).

The second class of DNA assembly methods exploits in vivo homologous recombination to assemble DNA directly in the cell (16–21). These techniques are attractive for their simplicity, as they require only a straightforward transformation step. In vivo homologous recombination has also been used extensively in the context of library generation for directed evolution applications; the highly efficient recombination machinery of organisms such as *Saccharomyces cerevisiae* has frequently been employed to build single-gene libraries containing  $10^4$ – $10^{10}$  variants (22–26). However, previously reported in vivo assembly techniques for multigene constructs have been low yielding, generating only tens to hundreds of recombinants at a time and making them impractical for the construction of libraries.

Thus, we sought to develop a high-yielding method for constructing multigene pathways that would harness the technical ease of in vivo recombination yet be efficient enough to create large libraries. Given that specific double-strand DNA breaks are known to promote repair by homologous recombination and have formed the basis of robust technologies to seamlessly manipulate genomes (27, 28), we hypothesized that coupling DNA cleavage by a homing endonuclease to DNA assembly could provide the needed boost in efficiency. Here we develop a method that utilizes endonuclease-stimulated homologous recombination for DNA assembly and demonstrate that we can easily and efficiently build large libraries of biosynthetic pathways in vivo.

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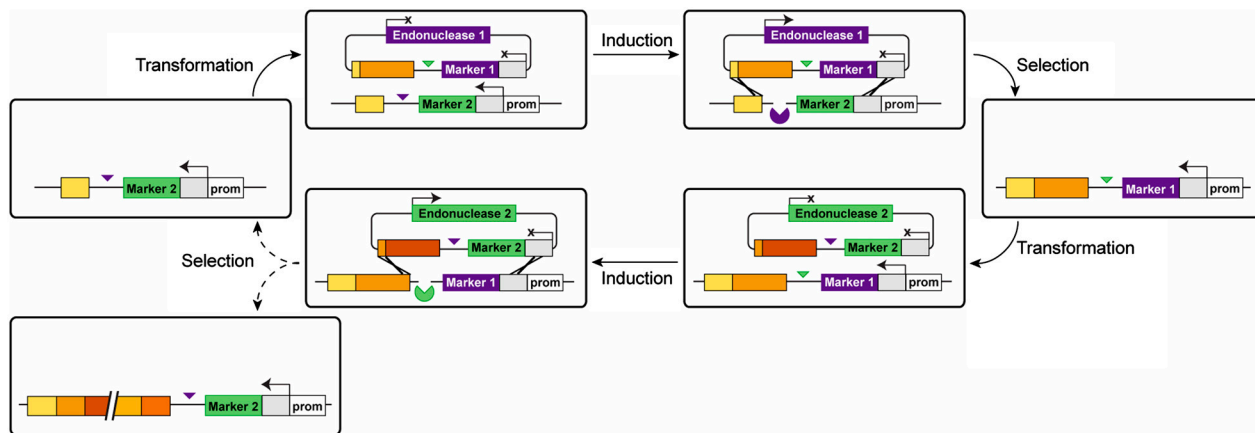
Conflict of interest statement: L.M.W. and V.W.C. are inventors on a patent filed regarding this method.

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**Fig. 1.** General scheme of Reiterative Recombination, showing two rounds of elongation. Each round of elongation is achieved by recombination between an acceptor module (shown here in the linear chromosome) and a donor module (in the circular plasmid). The two modules contain orthogonal homing endonuclease cleavage sites (triangles) adjacent to different selectable markers (purple and green). Both markers are downstream of a homology region (gray), but only the acceptor module contains a promoter (white) driving marker expression. Endonuclease cleavage of the acceptor module stimulates recombination, joining the fragments being assembled (orange) and replacing the acceptor module's endonuclease site and expressed selectable marker with those of the donor module. Repeating this procedure with a donor module of the opposite polarity returns the acceptor module to its original state, allowing the assembly to be elongated indefinitely.

## Results

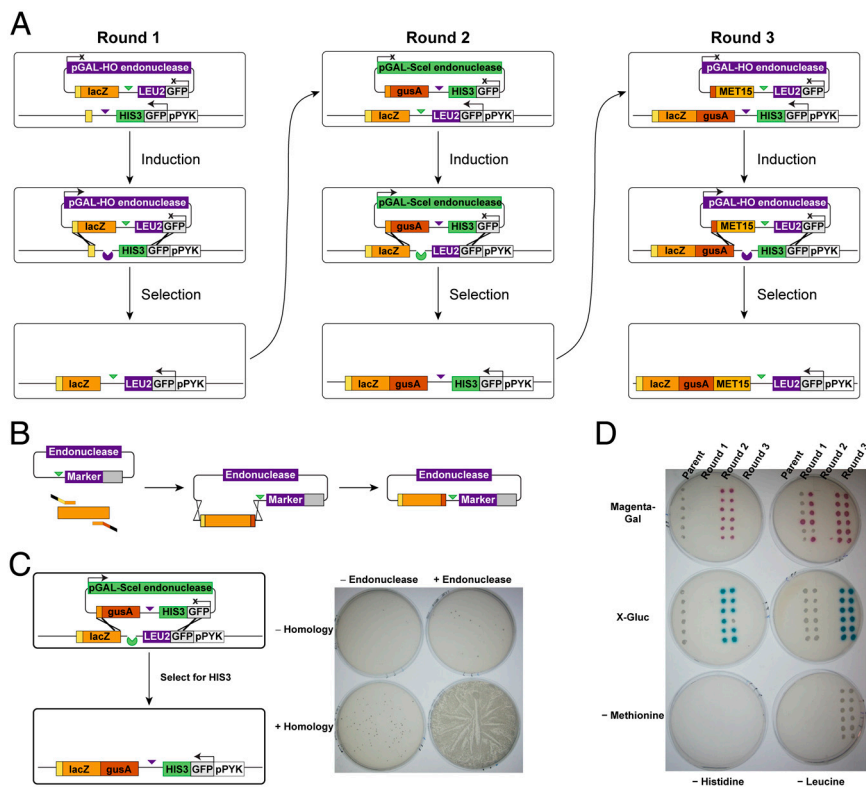
**Design and Construction of a Reiterative Recombination System.** Our DNA assembly system, “Reiterative Recombination,” elongates a construct of interest in a stepwise manner by employing pairs of alternating, orthogonal endonucleases and selectable markers. As shown in Fig. 1, endonuclease cleavage sites are placed between fragments of the construct of interest and selectable markers in “donor” and “acceptor” modules. Following endonuclease cleavage of the acceptor module, the donor module provides homology on either side of the double-strand break through a short region of overlap between the fragments to be assembled on one side and a homology region upstream of the marker on the other side. Repair by homologous recombination adds the donor module's fragment to the acceptor module's growing construct while simultaneously replacing the acceptor module's endonuclease cleavage site and selectable marker. Because only the acceptor module's marker is actively transcribed, recombinants can be readily identified. During the next round of elongation, the endonuclease cleavage site and selectable marker return to the original configuration, allowing assembly to proceed in a cyclical format.

We constructed an initial Reiterative Recombination system in *Saccharomyces cerevisiae*, which has highly efficient homologous recombination machinery, placing the acceptor module in the chromosome and donor modules in plasmids. For the orthogonal endonucleases, we turned to the two well studied *S. cerevisiae* enzymes employed throughout the homologous recombination literature, HO (29) and SceI (30), placing them under the control of the inducible *GAL* promoter on the donor plasmids. For the alternating markers, we used *HIS3* and *LEU2*, which complement the histidine and leucine auxotrophies of many laboratory yeast strains, adding endonuclease cleavage sites downstream of their terminators and fusing GFP genes to their N termini to provide upstream homology regions. The donor plasmids also contain the positive- and negative-selectable *URA3* marker, allowing cells to be cured of donor plasmids by growth on 5-fluoroorotic acid (FOA) after each elongation round. Only two modifications were necessary to prepare a standard strain with the appropriate auxotrophies for Reiterative Recombination: elimination of the endogenous HO cleavage site in the *MAT* locus with a silent mutation (31) via “pop-in/pop-out” gene replacement (32) and integration of the acceptor module. The resulting parental acceptor strain can in theory be used for the assembly of any desired DNA construct. However, if certain applications necessitate the

use of a specific background strain, only two established, robust integration steps are required to convert any strain with the appropriate auxotrophies into an acceptor strain.

**Proof-of-Principle of Reiterative Recombination.** We first employed Reiterative Recombination to sequentially integrate the reporter genes *lacZ* ( $\beta$ -galactosidase), *gusA* ( $\beta$ -glucuronidase), and *MET15* (complementation of methionine auxotrophy) using three rounds of assembly, creating an 8.5-kilobase construct (Fig. 2A). Subfragments for integration were PCR amplified as one or two overlapping pieces using primers that incorporated short regions of homology (30–40 bp) (*i*) to the preceding piece of the growing assembly and (*ii*) to the donor plasmid. PCR products were cotransformed with a digested, generic donor plasmid into the acceptor strain to generate intact donor plasmids by plasmid gap repair (Fig. 2B) (33). Our procedure thus eliminates any *in vitro* manipulation (e.g., subcloning) other than basic PCR. Galactose induction of endonuclease expression in the transformants led to a high rate of marker conversion only when both the endonuclease gene and the homology on both sides of the endonuclease cut site were present (Fig. 2C). Phenotypic analysis of recombinants following donor plasmid curing indicated that auxotrophies for histidine and leucine alternated with each round of elongation, as expected (Fig. 2D). Each newly integrated reporter (*lacZ*, *gusA*, or *MET15*) was functional in 75–100% of recombinants when >40 individual colonies from each round were assayed, and previously integrated reporters were maintained (Fig. 2D, Fig. S1, SI Text). We also confirmed that integration occurred in the expected manner by analyzing the purified genomic DNA of cured recombinants by PCR and restriction digestion (Fig. S2).

**Construction of Libraries of Biosynthetic Pathways via Reiterative Recombination.** To demonstrate the generality of Reiterative Recombination and its application to a biosynthetic pathway, we integrated codon-optimized versions of *Erwinia herbicola crtE* (round 1), *crtB* (round 2), and *crtI* (round 3) to generate a yeast strain capable of producing the isoprenoid pigment lycopene (Fig. 3A). We also integrated the selectable marker *TRP1* during round 3 to provide further verification of correct pathway construction (*vide infra*). After the third round of assembly, 99% of the resulting recombinants exhibited the expected orange phenotype, indicative of lycopene production (Fig. 3E). In parallel, as negative controls, we built pathways containing nonsense



**Fig. 2.** Reiterative Recombination reporter proof-of-principle system. (A) Details of the assembly process for the proof-of-principle system, in which the three reporter genes *lacZ*, *gusA*, and *MET15* were sequentially integrated into the chromosome. (B) Construction of donor plasmids by plasmid gap repair, in which a digested universal donor plasmid and PCR fragments with appropriate homology regions were cotransformed into the Reiterative Recombination strain and assembled via homologous recombination. (C) Results of the round 2 induction step are shown as a representative example. As negative controls, cells containing identical donor plasmids lacking the *Scel* endonuclease gene and/or the *gusA* fragment with *lacZ* homology were induced in parallel. A calculated  $6 \times 10^6$  cells were plated on SC(-Histidine) media to assay for selective marker conversion after a 12-h galactose induction. (D) Phenotypes of 12 unique cured colonies of assays for *lacZ* (Magenta-Gal), *gusA* (X-Gluc), and *MET15* [SC(-Methionine)] markers. In rows, recombinants are assayed for *lacZ* (Magenta-Gal), *gusA* (X-Gluc), and *MET15* [SC(-Methionine)] markers.

mutations in *crtB* and/or *crtI*, and the resulting strains did not produce lycopene (Fig. 3 B–D).

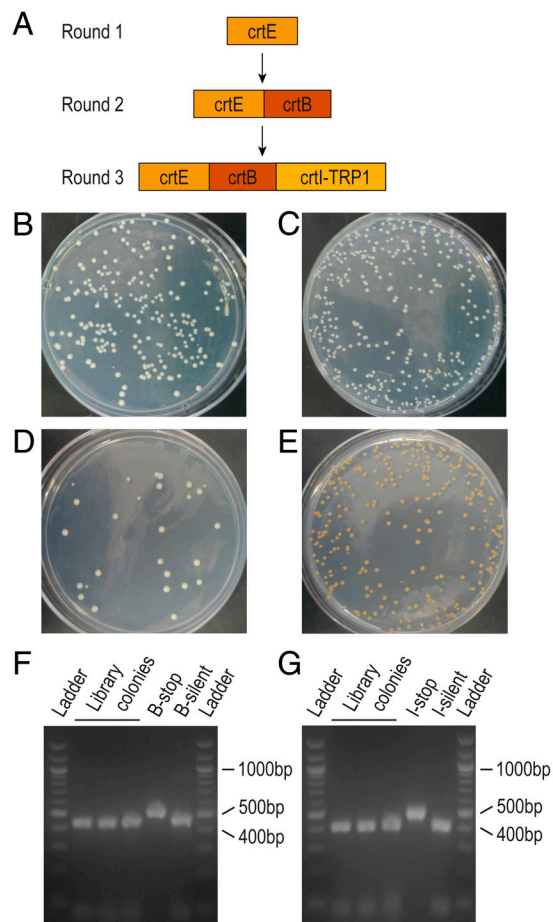
Given that every step of Reiterative Recombination proceeded with high efficiency in these proof-of-principle studies, we expected that we could generate larger libraries of pathways than attainable with other *in vivo* DNA assembly techniques, which generate only tens to hundreds of variants at a time. Using a basic yeast electroporation protocol, we can obtain as many as  $10^6$ – $10^8$  transformants per transformation (25); the induction, which is readily scalable, typically gives  $\gg 10^4$  recombinants per milliliter of culture. We therefore used the lycopene biosynthesis pathway to explicitly challenge Reiterative Recombination's ability to generate large libraries. We repeated rounds 2 and 3 of the lycopene pathway assembly, this time transforming various ratios of *crtB* and *crtI* alleles that contained either nonsense or silent mutations with diagnostic restriction sites. Initially, we did not recover lycopene-producing colonies from our libraries at the expected frequencies. Further analysis of the pool of cured recombinants obtained from various Reiterative Recombination rounds, both from the reporter proof-of-principle system and the lycopene pathway assembly, revealed that a small percentage of cured recombinants ( $<0.2\%$ ; *SI Text*, Table S1) acquired both the *HIS3* and *LEU2* markers. This subpopulation of cells was sufficient to skew the observed ratios of orange colonies after carrying the library forward for multiple rounds. While we are developing a next-generation Reiterative Recombination system that eliminates this problem entirely, we were immediately able to construct large libraries in this first-generation system by simply selecting for the *TRP1* marker at the end of the pathway (Fig. 3A) after the last round of assembly. This additional selection served

as a stringent final purification step for our libraries and, importantly, is a general solution that could be used for any desired library application. As shown in Table 1, we were readily able to recover lycopene-producing colonies at the expected frequencies from mock libraries of up to  $10^4$ . These colonies contained the expected silent mutations in *crtB* and *crtI*, demonstrating that they arose from the silent alleles rather than from mutation of the genes with nonsense mutations (Fig. 3 F–G, Fig. S3).

## Discussion

By providing a highly efficient method for the assembly of pathways *in vivo*, Reiterative Recombination opens the door to the routine construction of gene circuits, pathways, and libraries thereof in the cell. Reiterative Recombination's high efficiency, together with its technical straightforwardness, makes it a reliable method for building multigene constructs that is accessible to nonexperts without specialized equipment. While a handful of laboratories that are experts in the field have described landmark achievements in the realm of large-scale DNA assembly, these techniques have not yet been widely adopted by the scientific community. Reiterative Recombination distills the construction of individual pathways into a user-friendly process that requires only basic molecular biology tools.

The use of recyclable markers and endonucleases in Reiterative Recombination should also make it useful for the assembly and integration of very large DNA constructs. In this work, we have built constructs of only  $<10$  kilobases in three rounds of assembly. However, we anticipate no difficulty in continuing the procedure for more rounds, and we are currently working to construct significantly longer biosynthetic pathways. Recycling



**Fig. 3.** Assembly of the lycopene biosynthesis pathway using Reiterative Recombination. (A) Order of *crt* gene insertion. (B–E) Phenotypes of cured round 3 colonies containing wild-type *crtE* and (B) *crtB*-stop + *crtI*-stop, (C) *crtB*-stop + *crtI*-silent, (D) *crtB*-silent + *crtI*-stop, and (E) *crtB*-silent + *crtI*-silent. For (E), 315 out of 317 colonies had an orange phenotype; none of the other plates contained any orange colonies. (F, G) Restriction analysis of the three orange cured recombinants recovered from the  $10^4$ :1 (100:1 *crtB*stop:silent + 100:1 *crtI*stop:silent) lycopene library screen. Regions of the *crtB* (F) and *crtI* (G) alleles containing the diagnostic mutations were amplified by colony PCR and digested with EcoRV and BsmBI, respectively. Only alleles containing the silent mutations are cut by these enzymes. The plasmids with the *B*-stop, *B*-silent, *I*-stop, and *I*-silent alleles that served as PCR templates for the subfragments were PCR amplified and digested in parallel as controls. The ladder is a 100 bp DNA ladder from New England Biolabs.

markers in Reiterative Recombination eliminates the perennial problem of running out of selectable markers during complex strain constructions, which greatly complicates or limits the scope of more traditional methods for introducing multiple genes and libraries thereof into the same yeast strain (e.g., repeated integration steps and/or mating strategies) (34, 35). Furthermore, our assembly system's modular protocol minimizes the effort needed to design and execute each integration step. Finally, an important

aspect of Reiterative Recombination is the generality of its design; Reiterative Recombination systems could be developed in other organisms that have efficient endogenous or engineered recombination systems (36). Alternatively, standard molecular biology techniques could be used to shuttle pathways constructed in yeast into other organisms that are preferred hosts for specific applications (21).

Reiterative Recombination's robustness makes it capable of generating sizable libraries of multigene pathways (at least  $10^4$ ) containing diversity at multiple loci. Though methods for constructing multicomponent DNA constructs are proliferating, there are surprisingly few examples of using them to build libraries. The *in vivo* assembly methods described to date have proceeded with efficiencies that are simply too low ( $\leq 10^2$  colonies) to be useful for the one-pot assembly of collections of pathways. Even after modification and optimization, most *in vitro* assembly techniques based on restriction digestion and ligation or on enzymatic recombination typically display profound losses in cloning efficiency for  $\geq 3$  fragments ( $\leq 10^3$  colonies per reaction) (37, 38). Several particularly efficient *in vitro* approaches (8), notably *in vitro* recombination (12–14), could theoretically generate libraries of  $\sim 10^4$ . However, none of these *in vitro* assembly technologies directly address the problem of moving the resulting pathways into the cell efficiently enough to maintain library complexity, an especial challenge if constructs must be stably integrated in the chromosome. Tellingly, even the high-yielding isothermal assembly method has only once been used to construct a library that consisted of  $\sim 10^2$  two-gene constructs and was incompletely characterized (15). Our mock library experiment is key because it explicitly tests the library sizes Reiterative Recombination can generate and shows that members of the library are present in the expected proportions. To our knowledge, no other DNA assembly method's ability to create such libraries *in vivo* has been rigorously characterized in this way. In addition, though we only attempted to build libraries of up to  $10^4$ —due to the limits of our ability to readily screen large numbers of colonies for lycopene production visually—the high efficiency and straightforward scalability of the recombination step suggests that it is only the transformation efficiency of yeast ( $\sim 10^6$ – $10^8$  transformants per transformation) that will limit library size in Reiterative Recombination.

The development of highly efficient DNA assembly methods is an essential first step towards the combinatorial optimization of pathways *in vivo*. In spite of enormous advances in our understanding of systems-level biology in the past decade, our ability to rationally predict the effects of changes to cell circuitry remains limited (2, 5). Library approaches can provide a direct route for obtaining and refining functional *in vivo* systems (39–41). In the field of metabolic engineering, for example, researchers have repeatedly improved natural product yields and synthesized analogs by searching collections of isozymes (42, 43), mutant biosynthetic enzymes (3, 44), or promoters and regulatory regions that modulate the expression levels of genes that alter pathway flux (45, 46). Testing these multiple variables in the context of a pathway causes library sizes to rapidly swell (e.g., testing 100 mutants of enzyme A against 100 mutants of enzyme B is already

**Table 1. Mock screen for lycopene-producing strains via Reiterative Recombination**

Transformed DNA ratios		Library complexity	Colonies assayed	Orange colonies	Observed percentage of orange colonies	<i>P</i> *
<i>crtB</i> stop:silent	<i>crtI</i> stop:silent					
10:1	0:1	$10^1$	2,360	225	10%	0.5
100:1	0:1	$10^2$	587	5	0.9%	0.7
100:1	10:1	$10^3$	2,079	3	0.1%	0.4
100:1	100:1	$10^4$	18,450	3	0.02%	0.4

\*Because the plated cells represented a randomly selected aliquot ( $<0.1\%$ ) of the population, a 1-proportion z-test was used to test if the observed percentages of orange colonies were significantly different than the expected percentages. All *P*-values were greater than  $\alpha = 0.1$ , indicating that none were significantly different.

$10^4$  combinations). Pioneering efforts to combinatorially mutagenize the chromosome of *Escherichia coli* have driven home the message that it is essential to comprehensively explore this potential diversity to identify unexpected synergistic effects (40, 41). An assortment of methods for efficient combinatorial mutagenesis will therefore be needed to fully bring the power of directed evolution and other library optimization approaches to bear on metabolic engineering and synthetic biology problems.

We view Reiterative Recombination as part of an advancing wave of pioneering technologies for effecting large-scale modifications to the genome. One strategy is de novo genome synthesis, which allows complete customization of the genome. However, in spite of recent heroic feats in this area (16, 47) and the falling price of chemical DNA synthesis (48), such ambitious undertakings are neither technically nor economically feasible for most researchers. Alternatively, a second strategy is reprogramming well characterized host organisms, such as *E. coli* and *S. cerevisiae*, for designer functions through genetic engineering. This approach will require tools for incorporating two basic classes of genetic modifications: (i) the alteration of genes already in the genome and (ii) the introduction of multiple exogenous genes into the chromosome. To meet the first of these needs, altering the genetic background, classic mutagenesis techniques such as mutator strains can be useful for phenotypic optimization, but they do not provide control over the extent and location of mutations (49), unlike several notable, recently reported techniques. Exploiting sexual reproduction in *S. cerevisiae*, Suzuki et al. have used iterative cycles of mating and sporulation along with a quantitative GFP marker to gather up to 16 deletion mutations in an individual strain (50). Using an *E. coli* strain engineered with the  $\lambda$  Red recombination system (36), multiplex automated genome engineering automates the transformation of mutagenic single-stranded oligonucleotides  $\sim 90$  bases in length to create a powerful method for introducing specified deletions, point mutations, and very short insertions of  $\leq 30$  base pairs anywhere in the chromosome, but it has not been shown to be capable of efficient whole-gene insertion in its current form (see Note.) (40). Reiterative Recombination is one of several techniques that tackle the second issue, integrating exogenous pathways of genes into the chromosome (18–20). However, Reiterative Recombination is uniquely able to integrate pathways in a highly efficient manner to access large numbers of variant strains.

In conclusion, we foresee Reiterative Recombination becoming a powerful addition to the 21st-century molecular biology toolkit. Our method's simplicity and robustness will make it a user-friendly option for assembling seamless multigene constructs by any lab equipped for basic molecular biology. Reiterative Recombination's cyclical format means that it can be used to build pathways of indefinite length. Because our system is highly efficient, in contrast to other in vivo DNA assembly technologies, it can be used to assemble libraries of at least  $10^4$  pathways directly in the chromosome. Reiterative Recombination, as part of the expanding arsenal of cutting-edge cell engineering tools, will ensure the continued rapid development of synthetic biology as the scale of our ambitions increases and our applications move into the cell.

## Materials and Methods

**General Methods.** Standard methods for molecular biology in *S. cerevisiae* and *E. coli* were used (51, 52). Complete methods and additional details are provided in *SI Text*.

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**Fragment Design.** For clarity, a “fragment” refers to the total pathway-specific region of each donor plasmid, shown in orange in the figures. When convenient, fragments were divided into “subfragments” that were PCR amplified from different templates and assembled into the full fragment by plasmid gap repair upon transformation into yeast. Fragments contain 30 bp of homology to the donor plasmid and 20 bp of homology to the adjacent fragments, providing a total of 40 bp of homology for each integration event (Fig. S4). Overlapping ends of subfragments (within fragments) contained a total of 40 bp of homology. All regions of homology were incorporated with PCR primers. Illustrative examples are provided in the *Table S2*.

**Reiterative Recombination Protocol.** The protocol for an even-numbered round (e.g., round 2) of Reiterative Recombination is described. Descriptions of strains and plasmids are provided in *SI Text*, and plasmid maps are provided in *Fig. S5*.

1. Preparation of subfragments: Subfragments were amplified with primers that added appropriate homology to adjacent fragments and to the donor plasmid (see above and *SI Text*). All PCR products were gel purified.
2. Transformation: The PCR products were cotransformed with the digested donor plasmid (pLW2593) in a 100:1 molar ratio into the cured round 1 strain. Transformants were selected on synthetic complete media lacking leucine and uracil [SC(-Leucine, -Uracil)].
3. Induction: After 2 d of growth, transformants were lifted from the transformation plates, washed once with sterile water, resuspended in preinduction media [SC(Lactate, -Leucine, -Uracil)] to an OD<sub>600</sub> of 1, and shaken at 30 °C for 3 h. Cells were then harvested, washed once with sterile water, and resuspended in induction media [SC(-Uracil, 2% galactose, 2% raffinose)] to an OD<sub>600</sub> of 0.1. Cells were shaken at 30 °C for 12 h.
4. Selection: For control experiments, aliquots of the induction cultures were immediately plated on selective media [SC(-Histidine)] to determine the efficiency of marker switching. Colonies were counted after 2 d of growth. The remaining cells were inoculated into SC(-Histidine) liquid media, shaken at 30 °C for 1 d, and plated on SC(-Histidine, 0.1% FOA) to cure recombinants of the donor plasmid.
5. Reiteration: To begin the next round of Reiterative Recombination, after 2 d of growth, a single cured colony (in the reporter proof-of-principle experiment) or the pool of recombinants (in the lycopene pathway experiments) was lifted from the SC(-Histidine, 0.1% FOA) plates and inoculated into SC(-Histidine) liquid media to begin an overnight culture for the next transformation.

For odd rounds of Reiterative Recombination, pLW2592 was used as the donor plasmid, and the use of histidine and leucine in dropout media was reversed. All other aspects of the protocol remained the same. For library experiments, sufficient cells were carried through each step to ensure at least threefold coverage of the library.

**Note.** While this paper was under review, Isaacs, et al. reported that multiplex automated genome engineering could be used to insert double-stranded DNA encoding a selectable marker gene into the *E. coli* genome, but with efficiencies ranging from  $10^{-5}$  to  $10^{-7}$ , vs. efficiencies of  $>10^{-1}$  for almost all loci tested for point mutations introduced by single-stranded oligonucleotides (ref. 53).

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