

Recombineering: a homologous recombination-based method of genetic engineering

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Recombineering is an efficient method of *in vivo* genetic engineering applicable to chromosomal as well as episomal replicons in *Escherichia coli*. This method circumvents the need for most standard *in vitro* cloning techniques. Recombineering allows construction of DNA molecules with precise junctions without constraints being imposed by restriction enzyme site location. Bacteriophage homologous recombination proteins catalyze these recombineering reactions using double- and single-stranded linear DNA substrates, so-called targeting constructs, introduced by electroporation. Gene knockouts, deletions and point mutations are readily made, gene tags can be inserted and regions of bacterial artificial chromosomes or the *E. coli* genome can be subcloned by gene retrieval using recombineering. Most of these constructs can be made within about 1 week's time.

INTRODUCTION

Recombineering is an *in vivo* method of genetic engineering used primarily in *Escherichia coli* that uses short 50 base homologies^{1–5}. As recombineering is based on homologous recombination, it allows insertion, deletion or alteration of any sequence precisely and is not dependent on the location of restriction sites (Fig. 1). Linear DNAs, either double-stranded (ds), usually in the form of PCR products^{3,6–8}, or single-stranded (ss) synthetic oligonucleotides^{2,9} are introduced by electroporation and provide the homologous substrates (i.e., targeting constructs) to create genetic changes. Recombineering is catalyzed by bacteriophage-encoded homologous recombination functions, such as the coliphage λ Red system³ and the RecET system from the *Rac* prophage^{4,10}.

This protocol will emphasize modification of bacterial artificial chromosomes (BACs) and multicopy plasmids but the procedures described are generally applicable to other replicons. A basic knowledge of molecular and microbiological techniques is required to execute the recombineering protocols described here. These basic techniques are described in detail by Ausubel *et al.*¹¹. Recombineering protocols for manipulation of the bacterial and phage chromosomes are described elsewhere^{12,13}. Recombineering can also be used to modify episomal DNAs such as the low-copy plasmid derivatives of P1 and F that carry artificial chromosomes and are called PAC (P1 artificial chromosome)¹⁴ and BAC^{15,16}, respectively. Although multicopy plasmids may be the ideal choice of vector when the insert size is relatively small (up to 50 kb), PACs, which accommodate inserts of 50–100 kb, and BACs, which allow inserts of > 100 kb, are used for cloning large genomic fragments. A BAC is the vector of choice for cloning and manipulating large DNA fragments. BACs may contain genomic segments that include all of the extragenic *cis*-regulatory elements (promoter, terminator and enhancers) of a gene of interest. BACs are therefore ideal for generating transgenic mice because the insert size may allow expression of the cloned gene under the control of its own regulatory elements, mimicking the endogenous expression pattern. In addition, because of the ease of obtaining desirable BAC clones coupled with simple DNA purification protocols, BACs are widely used in gene mapping and functional studies, analysis of regulatory elements and expression of a transgene under the control of a heterologous promoter¹⁷. They are also used for genetic analysis of mutations that have been identified in human diseases^{18,19}. BACs are modified by recombineering in *E. coli*, and these modified constructs can then be used to generate knockout and knock-in mouse models using embryonic stem cell technology^{20,21}.

The recombineering protocols described here use the bacteriophage λ Red system that includes the phage recombination genes *gam*, *bet* and *exo*. The *gam* gene function, Gam, prevents an *E. coli* nuclease, RecBCD, from degrading linear DNA fragments^{22,23}, thus

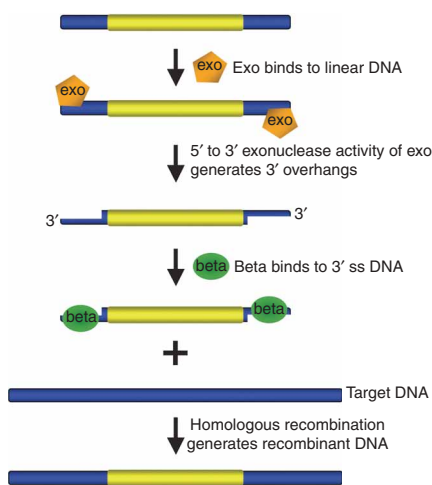


Figure 1 | Overview of bacteriophage λ recombination system used for recombineering. Exo has a 5'- to 3'-dsDNA exonuclease activity, which can generate 3'-overhangs on linear DNA. Beta binds the single-stranded DNA (3'-overhangs), promotes ss-annealing and generates recombinant DNA. An additional protein, Gam (not shown here), which prevents RecBCD nuclease from degrading double-stranded linear DNA fragments, is also required for dsDNA recombineering.

allowing preservation of transformed linear DNA *in vivo*. The *bet* gene product, Beta, is an ssDNA-binding protein that promotes annealing of two complementary DNA molecules^{24–26}, and the *exo* gene product, Exo, has a 5′ to 3′ dsDNA exonuclease activity^{27,28}. Working together, these latter two proteins insert linear DNA at the desired target, creating genetic recombinants (Fig. 1)^{29,30}. For dsDNA, Red Exo is thought to degrade from both 5′-ends, exposing ssDNA that is bound by Red Beta. Use of the phage λ Red system for *in vivo* genetic engineering was pioneered by Murphy *et al.*^{6,7}, who demonstrated dsDNA recombination with the Red functions expressed from the *lac* promoter, both on a multicopy plasmid and as an insertion on the *E. coli* chromosome. Murphy *et al.*^{6,7} used linear substrate DNA targeting homologies that were greater than 1 kb. Zhang *et al.*⁴ demonstrated that the phage RecET system catalyzes recombination using targeting homologies of only 40–60 bp. This short-length requirement allowed the homologies to be incorporated into PCR primers, substantially advancing the technology. The λ Red system was also shown to act on short homologies³. Ellis *et al.*² demonstrated that the λ Beta protein promotes efficient *in vivo* recombination with ssDNA, provided as 70-mer ss-oligos. Models for how recombineering occurs³¹ propose that the single-strand regions of the incoming linear DNA bound by the Beta protein are annealed to complementary single-strand gaps arising at the replication fork during DNA replication. Consistent with this model, an oligo able to anneal to the discontinuously replicated lagging strand gives a higher recombination frequency than its complementary ‘leading strand’ oligo².

When engineering DNA *in vivo*, it is important to provide a brief high-level pulse of the phage recombination proteins. Limited expression minimizes the toxic effects of the Gam protein³² and minimizes undesirable genome rearrangements between repetitive DNA sequences. Although the phage recombination functions have been produced from multicopy plasmids under control of the IPTG-inducible *lac* promoter^{6,7}, the *lac* promoter has a high basal level and requires the *lacIQ* repressor gene *in cis* for tight regulation. A better choice is the arabinose-inducible *pBAD* promoter^{33,34}. In *Ara*⁺ strains, the *pBAD* promoter can be tightly repressed by addition of glucose to the growth medium; however, in *ara* mutants, glucose-mediated repression is less effective, even with the *araC* gene on the plasmid³⁵. When the *pBAD* plasmids are used for recombineering³⁴, glucose is not added during cell growth and arabinose is usually added at least a generation before the cells are made competent for electroporation. This procedure, although convenient for induction, does not yield the tightest possible repression³⁵. In contrast, expression from the λ prophage system is based on the endogenous λ regulatory system, which is the natural method for expression of these recombination functions. The most recent studies on λ (see refs. 36,37) show that its repression system is uniquely strong: the λ repressor binds cooperatively at the three operator sites present at both the pL and pR promoters, and these two sets of repressor-bound operators interact with each other by protein–protein-mediated looping between pL and pR to generate a handcuff of 12 repressor proteins. To ensure this same tight repression during recombineering, both sets of operators are present on all of the prophage constructs, whether they are in the bacterial chromosome or on low-copy plasmids, and a temperature-sensitive repressor is expressed from the *cI857* gene. At low temperatures (30–34 °C), the repressor is active and the recombination genes are not expressed. When the temperature of

the bacterial culture is shifted to 42 °C for 15 min, the repressor is rapidly inactivated, and the recombination genes are expressed at high levels from the powerful λpL promoter. Repressor is renatured and tight repression is restored by lowering the temperature after 15 min. The short induction time minimizes adventitious recombination and cellular stress^{32,38}. An advantage of the natural phage system is that the λ repressor is autoregulated and, thus, better controlled than when recombination genes on multicopy plasmids are expressed from heterologous promoters, which are often leaky, causing unwanted expression and side effects. Red recombination does not require the *E. coli* RecA function³, and when recombineering is performed in a strain with a *recA* mutation, all extraneous homologous recombination is prevented.

Owing to its high efficiency and short homology requirements, recombineering can be used for a wide range of applications (Fig. 2). Recombineering can be used to insert selectable or nonselectable markers in plasmids, bacterial chromosomal DNA or BACs. It can be used to generate gene-targeting constructs to be used for making knockout or knock-in alleles in embryonic stem cells. This technology is also suitable for generating transgenic reporter constructs using BACs to express *lacZ*, fusion tags (e.g., *GFP*, tandem affinity, FLAG, HIS and so on), site-specific recombinases (e.g., *Cre*, *Flp*), selectable markers (e.g., neomycin resistance gene) or any cDNA under the control of a tissue-specific promoter or to make fusion proteins. It can also be used to generate subtle alterations in BACs or bacterial chromosomal DNA without the use of any selectable marker or site-specific recombination system.

In spite of being a very tractable and versatile technology, recombineering has limitations. For example, as very short regions of homology are sufficient for the recombination, manipulating regions containing repetitive sequences can be problematic. As most recombineering-based methods use PCR-amplified products as substrate DNA, the recombinant products may occasionally acquire mutations. However, confirming the integrity of the recombinant target DNA by sequencing helps to discard such products. Another limitation of recombineering is that the sequence of the target region must be known. However, because the entire genome of many organisms has been sequenced, this is not a limitation for most commonly used organisms.

Experimental design

Each recombineering experiment involves the following six steps, which are illustrated in the general flowchart (Fig. 2).

- (1) Generation of the appropriate linear targeting substrate DNA
- (2) Provision of the λ Red recombination genes
- (3) Induction of the λ recombination genes
- (4) Preparation of electrocompetent cells and electroporation of the linear targeting substrate DNA
- (5) Outgrowth following electroporation
- (6) Identification and confirmation of the recombinant clones.

Substrate DNA design and generation. Depending upon the application, different DNA substrates are required (Fig. 2). The creation of appropriate double-stranded and single-strand linear targeting substrates for specific applications is described below and their construction is detailed in Step 3 of PROCEDURE.

Double-stranded DNA recombination. Linear dsDNA substrates for recombineering consist of a region to be inserted flanked by two homology arms. The inserted region may be either a selectable

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marker or a nonselectable DNA that is used to replace a counter-selectable marker.

Recombineering can be used to insert a selectable marker (drug resistance gene or a prototrophic marker) into the bacterial chromosome or any episomal DNA. This is done by using linear DNA containing the desired selectable marker flanked by 50 bases of homology to the target site (Figs. 1 and 3). Such substrates can be made with the PCR by amplifying the selectable markers using a pair of chimeric primers, each about 70 bases in length. Each primer will have 50 bases at the 5'-end corresponding to the region to be targeted to provide homology and ~20 bases at the 3'-end to prime amplification of the selectable marker as shown in Figure 3. Although flanking 50 base homologies are more than sufficient for recombination, longer homologies of 150–200 bases have been used to further improve the targeting efficiency; however, this substantially increases the time and effort required^{6,21}. To use longer homologies, each homology arm is amplified independently by PCR and directionally cloned into a plasmid so as to flank the selectable marker already present in that plasmid. High-fidelity *Taq* DNA polymerase with proofreading ability (such as Invitrogen High Fidelity Platinum *Taq* or Roche Expand High Fidelity) is used for generating dsDNA PCR products for recombineering substrates; for confirming constructs, standard *Taq* polymerase can be used. Try not to use supercoiled DNA as template for PCR amplification, as residual intact plasmid will give a high background of transformants, making it difficult to identify actual recombinants. The TKC strain (Table 1) contains the three genes for antibiotic resistance to tetracycline, kanamycin and chloramphenicol, as well as their

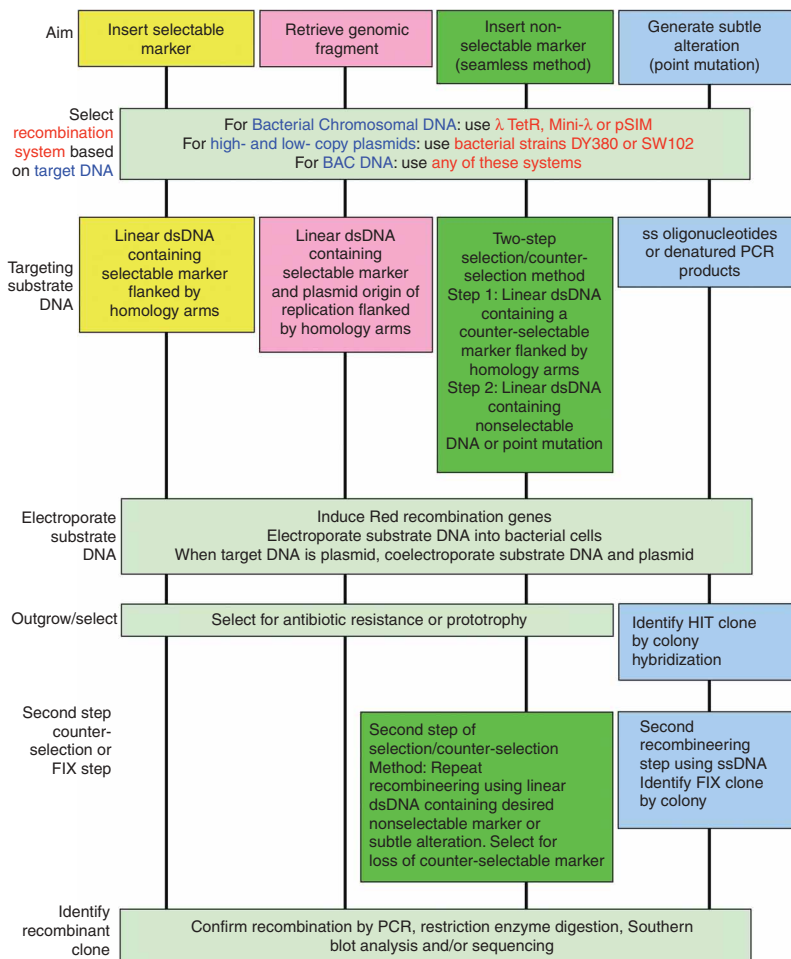
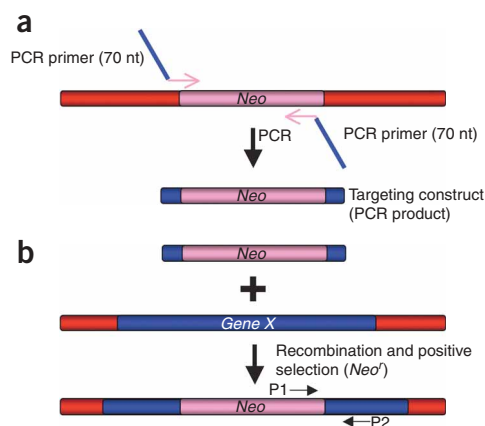


Figure 2 | A flowchart of recombineering procedures. Schematic representation of various steps involved in recombineering. An appropriate system should be selected on the basis of the choice of target DNA. The type of substrate DNA depends upon the choice of method used. The outgrowth procedures and methods to identify the recombinant clone are based on the use a selectable marker, selection/counter-selection method or lack of any selectable marker in the substrate DNA.



regulatory elements. This strain can be used to amplify any of these markers with the colony PCR technique using the primer pairs specified in Table 2 (see option A in Step 3 of PROCEDURE, Fig. 3a). Plasmid templates with selectable markers flanked by *loxP* and *FRT* sites are available through the NCI recombineering website (<http://recombineering.ncifcrf.gov/>; see option B in Step 3 of PROCEDURE, Fig. 3a). Drug cassettes with dual promoters that allow the use of the same selectable marker (e.g., neomycin, hygromycin and blasticidin) in both bacterial and mammalian cells are also available.

Figure 3 | Insertion of a selectable marker by recombineering. (a) Targeting construct can be generated by PCR to introduce the region of homology (in blue) and a selectable marker (e.g., *Neo*, kanamycin/neomycin-resistance gene). The PCR primers used to generate the targeting construct are 70-mer oligonucleotides with 50 nucleotides corresponding to the target site (e.g., *Gene X*, in blue) sequence to introduce the homology arm and 20 bases from the ends of the selectable marker (*Neo*, in pink). (b) The targeting construct is electroporated into the bacterial cells that are induced to express the phage recombination genes. Recombinant clones are selected as kanamycin-resistant colonies in this case and confirmed with PCR using test primers P1 and P2.

TABLE 1 | Bacterial strains used in recombineering.

Strain	Primary use	Relevant genotype	Reference	Available from
<i>For introduction of BACs</i>				
DH10B	Parent strain	<i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74 recA1 endA1 araD139 Δ(<i>ara, leu</i>)7697 <i>galU pgl</i>Δ8 <i>rpsL nupG</i></i>	55	Invitrogen
DY380	BAC recombineering with dsDNA	DH10B λ (<i>cI857ind1</i>) Δ {(<i>cro-bioA</i>) < > <i>tetRA</i> } (<i>TetR</i>) <i>gal490</i>	44	Court lab (court@ncifcrf.gov)
EL250	BAC recombineering: for removing selectable markers flanked by <i>frt</i> sites	DY380 (<i>cro-bioA</i>) < > <i>araC-PBAD Flpe</i>	44	The NCI recombineering website
EL350	BAC recombineering: for removing selectable markers flanked by <i>loxP</i> sites	DY380 (<i>cro-bioA</i>) < > <i>araC-PBAD Cre</i>	44	The NCI recombineering website
SW102	BAC recombineering: <i>galK</i> counter-selection	DY380 Δ <i>galK</i>	40	The NCI recombineering website
SW105	BAC recombineering: <i>galK</i> counter-selection	EL250 Δ <i>galK</i>	40	The NCI recombineering website
SW106	BAC recombineering: <i>galK</i> counter-selection	EL350 Δ <i>galK</i>	40	The NCI recombineering website
<i>For plasmid propagation and recombineering</i>				
DH5 α	Plasmid isolation	ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (<i>rk-, mk+</i>) <i>phoA supE44 thi-1 gyrA96 relA1</i>	56	Invitrogen
HME70	Plasmid recombineering: MMR-deficient strain for high-efficiency oligo recombination	W3110 Δ (<i>argF-lac</i>)U169 <i>galKtyr145UAG mutS</i> < > <i>cat</i> Δ (<i>srlA-recA</i>) :: <i>Tn10</i> { λ <i>cI857</i> Δ (<i>cro-bioA</i>)}	50	Court lab (court@ncifcrf.gov)
HME71	Plasmid recombineering	W3110 Δ (<i>argF-lac</i>)U169 <i>galKtyr145UAG</i> Δ (<i>srlA-recA</i>) :: <i>Tn10</i> { λ <i>cI857</i> Δ (<i>cro-bioA</i>)}	50	Court lab (court@ncifcrf.gov)
<i>Drug cassette amplification</i>				
TKC	Template for tetracycline, kanamycin and chloramphenicol	<i>tetA, cat, kan</i>		Court lab (court@ncifcrf.gov)
LE392	For propagation of λ Tet phage	<i>e14- glnV44 supF58</i> (<i>lacY1</i> or Δ <i>lacZY</i>) <i>galK2 galT22 metB1 trpR55 hsdR514</i> (<i>rK-mK+</i>)	NIH Strain Collection	Court lab (court@ncifcrf.gov)
DH10B-containing BACs		DH10B-containing BAC libraries		BACPAC Resources Center (BPRC)

Other strains for *E. coli* recombineering are listed elsewhere^{12,13}.

A nonselectable DNA fragment (e.g., a reporter gene, such as *lacZ*, a transgene, such as *cre*, *flp* or *GFP*, or a HIS-tag) can also be efficiently inserted into a genomic region using recombineering. There are two approaches to achieve this. The first approach is a

two-step selection/counter-selection method that allows insertion of the desired DNA without leaving other undesirable modifications, in a 'seamless' event (Fig. 4a). Generally, a counter-selectable cassette is first inserted at the site to be changed; this cassette is then

TABLE 2 | PCR primers and possible source of template for drug cassette amplification^a.

Gene cassette	Source	Primer sequence	Annealing temperature (°C)	Size (kb)
Ampicillin (<i>amp</i>)	pBluescript SK(+) (Stratagene)	5'-CATTCAAATATGTATCCGCTC-3' 5'-AGAGTTGGTAGCTCTTGATC-3'	53	1.2
Chloramphenicol (<i>cat</i>)	pPCR-Script Cam (Stratagene)	5'-TGTGACGGAAGATCACTTCG-3' 5'-ACCAGCAATAGACATAAGCG-3'	53	0.86
Kanamycin (<i>kan</i>)	<i>Tn5</i>	5'-TATGGACAGCAAGCGAACC-3' 5'-TCAGAAGAAGCACTCGTCAAGAAG-3'	55	0.95
Tetracycline (<i>tetA</i>)	<i>Tn10</i>	5'-TCCTAATTTTTGTTGACACTCTA-3' 5'-CTCTGGGTTATCAAGAGGG-3'	55	1.34

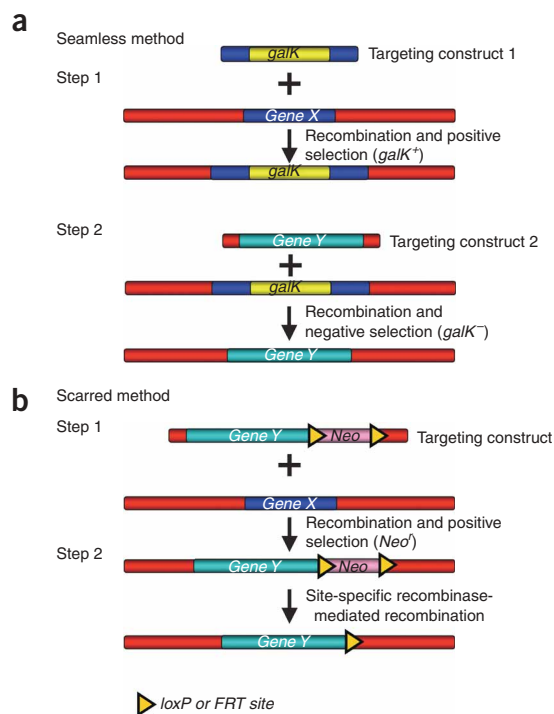
^aWhen amplified with these primer pairs, all drug cassettes will contain a promoter and all but *kan* will have a transcriptional terminator.



PROTOCOL

Figure 4 | Insertion of a nonselectable DNA fragment by recombineering.

(a) ‘Seamless’ method to insert nonselectable DNA fragment makes use of selectable markers that can be used for positive as well as negative selection (e.g., *galK*). In this two-step method, first the selectable *galK* marker is targeted to the site (*Gene X*) where the nonselectable DNA fragment (*Gene Y*) is to be inserted. In the second step, a targeting construct containing the nonselectable DNA fragment flanked by the same 50 bp of homology to the target site is electroporated into Gal⁺ bacterial cells containing the recombinant DNA from Step 1. Clones in which the *Gene Y* DNA fragment is correctly targeted are counter-selected for loss of the *galK* gene. (b) The scarred method: this method targets both the selected (*Neo*) and nonselected (*Gene Y*) DNAs jointly. In Step 1, the nonselectable DNA fragment (*Gene Y*) is introduced along with a selectable marker, *Neo*, which is flanked by *loxP* or *FRT* sites. Recombinants are selected for the presence of *Neo*. In Step 2, *Neo* is deleted by site-specific recombinase-mediated recombination (Cre for *loxP* sites and Flp for *FRT* sites). Unlike the ‘seamless’ method, a single *loxP* or *FRT* site is retained after recombination.



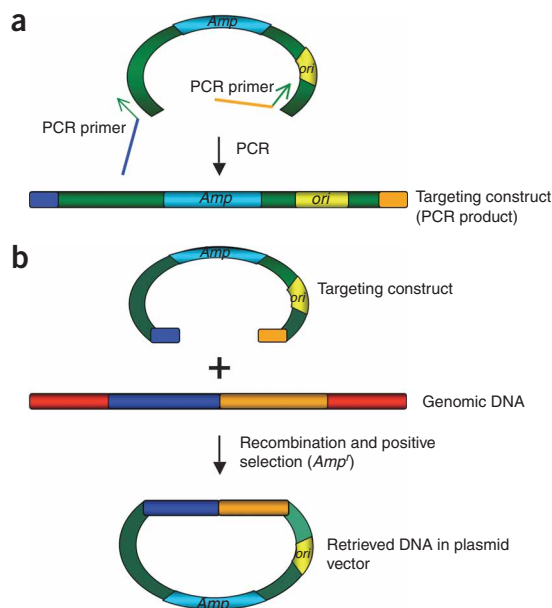
replaced with the desired alteration in a second recombineering reaction. In all such procedures, each step can be selected and the final construct will not have a drug marker or other genetic scar. A number of counter-selections are available: these include the *sacB*³⁹ gene linked with a drug marker, such as *cat* or *kan*, the *galK* gene⁴⁰, *rpsL*⁴¹, *thyA*⁴² and *tolC*⁴³. In many situations, such as when using *galK*, the same gene can be selected both for and against (Fig. 4a). The *galK* counter-selection has been optimized for use with BACs and uses strains that have been deleted for the *galK* gene at its normal chromosome location; such strains include SW102, SW105 and SW106. A detailed protocol for the *galK* counter-selection has been published⁴⁰. For convenience, the strains and plasmids used in the *galK* selection are listed in Tables 1 and 3. In the second approach, a selectable marker is linked with a nonselectable DNA fragment and the two are inserted together (Fig. 4b). If subsequent removal of the selectable marker is desired, it can be flanked with *loxP* or *FRT* sites, thus allowing excision by expression of the Cre or Flp proteins⁴⁴. Template drug cassettes with flanking *loxP* and *FRT* sites that can be used for PCR amplification are also available from the National Cancer Institute recombineering website. As a single *loxP* or *FRT* site remains after the genetic manipulation, we refer to this as the ‘scarred’ method (Fig. 4b).

Recombineering is now routinely used as a subcloning technique. Whereas classical methods of subcloning using restriction enzymes and DNA ligase allow manipulation of small DNA fragments, recombineering allows precise retrieval of even a large DNA segment from a genomic fragment in a BAC or from the bacterial chromosome^{21,44}. Recombineering allows the junctions of the subcloned fragment to be exactly determined on the basis of need rather than on chance location of restriction sites. For rapid subcloning by recombineering, the small retrieval vectors are generated with PCR using a pair of primers, each with 50 base homologies at the 5′-ends corresponding to the region to be retrieved and ~20 bases of plasmid sequence for PCR amplification (Fig. 5). As mentioned above, 150–200 bp homology arms can

TABLE 3 | Mini-λs and plasmids.

Mini-λ or plasmid	Relevant genotype	Antibiotics concentration (μg ml ⁻¹ of media)	Source or reference
<i>Primary use: express red functions for recombineering</i>			
Mini-λ Kan (kanamycin)	Contained in DH10B	30	31
Mini-λ Tet (tetracycline)	Contained in DH10B	12.5	31
Mini-λ Cat (chloramphenicol)	Contained in DH10B	12.5	31
Mini-λ Amp (ampicillin)	Contained in DH10B	30	31
pSIM5	pSC101ts gam exo bet chloramphenicol resistance (CmR)	12.5	45
pSIM6	pSC101ts gam exo bet ampicillin resistance (AmpR)	100	45
pSIM7	pBBR1 gam exo bet chloramphenicol resistance (CmR)	12.5	45
pSIM8	pBBR1 gam exo bet ampicillin resistance (AmpR)	100	45
pSIM9	pRK2 gam exo bet chloramphenicol resistance (CmR)	12.5	45
pSIM17	pSC101ts gam exo bet blasticidin resistance (BsdR)	50	45
pSIM18	pSC101ts gam exo bet hygromycin resistance (HygR)	50	45
pSIM19	pSC101ts gam exo bet spectinomycin resistance (SpecR)	100	45
<i>Primary use: amplify galK for counter-selection</i>			
pgalK	pBluescript SK- pEm7-galK ampicillin resistance (AmpR)	100	40

Figure 5 | Subcloning DNA fragments from genomic DNA. **(a)** To subclone or retrieve a genomic DNA fragment, generate a targeting construct by PCR. Each primer consists of 50 bases from the end of the genomic DNA that needs to be subcloned (blue and orange) and 20 bases from the plasmid sequence (in green) flanking the region of the origin of replication (*ori*) and an antibiotic resistance marker (*Amp*, ampicillin resistance gene). A linear plasmid DNA is used as template for PCR. **(b)** The linear targeting construct (PCR product) is electroporated into the bacterial cells that are induced to express the phage recombination genes. Recombination between the targeting construct and the genomic DNA results in the formation of a circular plasmid by gap repair. The circular plasmid contains the desired DNA fragment.



be cloned to improve retrieval efficiency if necessary²¹. A plasmid template must be used when amplifying the linear retrieval vector backbone, usually consisting of an origin of DNA replication and a selectable marker (option C in Step 3 of PROCEDURE, Fig. 5a). When the linear retrieval vector contains a selectable marker, however, a potential side reaction of end-joining can occur, generating a circular plasmid carrying only the selectable marker⁵. This is avoided by using a retrieval vector that contains only the origin of replication; in this approach, the selectable marker is first inserted adjacent to the target DNA to be retrieved. See Datta *et al.*⁴⁵ for details. The linear targeting construct recombines with the target DNA and retrieves the desired fragment by gap repair. This results in the generation of a circular plasmid (Fig. 5b). When gap repair is used for retrieving DNA, it is not necessary to know the DNA sequence of the entire piece to be retrieved, only that of the flanking region used as targeting homology. When the length of the fragment to be retrieved is less than 15 kb, a high-copy plasmid (e.g., pBluescript) can be used, but a lower-copy plasmid (e.g., pBR322) should be used for larger fragments⁴⁴. In a variation of this method known as ‘*in vivo* cloning’²⁵, a linear DNA to be incorporated onto the plasmid, often generated by PCR, is coelectroporated with the linear plasmid. DNA can also be transferred by retrieval from one BAC to another as has been described^{46,47}.

Single-strand DNA recombination. Recombineering can also be performed using synthetic oligonucleotides (see Step 2 in PROCEDURE) or short denatured PCR products^{2,8,9,48}. These oligos (ss-oligos) or ssDNAs can be used to create single base changes, insert or substitute short DNA sequences and generate deletions. Under optimal conditions, creation of small changes with recombineering using ssDNA is highly efficient and often completely eliminates the requirement for any selection. Two different variables contribute to creation of these optimal conditions. One variable is use of a ‘lagging-strand’ oligo, i.e., a single strand corresponding in sequence to the DNA chain that is replicated discontinuously^{2,9}. Which of the two complementary oligos corresponds to the lagging-strand oligo will depend on the direction of DNA replication through the region of the chromosome or episome to be modified, and often it is easier to just try both strands; one should recombine with a 20- to 30-fold higher efficiency than the other. The other important variable includes avoidance of the *E. coli* methyl-directed mismatch repair (MMR) system: preventing MMR increases the effective recombination frequency about 100-fold^{9,49}. As strains mutant for the MMR system acquire adventitious mutations, procedures that avoid the use of MMR mutants have been devised. The simplest of these tricks is to use an oligo that creates a C–C mismatch when annealed to the target DNA, as the

MMR system does not recognize and remove this particular mismatch. This method has limited utility, however, and other more generally useful two-step procedures are illustrated below.

A ‘hit and fix’ two-step recombineering approach⁴⁸, in combination with screening by colony hybridization, can be used to generate and find subtle changes in BAC DNA (Fig. 6). This approach is particularly useful for modifying DNA regions with repetitive motifs, which can be difficult to screen by PCR. This method uses short (~180 bp) dsDNA fragments generated by PCR that can be denatured to provide ss-oligonucleotide substrates for recombineering. In the first ‘hit’ step, a stretch of 20 nucleotides is changed, including the nucleotide(s) to be mutated in the final construct. The altered sequence is designed to contain recognition sites for a restriction enzyme. In the second ‘fix’ step, the bases that were modified in the first step are restored to the original sequence, with the exception of the desired mutation(s). As a run of nucleotides is changed at each step, the recombinant BACs can be detected by colony hybridization using a primer specific for the altered bases. Colony hybridization allows thousands of individual colonies to be screened at once. The presence or absence of the restriction site provides an additional tool to rapidly confirm both the ‘hit’ and ‘fix’ steps. The central 20-bp region can be the same for all ‘hit’ PCR products, allowing one 20 base universal probe to be used to identify the inserts at the ‘hit’ step. See **Box 1** for generation of ‘hit and fix’ substrates and Step 19D of PROCEDURE for identifying recombinants by the ‘hit and fix’ method.

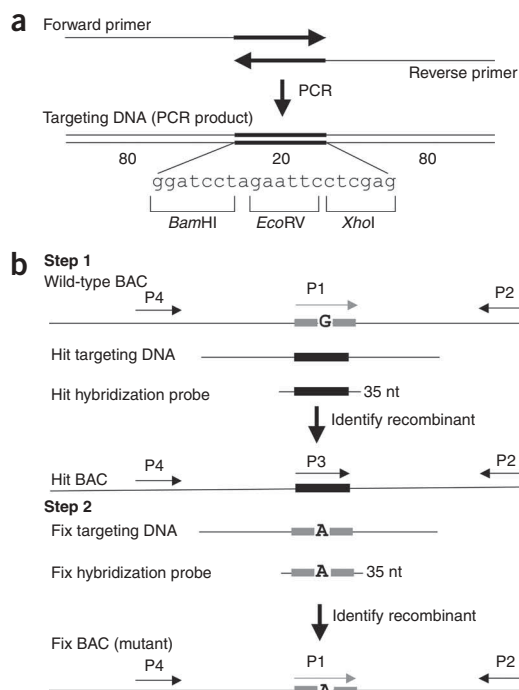
Oligonucleotides can also be used to precisely delete unwanted regions of DNA by designing a 70-mer lagging strand oligo that spans the portion to be deleted, with 35 bases of homology at either side. The efficiency of deleting large segments is lower than that of engineering small single-base changes, and a selection or screen will be needed to identify recombinants^{2,8,9,50}. As an example, a counter-selection can be used in combination with an oligo to generate deletions when needed. Alternatively, colony hybridization, using a 20-base probe spanning the deletion with ten bases on each side, can be used to identify recombinants.

Modifying multicopy plasmids. Recombineering is useful for making point mutations and small changes on plasmids^{3,5,50}. For inserting or deleting larger pieces of DNA without a selection, classical cloning methods may sometimes be easier, but the precision of making junctions is usually lost. The plasmid to be engineered can be introduced into the Red-expressing cells by co-electroporation with the linear DNA after the recombination functions are induced; alternatively, it may be resident in the recombineering host. Coelectroporation of the plasmid with the modifying DNA, as described in Step 16 of PROCEDURE, is often preferable, as it helps control the number of plasmids present at the time of recombineering and may minimize opportunities for plasmid multimer formation. Recombineering will occur only in cells receiving the plasmid, so when introducing the plasmid by coelectroporation with the linear substrate, enough plasmid molecules must be added to obtain a high transformation frequency, but not so high as to introduce large numbers of plasmid molecules per cell. This is generally about 10 ng of plasmid DNA per electroporation, but the appropriate amount of plasmid DNA to add may have to be determined empirically by generation of a transformation efficiency curve, as the transformation efficiency of plasmids may differ (see **Box 2**). Ideally, each cell should receive about one plasmid molecule. If the plasmid is very large and has a low copy number, coelectroporation may not be practical; in this case, a resident plasmid should be used for modification.

The intracellular copy number of commonly used plasmids such as pUC derivatives may exceed 500 copies per cell⁵¹. Such a high copy number means that most copies will not be altered during a recombineering reaction, and cells that contain recombinant plasmid molecules will also contain many unmodified parental molecules. To create pure clonal populations of the recombinant, it is necessary to separate the recombinant class from the unmodified class; this is done by isolating the plasmid DNA after recombineering and retransforming it at a low concentration of less than

Figure 6 | Two-step ‘hit and fix’ method to generate subtle mutations using ss short PCR product or oligonucleotides as targeting vector. **(a)** The ss oligonucleotides containing 160 bases of homology and 20 unique bases are generated by using two 100-mer oligonucleotides in a PCR. The two 100-mer oligonucleotides have 20 complementary bases (in this case, the 20 bp contains restriction sites *Bam*HI, *Eco*RV and *Xho*I) at the 3′-end. The 180 bp PCR product can be denatured to obtain ss oligonucleotides that can be used as targeting construct. **(b)** Schematic representation of the two steps involved in

‘hit and fix’ method to generate subtle alterations (e.g., G to A) without the use of a selectable marker. In Step 1, a 180-mer ss oligonucleotide is used to replace 20 nucleotides (gray box) around the target site with 20 heterologous nucleotides (black box). Recombinants can be identified by colony hybridization using an end-labeled 35-mer oligonucleotide that can specifically anneal only to the recombinant DNA. A primer set specific for the heterologous ‘hit’ sequence (P3 and P2) can be used to confirm the presence of recombinant clones by PCR. A second primer set (P1 and P2) can be used as a control to amplify only the nonrecombinant DNA. Generation of a correct recombinant clone can be confirmed by digesting with *Bam*HI, *Eco*RV or *Xho*I the PCR product (approximately 300–500 bp) of primers P2 and P4. In Step 2, the 20 nucleotides are restored to the original sequence, except for the desired mutation. Such clones can be identified by colony hybridization using a 35-mer oligonucleotide as probe and further confirmed by PCR amplification using primers P1 and P2, by testing for loss of the restriction sites inserted in Step 1, by digesting the PCR product of primers P2 and P4 and by sequencing.



one molecule per cell. If plasmid DNA has been deleted during the recombineering, often the plasmid mixture can be digested with a restriction enzyme that cuts only the parental plasmid species but not the desired recombinant, thus enriching for the recombinant class.

When making changes on plasmids, it is important to begin the procedure with a pure monomer plasmid species. By the same token, it is also important to use a host defective in RecA-mediated homologous recombination to prevent plasmid multimer formation by host recombination pathways. However, it has been observed^{13,50} that when plasmids are modified with recombineering,

BOX 1 | GENERATION OF ‘HIT’ AND ‘FIX’ PCR PRODUCTS

1. Synthesize a linear dsDNA cassette with PCR using two 100-mer oligonucleotide primers that overlap by 20 nucleotides (nt) at their 3′-ends, designing the primers so that the resulting DNA contains 80-nt homology arms flanking a 20-nt heterologous region. For each change to be created, the ‘fix’ DNA should contain the same homologies as the corresponding ‘hit’ DNA. Only the central region of the two PCR products will vary. The exact sequence of the ‘hit’ 20-nt central region is not critical as long as it is different from the wild-type sequence(s) and contains a restriction site not present in the flanking homology; however, we recommend using our universal 20 base sequence: 5′-GGATCCTAGAATTCTCGAG-3′.
2. For the ‘fix’ DNA, replace the central 20-bp sequence with the endogenous sequence but include the desired mutational change(s).
3. Verify the size of the PCR products (180 bp) by agarose gel electrophoresis and purify them with a PCR clean-up kit.

BOX 2 | DETERMINATION OF PLASMID DNA TRANSFORMATION EFFICIENCY ● TIMING 1 D

1. Make electrocompetent cells as described in the protocol (PROCEDURE Steps 9–15), adding a different amount of DNA to each of several electroporations (suggested range is 0.1, 1, 10 and 100 ng).
2. Outgrow the electroporation mixes for 2 h and plate tenfold serial dilutions on Petri plates, selecting for the plasmid drug resistance. Use a range of dilutions to obtain countable number of colonies for each transformation.
3. The next day, count colonies and generate a semilog graph of the number of transformants (ordinate) versus the amount of DNA added (abscissa).
4. From the graph, determine the minimum amount of DNA necessary to achieve maximal transformation efficiency (i.e., without adding excess DNA), and use this amount of DNA for each recombinering reaction.

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some larger multimeric circles of these plasmids arise. As these multimers form during and are dependent upon the recombinering reaction, they cannot be totally eliminated. There is anecdotal evidence that other replicons, including BACs, may also form larger-order multimers during recombinering. Ways to deal with these multimers are suggested in the Troubleshooting section.

Provision of the λ Red recombination genes. There are several ways to provide the prophage system that will express the phage recombination proteins in the bacterial cells where recombinering is performed (Fig. 6). All variations allow highly efficient recombination, and the choice of which to use will depend on the target DNA (see Fig. 2). When high- or low-copy plasmids have to be manipulated, bacterial strains containing the prophage recombination system on the chromosome (e.g., DY380 and its derivatives, such as SW102) should be used. When the bacterial chromosome or BAC DNA is modified, mobile recombinering systems, such as the pSIM vectors, mini- λ or the replication-defective λ phage

(λ TetR), can be introduced into the bacterial cells to be engineered (Fig. 7a–d). BAC DNA can also be transformed into bacterial strains containing the repressed recombination system. In all cases, it is advisable to check the integrity of the BAC DNA by restriction analysis before proceeding with the recombinering procedure. The individual options are discussed in more detail below.

Bacterial strains with defective prophage; DY380 and derivatives. A number of bacterial strains have been generated that harbor a defective λ prophage that is stably situated in the bacterial chromosome (see Step 4A of PROCEDURE, Fig. 7b and Table 1). Such host strains are ideal for manipulation of plasmid DNA, which can be introduced into these bacterial strains. Another major advantage of using the strains containing the integrated λ prophage is that a drug selection need not be applied to maintain the recombinering system. Strains routinely used for BACs include DY380⁴⁴, SW102 and their derivatives⁴⁰. To use these strains, the BAC or plasmid to be modified must be introduced into the recombinering strain by transformation (see Box 3). A mini-prep method described below

Figure 7 | Schematic representation of λ phage constructs used for recombinering. (a) The λ prophage with some of its genes is shown integrated in the bacterial chromosome. It is adjacent to the biotin gene *bioAB*. Bacterial DNA is shown as a blue bar; phage DNA is shown as a red bar; and a region containing a deletion and/or a substitution is shown as a white bar in b–d. The complete λ prophage is flanked by its attachment sites *att*, where integration occurred. The *int* and *xis* genes located in the *pL* operon encode functions to integrate and excise the phage DNA into and out of the bacterial chromosome. Two operons with their promoters *pL* and *pR* are indicated. Transcription of the promoters is controlled by the λ CI857 repressor. This repressor is temperature sensitive in that it is active and represses the promoter at 30–32 °C but is inactive at 42 °C allowing for transcription. The N protein functions as a transcription anti-terminator and prevents RNA polymerase termination of *pL* transcripts at terminators *tL1*, *tL2* and *tL3*. The red genes *exo*, *bet* and *gam* encoding the homologous recombination functions are also in the *pL* operon. The *kil* gene is adjacent to *gam* and, when expressed for over 1 h, kills the bacterial cell³². The replication genes *O* and *P* are in the *pR* operon. Cro functions as a partial repressor of the *pL* and *pR* operons when CI is inactive at 42 °C. The lysis and structural genes, *SRA–J*, are shown located beyond their regulator *Q*. The λ TetR phage used for recombinering is identical to the above complete prophage with the exception of four changes. The replication gene *P* has an amber mutation, the *cro* gene also has an amber mutation, and the *tetA* gene encoding tetracycline resistance replaces *rex*. (b) The defective prophages in DY380, SW102 and the HME strains have the lytic *pR* operon deleted from *cro* through the *bioA* genes as shown in brackets. The right *att* site is also deleted, preventing any excision of this prophage. In DY380 and SW102, *cro* through *bioA* is replaced by the tetracycline resistance cassette, *tetRA*. (c) The mini- λ phage DNA is shown with the lytic genes *cro* through *J* deleted. In different constructs of the mini λ , the *cro–J* region is replaced with various drug-resistant cassettes. Mini- λ has both *att* sites plus *int* and *xis*, which allows for its integration and excision. (d) The λ segment on the pSIM plasmids is shown. The plasmid backbone is not shown. The genes from *cro* to *att* are removed as well as genes beyond *tL3*, including *int* and *xis*. The red genes are connected directly to *pL* by a deletion that removes *kil* through *N*. The drug-resistant marker characteristic of each SIM plasmid replaces the *rex* gene adjacent to *CI857*. The basic features are conserved in all of these Red expression constructs. Red is left under the native phage-controlling elements for optimal expression and regulation. The *pL* promoter drives gene expression and is controlled by the temperature-sensitive but renaturable λ CI857 repressor. In all, the *cro* gene is inactive to maximize *pL* expression, and the replication genes are absent or inactive to prevent lethal effects on the cell.



BOX 3 | ELECTROPORATION OF BAC DNA INTO BACTERIAL STRAIN OF CHOICE ● TIMING 1 D

1. Pick an isolated colony from an LB plate and grow overnight in 3–5 ml of LB at 32 °C.
2. Next morning, add 0.5 ml of the culture to 25 ml of LB in a 250-ml flask and grow at 32 °C to an OD₆₀₀ of 0.50–0.60. Transfer the culture to a 50-ml Oak Ridge tube and spin at 6,000g in prechilled rotor for 10 min at 4 °C.
3. Wash the cell pellet with 20 ml of ice-cold H₂O once and then resuspend in 1 ml of H₂O and transfer to a chilled 1.5-ml tube. Spin at 10,000g for 20–30 s at 4 °C.
4. Wash the cells two more times with 1 ml of ice cold H₂O. Resuspend the cell pellet in H₂O in a final volume of 100 µl and keep on ice.
5. Mix 100 ng of BAC DNA with 50 µl of electrocompetent cells and chill on ice for 5 min then transfer into a 0.1-cm cuvette. Introduce the BAC DNA into the cells by electroporation (1.8 kV, 25 µF capacitance and 200 Ω resistance). Also do a control electroporation where no DNA is added. After electroporation, immediately add 1 ml of LB and transfer the cells to a standard sterile culture tube. Incubate cells at 32 °C for 1 h. Spin down cells for 20–30 s in a microcentrifuge. Resuspend the pellets in 200 µl of LB. Plate each aliquot of cells on a single LB plate containing chloramphenicol (12.5 µg ml⁻¹) and incubate for 20–22 h at 32 °C. Only the BAC DNA electroporation should have colonies.
6. Pick 5–10 individual colonies and grow in 3 ml of LB containing 12.5 µg ml⁻¹ chloramphenicol. Extract BAC DNA and perform restriction digestion with common enzymes like *EcoRI*, *BamHI*, *PstI*, *HindIII*, *EcoRV*. Run the sample on 0.8% agarose gel. Compare the restriction pattern with that of the original BAC DNA.

(see **Box 4**) works well for rapid extraction of BAC DNA and produces DNA that is suitable for restriction digestion, PCR analysis and sequencing⁵². For targeting both BACs and any multicopy plasmids, it is important to use *recA* mutant strains such as these.

Mobile recombineering systems; mini-λ and pSIM plasmids. There are mobile recombineering systems available that can be introduced into any bacterial strain to allow manipulation of the BAC or chromosomal DNA. Such mobile elements include ‘mini-λ’⁵³ and the plasmids collectively called the pSIM vectors⁴⁵ (see Steps 4B and 4C of PROCEDURE, respectively). Both mini-λ and the pSIM plasmids provide the same endogenous phage-controlling elements and Red recombination functions present in the above-mentioned defective prophage strains (see **Fig. 7c,d**); however, these must be introduced into the BAC-containing strain. Mini-λ consists of a defective nonreplicating, circular phage DNA, which, once introduced into the strain, integrates into the bacterial chromosome using site-specific recombination⁵³. The integrants are selected by the presence of the antibiotic resistance gene carried by the mini-λ. Once integrated, mini-λ is stable, replicates as part of the host chromosome and does not require drug selection for maintenance. The mini-λ includes the *pL* operon from which are expressed the *int* and the *xis* genes that catalyze the site-specific integration/excision events, as well as the *exo*, *bet* and *gam* recombination genes for recombineering (**Fig. 7c**). In addition to being easily

introduced into any bacterial cell, the mini-λ can be readily excised to cure the cells of the phage DNA. The excised mini-λ DNA circle can also be purified from the induced bacterial cells using a standard plasmid purification protocol. The pSIM vectors⁴⁵ consist of the elements of the prophage necessary for recombineering (see **Fig. 7d**) carried on a number of different plasmid origins. Especially useful is a pSC101 plasmid derivative with temperature-sensitive DNA replication; this plasmid has a low copy number and the *ts* replicon allows loss of the plasmid after recombineering is completed. These plasmids are available with a variety of selectable antibiotic resistance markers (**Table 3**). Unlike the prophage and the mini-λ, they require drug selection for stable maintenance.

λ phage carrying tetracycline resistance gene; λTetR. A further means of expressing the Red system is from a λ phage carrying a tetracycline resistance gene²⁰ (see Step 4D of PROCEDURE). This phage system combines the advantages of stability and mobility. The genotype of this phage is λ *cI857 ind1 rexAB<> tetAR cro^{TYR26am} P_{GLN59am}* and it is referred to here as λTetR (see **Fig. 7a**). The λTetR is easy to prepare as a high titer phage lysate; using this lysate, lysogens are readily created by infection of the desired host and TetR selection. The efficiency of lysogeny is greater than that of plasmid transformation, and once introduced into the BAC-containing strain, the prophage is very stable and drug selection is no longer required.

BOX 4 | SMALL-SCALE MINIPREP FOR BAC DNA PREPARATION ● TIMING 1.5 H

1. Inoculate a single colony of DH10B cells containing the BAC into 3 ml of LB containing chloramphenicol (12.5 µg ml⁻¹) and grow overnight at 32 °C (because of the presence of the Red system) in a shaking incubator at 200–250 r.p.m.
2. Next day, pellet 1.5 ml of culture by centrifugation in a 1.5-ml tube at 10,000g at room temperature (20–25 °C) for 1 min.
3. Add 100 µl of chilled alkaline lysis solution I. Resuspend by pipetting the cells up and down using the pipette tip. Place on ice for 5 min.
4. Add 200 µl of freshly prepared alkaline lysis solution II. Gently invert the tubes until the lysate is clear. Place on ice for 5 min.
5. Add 150 µl of alkaline lysis solution III and mix gently until a white precipitate appears. Place on ice for 5 min. Centrifuge the tubes at 10,000g for 5 min at 4 °C and then transfer the supernatant to a clean 1.5-ml tube.
6. Add two volumes of 95% ethanol to the supernatant. Mix and place on ice for 30 min. Centrifuge at 10,000g for 10 min at 4 °C. Discard supernatant and wash the pellet with 70% ethanol. Air-dry the pellet and resuspend in 30–50 µl of 1× TE (10 mM Tris-HCl, pH 8.0; 1.0 mM EDTA).

Induction of the λ recombination genes. The Red system is induced by incubating the mid-log bacterial culture in a 42 °C water bath shaking at 200 r.p.m. for 15 min as detailed in Steps 6–8 of PROCEDURE. It is important to note that air shakers are not satisfactory for this purpose because the heat transfer is less efficient with an air shaker. Immediately after the heat pulse, the cells should be placed in an ice-water slurry for quick chilling. Failure to rapidly chill the cells may result in premature decay of the recombination activity. Once the cells are induced for the Red functions, it is best to complete the procedure promptly.

Preparation of electrocompetent cells and electroporation of the linear targeting substrate DNA (see Steps 9–18 of PROCEDURE). The chilled cells are gently washed twice with cold sterile water to remove salts that will cause problems during electroporation. The washed cells are suspended in a small volume of water and mixed with the linear DNA substrate, then aliquoted into chilled electroporation cuvettes. Luria broth (LB) is added immediately after electroporation to maximize recovery of viable cells.

MATERIALS
REAGENTS

- One of the following is required to express the Red functions (see Experimental design for further details):
 - Bacterial strain containing defective λ prophage (see Table 1)
 - Mini- λ DNA (see Table 3)
 - pSIM plasmid (see Table 3)
 - λ TetR phage with titer of at least 10^9 plaque-forming units μl^{-1}
 - Ampicillin (A0166, Sigma)
 - Kanamycin (K0254, Sigma)
 - Chloramphenicol (C7795, Sigma)
 - Tetracycline (T7660, Sigma)
 - Hygromycin (H3274, Sigma)
 - Sterile distilled H₂O, chilled on ice
 - Minimal salts solution, such as M9
 - Plasmid DNA isolation reagents (12163, Qiagen maxiprep kit; 27104, Qiagen miniprep kit)
 - PCR purification kit (28104, Qiagen)
 - Gel extraction kit to purify DNA from agarose gels (28604, Qiagen)
 - High-fidelity Taq polymerase with proofreading ability (11304-029, Invitrogen)
 - Standard Taq polymerase (11342-020, Invitrogen)
 - dNTP mixture, 10 mM each, PCR grade, (10297-018, Invitrogen)
 - Agarose (SeaKem LE, ISC Bioexpress)
 - Restriction enzymes (New England Biolabs)
 - Glycerol (BP229-1, Fisher Scientific)
 - Chimeric primers for PCR amplification of recombinering substrates, 25 pmol μl^{-1} in H₂O (Integrated DNA Technologies)
 - Bacto-tryptone (Difco)
 - Yeast extract (Difco)
 - One of the following sources of pure linear DNA for recombinering (see Experimental design for further details), suspended in sterile H₂O:
 - Purified PCR product with 50 bp of homology to the target, ~ 100 ng μl^{-1}
 - 70-mer ss-oligo (salt-free but otherwise unpurified) with desired changes(s) at center and with ~ 35 bases flanking homology, 0.5 pmol μl^{-1} (Integrated DNA Technologies)
 - Plasmid backbone with flanking homology to DNA to be retrieved, ~ 100 ng μl^{-1}
 - Lysozyme (Roche Molecular Biochemicals)
- EQUIPMENT**
- Constant temperature bacterial incubator set at 30–34 °C (2005 Low temp Incubator, VWR)
 - Two shaking H₂O baths (200 r.p.m.) set at 30–32 °C and at 42 °C (G-76, New Brunswick Scientific)
 - Spectrophotometer and cuvettes (DU 530, Beckman-Coulter)
 - Electroporator (Genepulser II with Pulse Controller II, Bio-Rad)
 - Electroporation cuvettes with 0.1-cm gap (870582, Bio-Rad), labeled and prechilled

Outgrowth following electroporation. The cells are fragile after electroporation and require a minimal 30-min recovery period in LB before plating. An outgrowth period may also be needed to allow expression of cloned genes, such as those encoding antibiotic resistance. The details of the outgrowth procedure will vary according to the type of recombinering reaction performed and how the recombinants will be identified. The various types of outgrowth are described in Step 19A–D of PROCEDURE.

Identification and confirmation of the recombinant clones.

Drug-resistant recombinants are first selected using the appropriate antibiotic resistance and then analyzed with PCR to verify that the insertion has gone to the proper location. In certain cases, such as for multicopy plasmids, restriction analysis may be used to confirm the recombinant clones. Southern blots and sequencing may be used to analyze BAC or genomic recombinants. When a point mutation or other subtle change is made, DNA sequencing must be used to confirm correctness of recombinants. Recombinant identification is described in Steps 19A–D of PROCEDURE.

- Floor model low speed centrifuge at 4 °C (Avanti, J-25I, Beckman)
- Refrigerated microcentrifuge at 4 °C (Micromax, RF, IEC)
- Thermal cycler and accessories for PCR (MyCycler, Bio-Rad)
- Agarose gel electrophoresis apparatus (Bio-Rad)
- Sterile 125- and 250-ml Erlenmeyer flasks, preferably baffled (2540-00125 and 2540-00250 respectively, Bellco)
- Sterile 35- to 50-ml centrifuge tubes (03530, Thermo Scientific)
- 1.5-ml microfuge tubes (022363204, Eppendorf)
- 0.2-ml flat cap PCR tubes (TFI0201, Bio-Rad)
- Insulated ice buckets (35754-100, VWR)
- Sterile glass culture tubes (16 mm \times 150 mm) for overnight growth of bacterial cultures (89000-506, VWR)
- Stainless steel caps for culture tubes (60825-882, VWR)
- Pipettors of various volumes (Gilson) with aerosol-resistant sterile tips
- Petri plates, 100 mm \times 15 mm (25834-302, VWR)
- Computerized DNA analysis program (Gene Construction Kit, Textco Biosoftware; Vector NTI, Invitrogen)

REAGENT SETUP

M9 medium Mix 3.0 g of KH₂PO₄, 12.8 g of Na₂HPO₄ · 7H₂O, 1.0 g of NH₄Cl, 0.5 g of NaCl and 1 liter of distilled H₂O

LB Mix 10 g of Bacto-tryptone, 5 g of yeast extract, 5 g NaCl and 1 liter H₂O, pH 7.2. **▲ CRITICAL** Both M9 and LB should be autoclaved for sterility and stored at room temperature (20–25 °C).

Alkaline lysis solution I 25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA, pH 8.0, 2.5 mg/ml of Lysozyme and 100 $\mu\text{g}/\text{ml}$ RNase A

Alkaline lysis solution II 0.2 N NaOH and 1% Sarkosyl

Alkaline lysis solution III 5 M Potassium acetate, pH 4.8

EQUIPMENT SETUP

LB and Petri plates LB and Petri plates (100 mm \times 15 mm) containing 1.5% (wt/vol) Difco agar, with antibiotics as needed (see Table 4)

TABLE 4 | Antibiotics and concentrations ($\mu\text{g ml}^{-1}$) used in media for selection.

Antibiotic	Single copy ^a	Multicopy plasmids
Ampicillin	30	100
Kanamycin	30	50
Chloramphenicol	10	20
Tetracycline	12.5	25
Hygromycin	Not used in single copy	50
Spectinomycin	100	100
Blastocidin	50	50

^aUse these concentrations for drug markers on BACs or the *E. coli* chromosome and for single-copy plasmids.



PROCEDURE

Design and generation of linear DNA substrate for recombineering

1| Before beginning recombineering, precisely design your desired DNA sequence *in silico*. This is greatly facilitated by use of computerized DNA analysis programs, such as Gene Construction Kit (Textco Software) or Vector NTI (Invitrogen).

▲ **CRITICAL STEP** Design the desired molecule on the computer before ordering primers!

2| From the *in silico* design of the new DNA molecule, generate the sequence(s) of required primers or 70-mer ss-oligos (see Experimental design) and order or synthesize them as salt-free and otherwise unpurified.

▲ **CRITICAL STEP** Be sure to include the 50 bases of flanking homology to the 5'-ends of your primers (see Experimental design).

▲ **CRITICAL STEP** ss-oligos can be directly used as substrates for recombineering after appropriate dilution.

3| To generate dsDNA substrates for recombineering by PCR, different templates can be used as follows: option A for bacterial DNA (**Fig. 3a**), option B for plasmid DNA (**Fig. 3a**) and option C for plasmid origin amplification (**Fig. 5a**).

(A) PCR-amplification of dsDNA substrate for recombineering from bacterial DNA ● TIMING 3–4 h

(i) Antibiotic resistance genes containing tetracycline, kanamycin and chloramphenicol cassettes can be amplified from the TKC strain using 'colony PCR' and primer pairs listed in **Table 2**. To perform colony PCR, prepare a PCR without allowing any volume for the template DNA; for a typical 50- μ l PCR, using the high-fidelity Platinum *Taq* kit from Invitrogen, mix the following in a 0.2-ml PCR tube using sterile technique (for multiple PCRs, prepare a master mix and dispense aliquots as appropriate):

- 5 μ l of 10 \times PCR buffer
- 2 μ l of 50 mM MgSO₄
- 1 μ l of 10 mM dNTP mixture
- 1 μ l of each primer (25 pmol μ l⁻¹ in H₂O)
- 0.5 μ l of platinum *Taq* high fidelity
- 39.5 μ l of sterile distilled H₂O

(ii) Lightly touch a fresh colony of the *E. coli* strain with a sterile inoculating loop and mix cells into the PCR.

(iii) Use the following PCR program suitable for amplifying any of the drug cassettes referred to in **Table 2** (using optimal conditions for Invitrogen Platinum *Taq* high fidelity):

- Initial denaturation: 94 °C, 5 min
- Subsequent denaturation: 94 °C, 30 s
- Anneal primers: 53 °C, 30 s
- Extension: 68 °C, 1.5 min
- Repeat the last three steps 29 times
- Final extension: 68 °C, 10 min
- Hold at 4 °C

(iv) Visualize the PCR on an agarose gel as described elsewhere¹¹.

(v) Remove salt and clean up the PCR product by ethanol precipitation as described elsewhere¹¹ or by using a commercially available kit and following the manufacturer's instructions.

(vi) Suspend the product in small volume of H₂O so as to have a concentration of ~100 ng μ l⁻¹.

(B) PCR-amplification of dsDNA substrate for recombineering from plasmid DNA ● TIMING 3–4 h

(i) Plasmid templates must be linearized before PCR amplification. Digest plasmid DNA with a restriction enzyme at sites present in the vector sequence but absent in the sequence to be amplified¹¹.

(ii) Run an aliquot of the DNA on an agarose gel to verify complete digestion¹¹.

▲ **CRITICAL STEP** Be aware that super-coiled plasmid migrates at ~70% of the size of a full-length linear species.

(iii) If necessary, cut the band containing the linearized DNA from the gel and extract the DNA¹¹.

(iv) Resuspend the linear DNA in 20–30 μ l TE (pH 8.0), check the concentration using a spectrophotometer (read absorbance at 260 nm) and dilute to 0.5–1.0 ng μ l⁻¹. A purified fragment containing just the drug cassette can be stored and reused as template.

(v) Amplify the drug cassette with a pair of chimeric primers, using PCR conditions based on information in **Table 2** and a *Taq* DNA Polymerase with proofreading ability.

(vi) Examine the PCR product by agarose gel electrophoresis¹¹.

(vii) Gel-purify the desired band¹¹ to eliminate contamination of supercoiled plasmid.

(viii) If linear plasmid DNA was used as template and the product was not gel-purified, digest the PCR with 3–5 U of the modification-dependent restriction enzyme *DpnI* per μ g DNA (at 37 °C for 2 h), which will cut the plasmid DNA but not the unmodified PCR product. *DpnI* digestion may not totally eliminate plasmid background, however.

▲ **CRITICAL STEP** Do not expose the PCR product to direct UV light, which may damage it and result in abnormal recombination frequencies.



(ix) Clean up the PCR product as described in Step 3A(v).

▲ **CRITICAL STEP** Do not use supercoiled DNA as template for PCR amplification. To avoid having to gel-purify the DNA or treat with *DpnI*, use the TKC strain as a template when possible.

(C) Generation of dsDNA substrate for recombineering by PCR amplification of plasmid origin for gene retrieval

● **TIMING 3–4 h**

- (i) Digest the plasmid with one or more restriction enzyme(s) that do not cut within the region to be amplified.
- (ii) Amplify the origin and selectable marker from the linear plasmid by PCR using the primers generated in Step 2 (reaction conditions will need to be established empirically). Use the least amount of linear plasmid possible for the PCR template, to minimize residual uncut plasmid.
- (iii) Digest the completed PCR with *DpnI* to further remove the template plasmid as in Step 3B(viii).
- (iv) Purify the PCR product to remove salt before proceeding (as in Step 3A(v)). The amplified product will be a linear plasmid with flanking homology to either side of the region to be rescued from the chromosome. It is possible to avoid the potential side reaction of plasmid end-joining by using an alternative approach: amplify a retrieval vector containing only the origin of replication, after first inserting a selectable marker different than the one on the starting plasmid next to the target DNA to be retrieved⁴⁵. See **Box 1** for generation of ‘hit’ and ‘fix’ PCR products.

? **TROUBLESHOOTING**

Provision of the λ Red recombination genes

4| Provide the λ recombination genes from the defective prophage present in DY380 and its derivatives (option A), the mini-λ (option B), a pSIM plasmid (option C) or the replication-defective λTet phage (option D).

▲ **CRITICAL STEP** It is critical to realize that in all cases, the Red genes are under control of a temperature-sensitive repressor, and strains containing them should always be propagated at a low temperature (30–32 °C) except during induction of the Red system.

(A) Provision of the λ Red recombination genes from DY380 and derivatives

- (i) DY380 and derivatives already contain the Red functions and so require no further preparation before using for recombineering. See **Box 3** for electroporation of BAC DNA into bacterial strain of choice and **Box 4** for small-scale miniprep for BAC DNA preparation.

(B) Provision of the λ Red recombination genes from Mini-λ ● TIMING 2 d

- (i) To prepare the mini-λ DNA, inoculate a single colony of *E. coli* with an integrated mini-λ prophage into 125 ml of LB containing appropriate antibiotics in a one-liter flask and culture overnight at 30–32 °C.
- (ii) The next day, incubate the culture at 42 °C for 15 min to induce excision of the mini-λ DNA circles.
- (iii) Chill the culture on ice for 15 min and extract the mini-λ DNA using a standard plasmid midi-prep purification kit (e.g., Qiagen) as described previously⁵³.
- (iv) Introduce the mini-λ into the desired *E. coli* strain that will be used for recombineering by mixing 1 μl of mini-λ DNA (25–35 ng) with 25 μl of electrocompetent bacterial cells and introduce the DNA by electroporation. Also do a control electroporation of cells alone without added DNA.
- (v) Grow the transformed cells at 32 °C for 1 hour in LB medium and plate dilutions on an LB agar plate containing the appropriate antibiotic to select the strain with mini-λ (see **Table 4**, use antibiotic concentration indicated for single-copy plasmids). Incubate overnight at 32 °C.
- (vi) Pick isolated colonies for recombineering and test for the presence of the prophage by confirming that the strain is temperature sensitive for colony formation on antibiotic plates at 42 °C.

(C) Provision of the λ Red recombination genes from pSIM plasmids ● TIMING 1 d

- (i) Introduce the pSIM plasmid DNA into the bacterial strain where recombineering will be performed by selecting for the appropriate antibiotic resistance of the plasmid and plating the cells at 30–32 °C (see **Table 4**, use antibiotic concentration indicated for multicopy plasmids).
- (ii) Purify a single colony 30–32 °C and grow an overnight culture from it with the appropriate antibiotic using methods described elsewhere¹¹.

(D) Provision of the λ Red recombination genes from replication-defective λTet phage ● TIMING 1 d

- (i) Grow a 1-ml culture of the appropriate bacterial strain that will be used for recombineering overnight in LB, adding chloramphenicol (10 μg ml⁻¹) if a BAC is present.
- (ii) The next day, pellet the cells by centrifuging at 10,000g at 20–25 °C for 1 min in a microfuge and suspend them in 0.1 ml of 10 mM MgSO₄.
- (iii) Add 1 μl of high titer phage stock (at least 10⁹ ml⁻¹) to the cells and incubate at 32 °C for 20 min.
- (iv) Add 1 ml of LB and incubate for 1 h at 30–32 °C, then plate a series of tenfold dilutions on LB-Tet plates (tetracycline concentration: 12.5 μg ml⁻¹) and incubate at 30–32 °C overnight.
- (v) Purify tetracycline-resistant colonies by streaking the bacteria and picking isolated colonies on LB-Tet plates.

? **TROUBLESHOOTING**

PROTOCOL

5| Inoculate the bacterial strain containing the Red functions from a single colony into 3–5 ml of LB medium. Incubate the culture at 32 °C overnight with aeration.

▲ **CRITICAL STEP** Maintain antibiotic selection for the BAC (chloramphenicol 10 µg ml⁻¹), and if a pSIM plasmid is used, also include the antibiotic for the pSIM (see **Table 4**, use antibiotic concentration indicated for multicopy plasmids).

■ **PAUSE POINT** Once the recombineering strains are created, they can be frozen and stored indefinitely for future use at –70 °C as a glycerol stock prepared by mixing 1 ml of a freshly grown overnight culture with 1 ml of 50% glycerol (dilute glycerol in water (vol/vol) and autoclave).

Induction of the λ recombination genes ● **TIMING 3 h**

6| Add 0.5 ml of the overnight culture to 35 ml of LB medium in a 250-ml baffled Erlenmeyer flask. Supplement with appropriate antibiotic to maintain plasmids, if needed. Use antibiotic concentration indicated in **Table 4**.

▲ **CRITICAL STEP** Dilute the overnight culture by at least 70-fold.

7| Place the flask in the 32 °C H₂O bath and grow cells with shaking for about 2 h or until the cells reach an OD₆₀₀ of 0.4–0.6.

▲ **CRITICAL STEP** The growth time may vary with different strains. The cells are ready when the OD₆₀₀ is 0.4–0.6. It is important not to overgrow the cells, as stationary-phase cells are not optimal for recombineering.

8| Transfer half of the culture to a 125-ml Erlenmeyer flask and place the flask to shake in a 42 °C H₂O bath; keep the other flask at 32 °C. Shake for 15 min at 200–220 r.p.m. The culture at 42 °C is induced for recombination functions and the 32 °C culture is the uninduced control.

? **TROUBLESHOOTING**

Preparation of electrocompetent cells and electroporation of the linear targeting substrate DNA ● **TIMING 2 h**

9| Immediately after the 15-min induction, rapidly chill both cultures in an ice-water slurry; swirl the flasks gently. Leave on ice for 5–10 min. Label and chill two 35- to 50-ml centrifuge tubes for each set of induced and uninduced cells.

10| Transfer both the induced and uninduced cultures to the centrifuge tubes and centrifuge at 4,600g (6,700 r.p.m. in a Sorvall SA-600 rotor) for 7 min at 4 °C. Using sterile technique, aspirate or pour off supernatant.

11| Add 1 ml of ice-cold sterile distilled H₂O to the cell pellet and gently suspend cells with a large pipette tip (do not vortex). Add another 30 ml of ice-cold distilled H₂O to each tube, seal and gently invert to mix, again without vortexing. Centrifuge tubes again as mentioned in Step 10.

12| Promptly decant the 30-ml supernatant very carefully from the soft pellet in each tube and gently suspend each cell pellet in 1 ml of ice-cold distilled H₂O.

▲ **CRITICAL STEP** Remove tubes from the centrifuge promptly at the end of spin. The pellet is very soft and care should be taken not to dislodge it or lose the cells, especially when processing multiple tubes. If necessary, leave a little supernatant in the tube.

? **TROUBLESHOOTING**

13| Transfer the suspended cells to pre-chilled microcentrifuge tubes. Centrifuge at 10,000g in refrigerated microcentrifuge for 30 s at 4 °C. Carefully aspirate the supernatant and suspend cells in 1 ml of ice-cold H₂O, centrifuge again and aspirate supernatant, being extremely careful with the pellet.

14| Suspend the cell pellet in 200 µl of sterile cold distilled H₂O and keep on ice until used.

▲ **CRITICAL STEP** For highest efficiency, use freshly processed cells.

15| To introduce the linear DNA recombineering substrate (from Step 3) into the electrocompetent cells (from Step 14) by electroporation, chill the desired number of labeled 0.1-cm electroporation cuvettes on ice. Turn on the electroporator and set to 1.80 kV, 25 µF capacitance and 200 Ω resistance.

? **TROUBLESHOOTING**

16| In labeled microcentrifuge tubes on ice, mix 1 µl of DNA from Step 3 (~100 ng of salt-free PCR fragment or 0.5 pmol (~100 ng) ss-oligo) with 50 µl of electrocompetent induced or uninduced cells from Step 14. When modifying a multicopy plasmid introduced by coelectroporation, also add 1 µl of the multicopy plasmid DNA (~20 ng µl⁻¹) at this point. Include the following controls:

- Induced cells without DNA. If colonies are present on this control plate, either the selection is not working properly or the cells have a high reversion frequency for the property selected.
- Uninduced cells plus DNA. This is a control to estimate the number of background colonies due to some contaminating factor in the DNA, such as intact plasmid template from a PCR.

▲ **CRITICAL STEP** Never leave the DNA–cell mixes on ice for more than ~10 min.

? **TROUBLESHOOTING**

17| Transfer the DNA–cell mixes to chilled electroporation cuvettes as quickly as possible and introduce the DNA into the cells by electroporation (according to settings outlined in Step 15).

▲ **CRITICAL STEP** Make sure that the time constant is greater than 5 ms for optimal results. Lower time constants generally indicate impurities or salts in the cells or the DNA.

? **TROUBLESHOOTING**

18| Immediately after electroporation, add 1 ml of LB medium to the cuvette. Do this before proceeding to the next electroporation. After the electroporations are completed, transfer the electroporation mixes in LB to sterile culture tubes and incubate with shaking at 32 °C for a minimum of 30 min.

▲ **CRITICAL STEP** Failure to allow 30 min outgrowth gives poor cell viability.

Outgrowth following electroporation, identification and confirmation of the recombinant clones ● **TIMING 1 d to 2 weeks**

19| The outgrowth and screening procedures will vary according to the type of recombinering reaction: use option A to select recombinant clones by antibiotic resistance or prototrophy, option B to select recombinant clones containing a genomic fragment subcloned by recombinering, option C to select modified multicopy plasmids and option D to screen ‘hit and fix’ recombinants by colony hybridization.

(A) Outgrowth and selection of recombinant clones by antibiotic resistance selection or prototrophy ● **TIMING 2 d**

- (i) Continue outgrowth in 1 ml of LB without any antibiotics for at least 2 h at 30–32 °C to allow for expression of the antibiotic resistance or metabolic gene to be selected.
- (ii) Following the outgrowth, make six 1:10 serial dilutions of the experimental cultures in a buffered medium lacking a carbon source, such as M9 salts.
- (iii) To select recombinants, spread 0.1 ml of the undiluted culture and the 10⁻¹ and 10⁻² dilutions on plates selective for the recombinant at 30–32 °C. Also assay total viable cells by plating 0.1 ml of the 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilutions on LB; determining this viable cell count will allow calculation of a recombinant frequency. If the number of viable cells is too low (less than 10⁷ ml⁻¹), recombinants may not be found. For the control cultures, both the uninduced (32 °C) and the induced (42 °C) to which no DNA was added, plate 0.1 ml of the undiluted culture on a single selective plate.

▲ **CRITICAL STEP** Do not add chloramphenicol for BAC maintenance while outgrowing or selecting the recombinant on plates.
- (iv) Incubate plates until colonies appear (generally 22–24 h for rich plates at 32 °C). Generally no or only a few colonies are obtained on the control plates, indicating that most of the colonies on the experimentally induced plus DNA plates represent true recombinant clones.
- (v) Once potential recombinant clones are identified, confirm the construct by PCR analysis, restriction digestion analysis and sequencing where necessary. For confirming insertion of a dsDNA, such as a drug cassette, use two pairs of primers, each pair having one primer outside the targeted flanking region and one primer in the inserted DNA, and amplify the two junctions. Another PCR, using the two outside flanking primers, should also be performed to confirm the absence of the gene to be removed, thus ruling out the possibility of a duplication event⁵⁴.

? **TROUBLESHOOTING**

(B) Outgrowth and selection of recombinant colonies containing genomic fragments retrieved onto a plasmid by subcloning using recombinering: ● **TIMING 2 d**

- (i) Following electroporation, add the 1 ml cell mixture to 9 ml of LB medium and grow the culture overnight nonselectively at 32 °C.
- (ii) The next day, isolate plasmid DNA using a standard miniprep protocol. It is not necessary to visualize this DNA by agarose gel electrophoresis.
- (iii) Introduce the plasmid DNA into a high-efficiency cloning strain by transformation, selecting the drug resistance of the plasmid or the retrieved selectable marker. Use a low concentration of DNA to minimize uptake of multiple plasmids into the same cell.
- (iv) Purify drug-resistant clones.
- (v) Grow 5 ml of overnight cultures from ~20 isolates with antibiotic selection.
- (vi) Isolate plasmid DNA and examine by restriction analysis.

? **TROUBLESHOOTING**

(C) Modifying multicopy plasmids ● **TIMING 3 d**

- (i) Outgrow the electroporated cultures in at least 1 ml of LB with aeration for at least 2 h at 30–32 °C before applying selection.
- (ii) After outgrowth, add 9 ml of LB and drug for plasmid selection and grow overnight at 30–32 °C with aeration.
- (iii) Isolate plasmid DNA using a standard mini-prep procedure.
- (iv) When possible, eliminate parental plasmid by cutting with a restriction enzyme that does not cut the recombinant molecule.
- (v) Introduce plasmid DNA by transformation into a *recA* strain of *E. coli* (e.g., DH10B) at a low DNA concentration (less than one plasmid per cell). Generally, the amount of DNA should be around 0.1 ng. See **Box 2** for determination of plasmid DNA transformation efficiency.
- (vi) Select or screen for desired phenotype. Possible methods of screening include restriction digestion, identification of plasmids with altered size as assayed by migration on agarose gels, sequencing and PCR analysis.

BOX 5 | TESTING COLONY PURITY BY RESTRICTION DIGESTION

For this approach, the region including the modification site is amplified using PCR primers hybridizing outside the region of homology. The purified PCR product should be digested with a restriction enzyme recognizing the 'hit' cassette (Fig. 6). Complete digestion of the PCR product indicates that the recombinant BAC is correct and is a pure culture.

▲ CRITICAL STEP Some of the recombinant plasmid molecules will have undergone multimer formation. Avoid these. If monomer recombinants are not apparent, a recombinant multimer can be digested with a restriction enzyme having a unique site in the monomer plasmid and the resulting monomer DNA can be ligated at a low DNA concentration.

(vii) Confirm the desired change by sequencing where necessary.

? TROUBLESHOOTING

(D) Screening 'hit and fix' recombinants with colony hybridization ● TIMING 2 weeks

(i) After 30 min of outgrowth at 32 °C, dilute the cell suspension 10^{-2} and 10^{-3} in LB. Spread 200 μ l of each diluted suspension onto a 150 mm \times 10 mm (large) agar plate containing appropriate antibiotic for BACs selection. Try to achieve uniform distribution of colonies throughout the plate. Incubate plates for 18–22 h at 32 °C. Approximately $1-5 \times 10^3$ colonies are expected.

(ii) Identify recombinant clones by colony hybridization using a γ - 32 P-end labeled (or a nonradioactively labeled) oligonucleotide (35-mer) containing the 20 nt heterologous sequence as described in detail by Ausubel *et al.*¹¹

(iii) Using a bacteriological loop, pick bacterial colonies giving a positive signal and suspend them in 1 ml of LB media. Screen at least 3–5 positive areas for each BAC modification. As the recombinant colonies also contain parental cells and because many colonies are close or touching each other on the original plate, a second screening step is required.

(iv) Make 10^{-2} , 10^{-3} and 10^{-4} dilutions of a bacterial suspension for each colony picked.

(v) Plate 50 μ l of each dilution on a 15 mm \times 100 mm agar plate containing the appropriate antibiotic for BAC selection. Incubate the plates at 32 °C for 18–22 h.

(vi) Select plates with 20–50 clearly isolated colonies and repeat colony hybridization.

(vii) Pick two well-isolated positive colonies from each plate for further analysis. Confirm the purity of the clones by colony PCR. Set up two PCRs for each clone using primers (20 nt) that will specifically amplify either the recombinant ('hit' clone) or the nonrecombinant DNA (Fig. 6). Test a total of ten individual colonies corresponding to the 3–5 positive areas on the original plate for each BAC modification.

▲ CRITICAL STEP Only one of the two reactions should be positive if the colony is pure. Occasionally, some colonies will amplify PCR products with both sets of primers, implying that at least one modified and one unmodified copy of the BAC is present. These colonies may be due to BAC multimer formation and should be discarded.

(viii) Once correctly targeted pure 'hit' colonies have been identified, repeat the preceding Steps 19D (ii–vii) used for the 'fix' step, beginning with the newly created 'hit' strain.

▲ CRITICAL STEP When using either the mini- λ or the pSIM plasmids for recombineering, streak the correct 'hit' clones on the appropriate antibiotic plate to determine whether they have retained the λ Red system. If necessary, reintroduce the Red system before proceeding.

(ix) Confirm the presence of correct mutation after the 'fix' step by sequencing.

(x) Digest both the original and modified BACs with a few commonly used restriction enzymes (e.g., *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I and so on). Confirm the integrity of the new BAC by comparing restriction digestion pattern with the original BAC. See **Box 5** for testing colony purity by restriction digestion and **Box 6** for detection of selectable marker flanked by *loxP* or *FRT* sites.

? TROUBLESHOOTING

BOX 6 | DELETION OF SELECTABLE MARKER FLANKED BY *loxP* OR *FRT* SITES

1. Grow an isolated colony of the recombinant strain containing the correct insertion of the transgene with its selectable marker flanked by *loxP* or *FRT* sites at 32 °C overnight in 3 ml of LB containing appropriate antibiotics.

2. Dilute 1 ml of the culture into 50 ml of LB containing antibiotics in a 250-ml flask and grow at 32 °C.

3. Once the culture reaches an OD₆₀₀ of 0.3, add 500 μ l of 10% filter-sterilized Arabinose to a final concentration of 0.1%. This induces the *flp* gene in SW105 or the *cre* gene in SW106.

4. Grow the culture at 32 °C until it reaches an OD₆₀₀ of 0.5. Plate 100 μ l of 10^{-2} , 10^{-3} , 10^{-4} dilutions on LB plates containing chloramphenicol (12.5 μ g ml⁻¹) to maintain the BAC and grow overnight at 32 °C.

5. Screen for clones that have lost the selectable marker by site-specific recombination, these can be identified by antibiotic sensitivity. Streak approximately 50–100 colonies in duplicate on LB plates and LB containing the drug. Colonies that grow on LB plates but not in the presence of the drug are the desired clones that have recombined and retain only a single *loxP* or *FRT* site.

6. Confirm the loss of the selectable marker by PCR and sequencing.

● **TIMING**

Steps 1–3, design and generation of linear dsDNA substrates for recombineering by PCR: 3–4 h
 Steps 4 and 5, introduction of the λ Red recombination system: 1–2 d
 Steps 6–8, cell growth and induction of the λ Red genes: 3 h
 Steps 9–14, preparation of electrocompetent cells: 1 h
 Steps 15–18, electroporation of the linear DNA: < 1 h
 Step 19: outgrowth, identification and confirmation of the recombinant clones: 1 d to 2 weeks

? **TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 5**.

TABLE 5 | Troubleshooting table.

Step	Problem	Possible cause(s)	Possible solution(s)
19A	No recombinants obtained with drug selection	Inadequate outgrowth time	Allow greater than 2 h outgrowth if needed. Increase LB volume so that more cell doublings occur
	No recombinants or transformants with double drug selection	Double drug selection issues	Try to use only one drug selection at a time rather than combining drugs. Do not maintain selection for BAC or pSIM plasmid when selecting drug-resistant recombinants
19B	No DNA retrieved, only empty vector	Vector is recircularizing by microhomologies	Either redesign the primers or place a selectable marker next to the DNA to be retrieved and retrieve with plasmid <i>ori</i> only
19C	Difficulty obtaining pure clones: multicopy plasmids	Contamination with parental plasmid	Transform DNA prep at low DNA concentration (less than one molecule per cell), select or screen for recombinant
		No recombinants	Poor plasmid transformation efficiency
	Multimers with plasmid recombineering	Heteroallelic multimer	Either screen other candidates or digest heteroallelic multimer with a unique cutter and religate at a low DNA concentration
		Inherent nature of recombineering	Screen other isolates Use restriction digestion to convert to monomer, retransform and screen for recombinant plasmids
19A–C	Colonies in uninduced control reactions	Use of RecA ⁺ host	Use only a <i>recA</i> mutant host for plasmid modification
		Plasmid was a multimer initially	Start with a pure monomer plasmid species
		Incomplete digestion of plasmid template used for PCR, so intact plasmid is transformed (Step 3)	Digest plasmid before using as template, digest PCR product with <i>DpnI</i> following amplification
		Leaky selection	Alter selection, e.g., increase drug concentration
19A–D	No recombinants obtained	Contamination	Use sterile technique Test solutions for source of contamination and discard if necessary
		Problem with bacterial strain (Step 4)	Never place recombineering strains at temperatures above 35 °C except during induction For strains containing defective prophage and λTet, verify that strain is temperature sensitive by plating dilutions at 32 and 42 °C
			For strains containing mini-λ and pSIMs, verify drug resistance

(continued)



TABLE 5 | Troubleshooting table (continued).

Step	Problem	Possible cause(s)	Possible solution(s)
			Do control recombinering reaction (i.e., drug cassette insertion) to check strain
		Cell loss during washing for electroporation (Step 12)	Titer viable cells as well as plating for recombinants—if viables are less than 10^7 ml ⁻¹ , recombinants may not be obtained. Be careful not to lose cells during H ₂ O wash steps
		Problem with induction procedure (Step 8)	Using shaking H ₂ O baths for both 32 °C growth and 42 °C induction, do not use air shaker. Confirm temperatures of H ₂ O baths. Do not exceed 15 min, 42 °C induction. Be sure to quick-chill flasks in ice bath after induction. If scaling up procedure, maintain 1/10 media/flask ratio
		Problem with linear DNA targeting construct (Step 3)	Remake PCR product or obtain new oligos
		Problem with electrocompetent cells (Steps 15–17)	Test cells by transforming supercoiled plasmid Use correct electroporation conditions. Be sure that the time constant for electroporation is ~5 ms. Add LB immediately after electroporation
		Drug concentration too high (see Table 4)	Be sure to use antibiotic concentrations for single-copy molecules when appropriate
19A–D	Difficulty in obtaining pure clones: BAC, PAC or <i>E. coli</i> chromosome	Incomplete colony purification	Restreak for single colonies and retest
	Difficulty in obtaining pure clones: BAC or PAC	Possible multimer formation	Screen more candidates

Troubleshooting guide for Recombineering (also see <http://recombineering.ncicrf.gov/faq.asp>).

ANTICIPATED RESULTS

Recombineering is an efficient and relatively straightforward method of *in vivo* genetic engineering that is rapidly becoming routine for modification of the bacterial genome, plasmids, PACs and BACs. As recombineering allows DNA to be precisely inserted, altered or deleted, it is very useful for generation of various genomic constructs for functional studies. Linear DNA, both PCR products and synthetic oligonucleotides (oligos) can be used as substrates to create null mutations in genes as well as more subtle genetic changes. Generally, recombinants are obtained at a frequency of 10^{-4} – 10^{-5} when dsDNA is used to make insertions. For optimized recombineering with ss-oligos or largely homologous denatured PCR fragments, the frequency of recombinants is in the range of 0.1–10%. The frequency of cloning by gene retrieval is lower, in the range of 10^{-5} – 10^{-6} . Insertion of reporter genes at the 5′- or 3′-ends of genes cloned in BACs can be easily achieved. There are a number of critical steps in the recombineering procedure; each step should be optimized. Deviations from the protocol may result in suboptimal results; the overall effect of not optimizing each step can be a reduction in recombination efficiency and even failure to generate recombinants.

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