Recombineering: Genetic Engineering in Bacteria Using Homologous Recombination

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ABSTRACT

The bacterial chromosome and bacterial plasmids can be engineered in vivo by homologous recombination using PCR products and synthetic oligonucleotides as substrates. This is possible because bacteriophage-encoded recombination proteins efficiently recombine sequences with homologies as short as 35 to 50 bases. Recombineering allows DNA sequences to be inserted or deleted without regard to location of restriction sites. This unit first describes preparation of electrocompetent cells expressing the recombineering functions and their transformation with dsDNA or ssDNA. It then presents support protocols that describe several two-step selection/counter-selection methods of making genetic alterations without leaving any unwanted changes in the targeted DNA, and a method for retrieving onto a plasmid a genetic marker (cloning by retrieval) from the *Escherichia coli* chromosome or a co-electroporated DNA fragment. Additional protocols describe methods to screen for unselected mutations, removal of the defective prophage from recombineering strains, and other useful techniques. *Curr. Protoc. Mol. Biol.* 106:1.16.1-1.16.39. © 2014 by John Wiley & Sons, Inc.

Keywords: recombineering • bacteria • homologous recombination • bacteriophage λ • λ Red system • RecET • Rac prophage • selection/counter-selection

INTRODUCTION

Over the past decade, an in vivo technology has emerged that is precise, rapid, efficient, and more practical than standard in vitro recombinant genetic engineering techniques. This technology, termed recombineering, allows DNA sequences to be modified without regard to the presence or location of restriction sites, which are an essential component of classical genetic engineering. The bacterial chromosome as well as bacterial episomes can be directly engineered in vivo by homologous recombination using linear DNA donor substrates, such as PCR products or synthetic single-stranded oligonucleotides (ssDNA oligos). Gene knockouts, replacements, deletions, and point mutations can be made; genes can be modified with tags, reporter fusions, and almost any imagined construction. This in vivo genetic engineering is possible because bacteriophage-encoded recombination functions efficiently recombine sequences with homologies as short as 50 nucleotides (nt). The linear DNA donor substrate containing the desired change is introduced by electroporation into bacterial strains that express the recombination functions.

Current Protocols in Molecular Biology 1.16.1-1.16.39, April 2014
Published online April 2014 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471142727.mb0116s106
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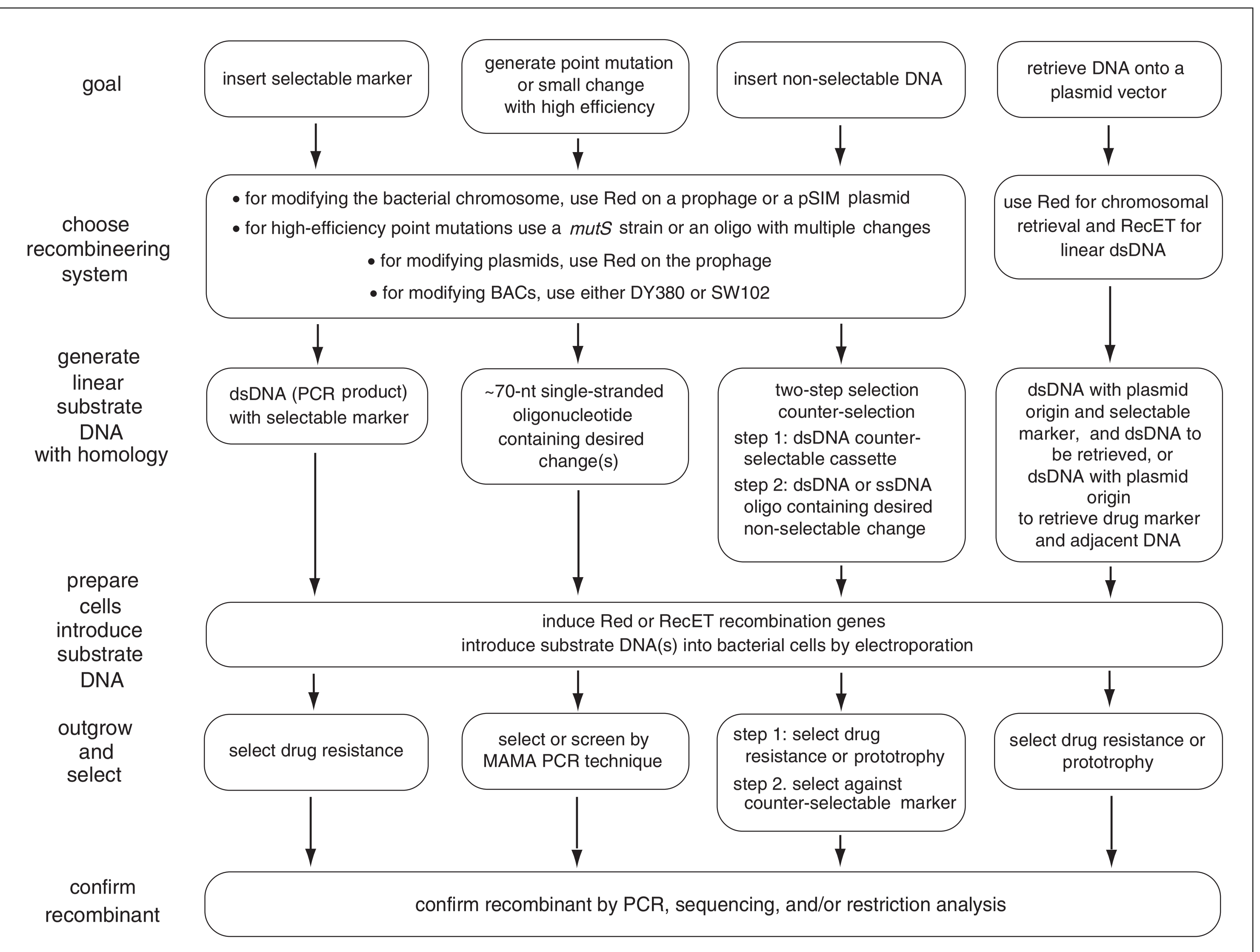

Figure 1.16.1 A general flow chart for recombineering. The appropriate recombineering system and linear DNA substrate should be chosen depending on the desired goal. While preparation of the bacterial cells and introduction of linear DNA by electroporation is similar for all procedures, treatment of the cells following the recovery period will vary.

The general flow chart in Figure 1.16.1 will help the researcher determine which recombineering pathway is most appropriate for the desired construct. The steps are summarized below. First, define the goal of the recombineering project, which may be to insert a selectable marker, create point mutations or other small alterations, insert a non-selectable segment of dsDNA, or retrieve dsDNA onto a plasmid vector. Depending on the goal, choose the appropriate recombineering system and generate the appropriate linear DNA substrate. Linear dsDNA substrates are generally made using PCR, while linear single-stranded substrates are synthetic oligos ~70 nt in length with the desired alteration(s) centrally located. The linear DNA is introduced into electrocompetent bacteria (*E. coli*) pre-induced for the recombination functions. After electroporation, the cells are allowed to recover for a period of time before looking for recombinants. Recombinant colonies may be identified by a selection or simply by screening.

In this unit, Basic Protocol 1 describes preparation of electrocompetent cells pre-induced for the recombination functions. The Court laboratory phage λ–based system uses the λ *pL* promoter to express these functions. The competent cells are then transformed with dsDNA or ssDNA (see Basic Protocol 1). Support Protocol 1 describes several variations of a two-step selection/counter-selection method of making precise genomic alterations without leaving any unwanted changes. In this support protocol, the first of the two steps is a recombineering reaction that inserts a selectable DNA cassette either consisting of, or accompanied by, a counter-selectable marker near or within the sequence to be modified. The second step is a subsequent recombination that replaces the counter-selectable cassette with the desired genetic alteration. This leaves the engineered sequence otherwise unaltered and thus allows the same selection and counter-selection to be reused to make further modifications. Support Protocol 2 describes a method for retrieving a genetic marker (cloning) from the *E. coli* chromosome or a co-electroporated DNA fragment onto a plasmid. Whereas the above protocols generally use selection to identify the recombinants, Support Protocols 3 and 4 describe methods to screen for unselected mutations. Support Protocols 5 and 6 detail modifications necessary when using recombineering to introduce changes into multicopy plasmids.

Basic Protocol 2 describes the removal of the defective prophage that expresses the recombineering functions in Basic Protocol 1. Basic Protocol 3 is for the plasmid-based phage systems. It describes a simple method for curing temperature-sensitive recombineering plasmids after engineering is complete. Finally, Alternate Protocols 1 and 2 present methods for recombineering with an intact prophage and for introducing mutations onto bacteriophage λ, respectively.

STRATEGIC PLANNING

General Considerations and Key Points

First, before attempting to modify the *E. coli* chromosome, a plasmid, or a bacterial artificial chromosome (BAC), the design of the desired final construct should be determined. A DNA-analysis program such as Gene Construction Kit (GCK; Textco Software; *http://www.textco.com/*) or Vector NTI (Invitrogen) is invaluable for this task. Having the sequence of both the original genome arrangement and the desired final construct as electronic files facilitates design of oligonucleotides to be used as primers for PCR recombination substrates or as ssDNA substrates. Possession of the desired DNA sequence in silico also allows rapid design of primers to analyze and verify potential recombinants.

During the design process, the researcher should be aware of complications in the final construct due to gene regulation issues. Bringing in a promoter with an antibiotic cassette can help in establishing drug resistance; however, transcription from this promoter can extend beyond the drug marker and affect distal genes. The authors have designed

several drug cassettes with their promoter, open reading frame, and transcription terminator region, as described in Yu et al. (2000); primers for amplification are listed in Table 1.16.3. During the design process, the researcher should also be careful of possible polarity effects caused by premature termination within a coding sequence and inhibition of expression of downstream genes in a multicistronic transcript (Adhya and Gottesman, 1978; Bubuenenko et al., 2007). The researcher should also be careful to design the final construct in a way that avoids creating unwanted fusion proteins.

Second, the investigator must decide whether to use dsDNA or ssDNA linear substrates to make the desired construct. Generally, insertion or removal of segments of DNA longer than ~20 nt requires a means of selection, since the efficiency of such reactions is not high enough to screen for recombinants: the frequency of such recombinants is $\sim 10^{-4}$- to 10^{-5}. For example, to knock out a gene on the *E. coli* chromosome or on a BAC, a dsDNA cassette encoding drug resistance is routinely used. On the other hand, if point mutations or changes of only a few bases are desired, a synthetic oligo of ~70 to 100 nt can be used to create them. Creating these small changes with ssDNA recombineering in wild-type *E. coli* containing the Court laboratory defective λ prophage gives efficiencies of 0.1% to 1% (Ellis et al., 2001; Costantino and Court, 2003), and if host methyl-directed mismatch repair (MMR) is inactivated, either by mutating the mismatch repair system or by using oligos that escape MMR, a 25% to 50% recombination frequency is achievable (Costantino and Court, 2003; Sawitzke et al., 2011). Such a high frequency means that, for oligo recombination, it is possible to create recombinants without a selection and find them by screening methods, such as using a PCR technique. A *mutS* mutation is routinely used to eliminate MMR, although we have also created and tested *mutH*, *mutL*, and *uvrD* mutants, which give similarly increased recombination frequencies (Costantino and Court, 2003). These four genes encode various components of the MMR system (Schofield and Hsieh, 2003).

Third, with the above considerations in mind, the investigator must design the linear DNA substrate to be used to generate recombinants, depending on the desired change. For a sizeable insertion, such as a drug cassette, a PCR product is generated that contains the cassette to be inserted flanked at each end by 50 base pairs of homology to the chromosomal or plasmid target. This homology is provided at the 5′ end of each synthetic primer. Following this region of target homology, ~20 bases of homology to the drug cassette provides the primers to amplify the cassette sequence. Thus, two primers are designed that should each be ~ 70-nt long: the 5′ ends provide homology to the targeted region and the 3′ ends provide priming homology to the cassette (see Fig. 1.16.2). Careful primer design is crucial.

Fourth, for oligo recombination, the ssDNA oligo should be ~70-nt long with the alteration(s) centrally located in the oligo. High efficiencies of oligo recombination require that the oligonucleotide correspond to the lagging strand of DNA replication, which is ~15- to 20-fold more efficient than its complement for ssDNA oligo recombineering (Ellis et al., 2001). Thus, it is helpful for the researcher to determine the direction of replication through the region of interest and use the oligo that corresponds to the lagging strand (see Fig. 1.16.3). Alternatively, the researcher can choose not to determine the direction of DNA replication through the region to be modified, but instead use both possible complementary oligos separately to do the recombination—one will be the lagging strand and give a higher efficiency than the other.

Finally, the investigator should bear in mind that it is possible to use ssDNA oligos to make deletions of large chromosomal regions, but the efficiency of this reaction is only ~0.01% to 0.1%, meaning that such changes cannot easily be screened for. Thus, when a large net deletion is the desired goal, a two-step selection/counter-selection

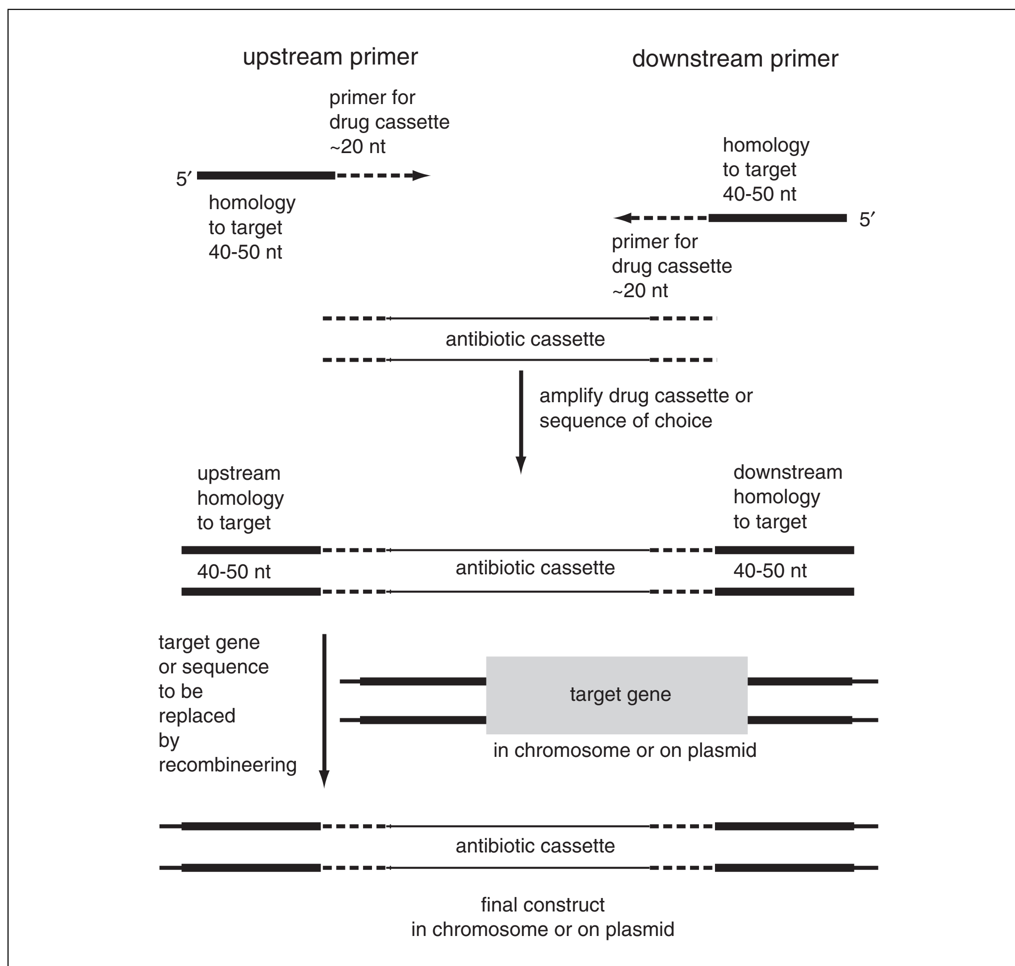

Figure 1.16.2 Targeting of an antibiotic cassette. Two primers with 5′ homology to the target are used to PCR amplify the antibiotic cassette. The PCR product is introduced by electroporation into cells induced for the Red recombineering functions. The Red functions catalyze the insertion of the cassette at the target site, which may be on the bacterial chromosome or on a plasmid.

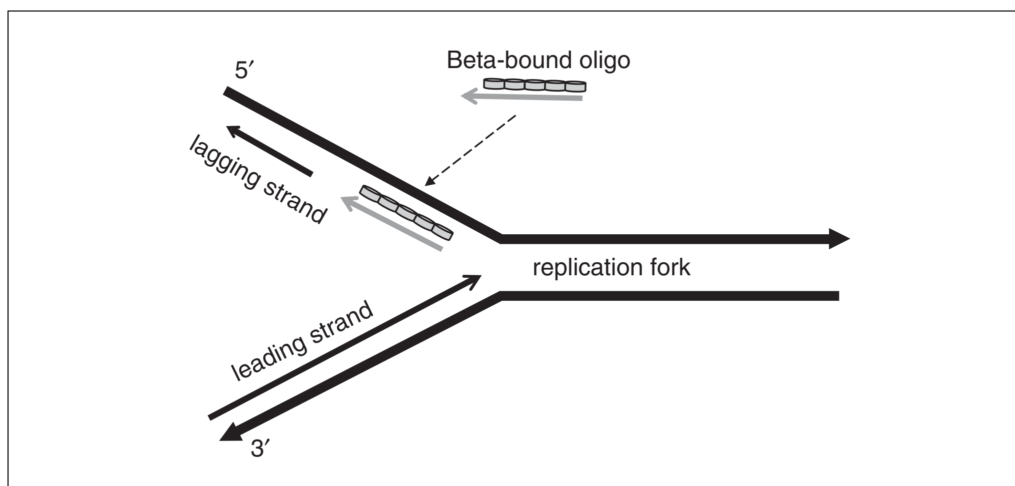

Figure 1.16.3 A schematic of the DNA replication fork. The leading and lagging strands are indicated. A DNA oligonucleotide, coated by the λ Beta protein, is shown annealed to the lagging strand template at a gap in the discontinuously replicated lagging strand.

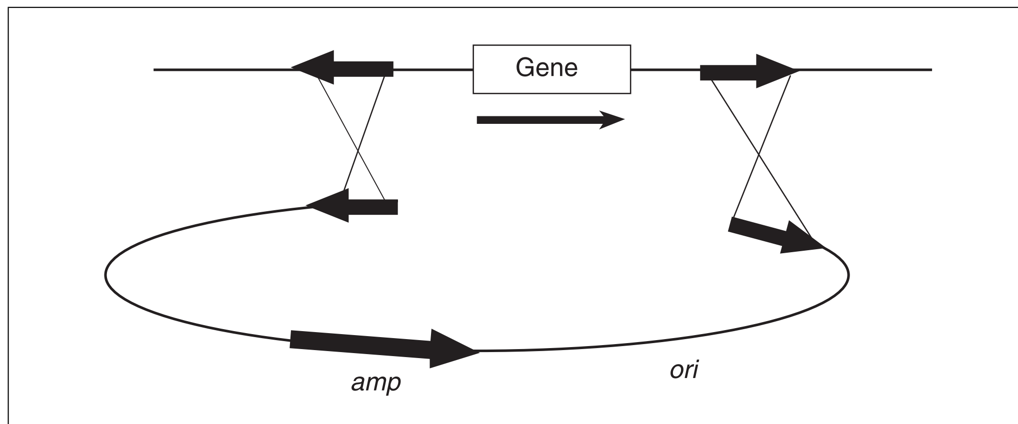

Figure 1.16.4 Cloning genes by gap repair of a plasmid. A linear plasmid with flanking homology to the target at the ends (indicated by dark arrows) is generated by PCR. The plasmid is introduced by electroporation into cells expressing the Red functions, which catalyze recombination of the vector with the target site, resulting in incorporation of the gene onto the plasmid.

(see Support Protocol 1) should be used. Here the first step is insertion of the dsDNA selectable/counter-selectable cassette in the segment of DNA to be deleted. For the second step, a 70-nt long oligo can be used; one half of the oligo (i.e., ~35 nt) is homologous to one end point of the desired deletion, while the other half of the oligo is homologous to the other end point. The oligo will recombine with both non-contiguous regions of homology and delete the intervening DNA, including the counter-selectable marker.

Rescuing Genes onto Plasmids

The researcher should also bear in mind that recombineering allows other, more specialized operations. Recombineering makes it possible to "retrieve", i.e., "clone" gene(s), either from the bacterial chromosome, a BAC (Lee et al., 2001), or a linear piece of DNA (Fig. 1.16.4), onto a plasmid backbone. For cloning by retrieval, the investigator must first add the terminal homologies necessary for recombination to the plasmid backbone. This is done with PCR, using chimeric primers that amplify the plasmid replication origin in conjunction with an adjacent selective marker; these chimeric primers also contain 50-nt homologies at their 5′ ends that target the DNA sequence to be retrieved. The homology design also ensures that the target sequence will be incorporated onto the circular plasmid in a particular orientation (Fig. 1.16.4). A minor variant of this protocol can be used if the region to be retrieved is already adjacent to a selectable marker. In this case, both the marker and the region of interest can be retrieved onto a vector backbone consisting of only the plasmid replication origin (i.e., lacking a selectable marker). Experiments are underway to determine whether the Red or the RecET system is more efficient for retrieval from the *E. coli* chromosome. However, because the RecET system is able to recombine two linear DNA molecules at a higher efficiency than the Red system, it is the system of choice to use for incorporation of a linear DNA fragment onto a linear plasmid backbone (Fu et al., 2012).

Modification of Existing Plasmids

Recombineering also makes it possible to modify multicopy plasmids, at frequencies equivalent to those found for modifications of the *E. coli* chromosome (Thomason et al., 2007). For example, point mutations can be made at a frequency of ~50% of total viable cells when mismatch repair is blocked. Thus, these recombinants can readily be detected by simple screening procedures. However, the insertion or removal of large DNA segments occurs at a much lower frequency that necessitates selection for the recombinant.

Recombineering with multicopy plasmids requires some modification of Basic Protocol 1. Different plasmids vary widely in their intracellular copy number, ranging from low-copy vectors with only a few molecules per cell, such as pSC101, to pUC-based plasmids that routinely have ~500 copies per cell. After recombineering, cells containing recombinant plasmids will also contain unmodified parental plasmids. In all cases, the recombinant class of molecules can be separated from the unmodified parental class by plasmid DNA isolation and retransformation at a DNA concentration of less than one molecule per cell; this creates pure clonal populations of the recombinant and parental plasmid species in different cells. If the desired plasmid recombinant is designed so that it lacks a restriction site present on the parent, the investigator can enrich for the recombinant class by destroying the parental population by restriction digestion prior to transformation.

Another complexity arising when plasmids are modified with recombineering is that circular multimeric plasmid species can be formed during the recombination reaction. In the absence of linear substrate DNA, expression of the Red proteins alone does not cause plasmids to multimerize under the conditions given here (Thomason et al., 2007), but when linear single-stranded or double-stranded substrate DNA modifies the target sequence, generating the recombinant, circular multimeric species are often formed. Multimer formation is thus a hallmark of a recombinant plasmid molecule (and could, in principle, be used to identify recombinant species). A host defective in RecA-mediated recombination eliminates host-mediated plasmid-by-plasmid recombination, and thus one route to multimer formation, so it is useful to use *recA* mutant strains for plasmid engineering.

For plasmid modification by recombineering, a decision should be made as to whether the plasmid will be introduced into the Red-expressing cells by co-electroporation with the linear DNA after the recombination functions are induced, or whether the plasmid should already be established in the bacterial strain. Co-electroporation offers the advantage that it helps control plasmid copy number at the time of recombineering and minimizes opportunities for plasmid multimer formation. However, for very large plasmids of low copy number, co-electroporation may not introduce plasmids into enough cells to be feasible.

Recombineering Expression Systems

To perform recombineering, a bacterial strain that expresses a bacteriophage generalized recombination system is required. Phage λ Red and RecET from the endogenous *E. coli* Rac prophage are two such systems used for engineering *E. coli*. The two systems do not yield similar recombination efficiencies for a given particular type of recombination reaction, and so should not be substituted for each other without careful consideration; restated, each system has an optimal use. While the λ Red system is superior for targeting the *E. coli* chromosome and episomes replicating in *E. coli* (Datta et al., 2006), the RecET system is superior for recombining two linear DNA molecules to generate a new, intact plasmid (Fu et al., 2012). The Court laboratory makes available bacterial strains allowing work with each system (see Table 1.16.1).

Each recombination system uses proteins that perform similar functions. Both λ Exo and Rac RecE are 5′→3′ double-stranded DNA (dsDNA)-dependent exonucleases (Little, 1967; Carter and Radding, 1971; Joseph and Kolodner, 1983). These nucleases will initiate degradation from 5′ ends of a linear dsDNA, creating either a partially duplex molecule with 3′ single-stranded DNA (ssDNA) overhangs, or if the dsDNA is short enough, a ssDNA molecule in which the complementary strand is entirely degraded. These ssDNAs are the substrates of the λ Beta and Rac RecT proteins, which are ssDNA-annealing proteins (Muniyappa and Radding, 1986; Hall et al., 1993). During

Table 1.16.1 Bacterial Strains Commonly Used for Recombineering

Strain	Genotype
DY329	W3110 Δ*lacU169 nadA::Tn10 gal490 pgl*Δ8 λ*c*I*857*Δ*(cro-bioA)* (TetR)
DY330	W3110 Δ*lacU169 gal490 pgl*Δ8 λ*c*I*857* Δ*(cro-bioA)*
DY331	DY330 *srlA::Tn10* Δ*recA*
DY378	W3110 λ*c*I*857* Δ*(cro-bioA)*
DY380[a]	*mcrA* Δ*(mrr-hsdRMS-mcrBC)* ϕ80d*lacZ*ΔM15 *lacX74 deoR recA1 endA1 araD139* Δ*(ara, leu)7697 galU gal490 pgl*Δ8 *rpsL nupG* λ*(c*I*857ind1)* Δ{*(cro-bioA)<>tetRA*} (Tet^{R})
HME5	W3110 Δ*lacU169* λ*c*I*857* Δ*(cro-bioA)*
HME6	W3110 Δ*lacU169* $galK_{TYR145UAG}$ λ*cI857*Δ(*cro-bioA*)
HME45[b]	W3110 *gal490 pgl*Δ8 λ*c*I857 Δ*(cro-bioA)*
HME63	HME6 *mutS<>amp*
HME64	HME6 *uvrD<>kan*
HME68	HME6 *mutS<>cat*
LT1713	HME6 [λ (*int-cIII*)< >*gam recE recT*]
SIMD49	HME6 [λ (*int-cIII*)< >*recT*]
SIMD63	HME6 [λ (*int-cIII*)< >*recE recT*]
SIMD89	HME6 [λ (*int-cIII*)< >*recT*] *mutS<>cat*
SW102	*mcrA* Δ*(mrr-hsdRMS-mcrBC)* ϕ80d*lacZ*ΔM15 *lacX74 deoR recA1 endA1 araD139* Δ*(ara, leu)7697 galU pgl*Δ8 *rpsL nupG* λ*(c*I*857ind1)* Δ{*(cro-bioA)<>tetRA*} (Tet^{R}) Δ*galK*
T-SACK	W3110 *araD<>tetA-sacB amp fliC<>cat argG::Tn5*
TUC01	HME45 *int<>cat-sacB*

[a]DH10B derivative (Invitrogen).
[b]Gives less background on low concentrations of chloramphenicol than DY378.

recombineering, Beta and RecT bind to and protect the ssDNA while annealing it to complementary ssDNA in the cell. Additionally, both proteins have been reported to have a strand invasion activity. The Beta invasion activity requires DNA with an 80% A-T content (Rybalchenko et al., 2004), while the RecT activity is independent of A-T content (Noirot and Kolodner, 1998). The λ Gam protein inhibits both the *E. coli* RecBCD (Karu et al., 1975) and SbcCD exonucleases (Kulkarni and Stahl, 1989), preventing those exonucleases from degrading the linear dsDNA substrates. The Rac prophage does not have a gene product analogous to λ Gam. Because the exonuclease proteins act on dsDNA rather than ssDNA, they are required for dsDNA recombineering reactions, but are unnecessary when ssDNA is the linear substrate, i.e., only the single-stranded annealing proteins are required for ssDNA recombineering.

The genes for each system can be expressed from an endogenous promoter or from a heterologous-regulated promoter. Expressing the three λ Red genes, *exo*, *bet*, and *gam*, from their endogenous phage promoter, *pL*, confers the advantage of tight regulation and coordinate high-level expression, which results in higher recombination frequencies. This is an important advantage, since in many cases, high recombination frequencies will be essential in obtaining a desired recombinant. For the λ Red system, the authors routinely use a defective λ prophage located on the *E. coli* chromosome, but they have also transferred the critical elements of this prophage to a number of different plasmids (Thomason et al., 2005; Datta et al., 2006; also see Commentary). In both situations, the

Table 1.16.2 Plasmids Containing the Red System Under *cI857* Control[a]

Plasmid designation	Drug resistance	Plasmid origin	Copy number/cell
pSIM5	Chloramphenicol	pSC101*ts*	16
pSIM6	Ampicillin		
pSIM7	Chloramphenicol	pBBR1	30-40
pSIM8	Ampicillin		
pSIM9	Chloramphenicol	pRK2*ts*	20-40
pSIM10	Hygromycin		
pSIM17	Blasticidin	pSC101*ts*	16
pSIM18[b]	Hygromycin		
pSIM19	Spectinomycin		

[a]Note that plasmids require higher drug concentrations for retention than those for single copy insertions given in Basic Protocol 1.
[b]Be aware that this plasmid has homology to AmpR (but is not AmpR). This can result in a background of incorrect recombinants if targeting an Amp-cassette into cells containing this plasmid.

phage recombination functions are under control of the bacteriophage λ CI repressor, which affords extremely tight regulation. Three operator sites are present at each of the λ *pL* and *pR* promoters flanking the repressor gene; the repressor protein, CI, binds cooperatively to these operator sites and blocks transcription from these promoters. CI-mediated looping between the two sets of operators provides an additional level of repression and in combination with autoregulation ensures tight and uniform repression. The prophage λ constructs used for recombineering, whether resident on the *E. coli* chromosome or on plasmids, encode a temperature-sensitive version of this CI repressor, CI857, which represses expression from the powerful λ *pL* promoter at low temperatures (30°C to 32°C) by binding to both sets of operators. To express the recombination proteins at high levels, the temperature of the bacterial culture is quickly but temporarily raised to 42°C, resulting in rapid inactivation of the repressor and expression of recombination functions. Lowering the temperature after a 15-min induction allows CI to renature with consequent restoration of tight repression. This burst of expression is sufficient to catalyze high levels of recombination while minimizing unnecessary expression, perpetuation of long-term recombination activity, and stress on the cells from the continuing high temperatures.

In the other λ Red expression systems, the recombination genes are expressed from non-λ promoters on multicopy plasmids. One such plasmid is pKM208 (Murphy and Campellone, 2003), which has the Red genes expressed from *pTAC*. Plasmid pKM208 contains the *bla* gene for ampicillin drug selection, *lacI* to lower expression until depression of the *lac* repressor with IPTG, and a temperature-sensitive origin of DNA replication to allow isolation of cells that lack the plasmid ("curing") after use by growth at high temperature. The authors have found that pKM208 yields similar recombination frequencies to those obtained with the prophage system, although repression of this plasmid is incomplete, with consequent constitutive recombination activity. Other plasmids using the arabinose-inducible *pBAD* promoter are also used to express the Red proteins (Datsenko and Wanner, 2000). In the authors' laboratory, the Datsenko and Wanner plasmids give about ten-fold lower recombination levels than the prophage constructs (Datta et al., 2006). Most of the plasmid constructs from the authors (Table 1.16.2) also have temperature-sensitive origins of DNA replication to facilitate curing. The plasmid-based systems have the advantage of portability to different strains—they can be transferred among different *E. coli* strains, or to *Salmonella typhimurium*, and presumably to other Gram-negative bacteria. Still, using the prophage system located on the bacterial

chromosome requires no drug-resistance markers and is the system of choice if the object is to engineer a plasmid.

By contrast, since expression from the endogenous Rac prophage gives only low-level recombination, the required enzymes for the RecET system are usually expressed from multicopy plasmids and under the control of heterologous promoters, such as the arabinose-inducible *pBAD* promoter (Zhang et al., 1998). The Court laboratory has replaced the Red genes with the full-length *recE* and *recT* genes (including λ *gam*) on the defective prophage. A similar strain carrying only *recT* also exists (Datta et al., 2008) (Table 1.16.1).

Cleaning Up After Recombineering

For the Court laboratory's phage-based systems, after the desired construct is obtained, the defective prophage carrying the recombination genes can be removed, either by recombination as described in Basic Protocol 2 or by P1 transduction (*UNIT 1.17*), using a non-lysogenic donor, selecting for growth in the absence of biotin (Bio^+). Alternatively, engineered alleles on the chromosome can be moved into a different host by P1 transduction (*UNIT 1.17*), provided there is a selection for them. For plasmid-borne systems, plasmid replication is temperature-sensitive and growth at high temperature causes plasmid loss as detailed in Basic Protocol 3.

BASIC PROTOCOL 1

MAKING ELECTROCOMPETENT CELLS AND TRANSFORMING WITH LINEAR DNA

This protocol describes the preparation of electrocompetent cells that are preinduced for the recombination functions and transformation with appropriate DNA to create the desired genetic change. As noted in Strategic Planning, the phage recombination functions are repressed by the phage λ temperature-sensitive CI857 repressor, so that they are not expressed when the cells are grown at low temperatures (30° to 32°C) but are highly expressed when the culture temperature is shifted to 42°C. See Commentary for additional considerations before performing the procedure.

Materials

Purified PCR product with ~50 bases of flanking homology or oligonuclotide primers with ~35 bases of flanking homology on either side of desired change (also see *UNIT 15.1*)

Bacterial strain expressing the defective lambdoid prophage recombination system λ Red (Table 1.16.1; strains available from Court Laboratory Website: *http://redrecombineering.ncifcrf.gov/*)

LB medium and plates (*UNIT 1.1*), without antibiotic

Medium lacking carbon source: M9 medium (*UNIT 1.1*) or 1× TM buffer (*APPENDIX 2*)

Selective plates (*UNIT 1.1*)—minimal plates if selecting for prototrophy *or* rich plates containing antibiotic (depending on drug cassette used):

- 30 μg/ml ampicillin
- 30 μg/ml kanamycin
- 10 μg/ml chloramphenicol
- 12.5 μg/ml tetracycline
- 50-100 μg/ml spectinomycin

30° to 32°C incubator

30° to 32°C shaking incubator or roller

32° and 42°C shaking water baths

125- and 250-ml Erlenmeyer flasks, preferably baffled

Refrigerated, low-speed centrifuge with Sorvall SA-600 rotor (or equivalent)

35- to 50-ml plastic centrifuge tubes
1.5-ml microcentrifuge tubes
Refrigerated microcentrifuge
0.1-cm electroporation cuvettes (Bio-Rad), chilled
Electroporator (e.g., Bio-Rad *E. coli* Pulser)
Micropipettor and 1000-μl pipet tips
18 × 150-mm sterile borosilicate glass culture tubes
15-ml sterile culture tubes, optional

Additional reagents and equipment for PCR (*UNIT 15.1*), agarose gel electrophoresis of DNA (*UNITS 2.5A & 2.6*), purification of DNA by ethanol precipitation (*UNIT 2.1A*; optional; commercially available PCR cleanup kit may be substituted), electroporation (*UNIT 1.8*), isolation of bacterial colonies by streaking (*UNIT 1.3*), restriction enzyme digestion (*UNIT 3.1*), and DNA sequencing (Chapter 7)

Prepare DNA for transformation

1. Design and procure the oligos to use for PCR-mediated generation of a dsDNA product, or for use in single-stranded oligo engineering.

   The sequences of the primers used to amplify the common drug cassettes are listed in Table 1.16.2. Remember to add the homologous targeting sequence to the 5′ ends of the oligos.

   UNIT 15.1 *describes general considerations for primer design.*

2. Make the PCR product (*UNIT 15.1*) and examine it by agarose gel electrophoresis (*UNIT 2.5A*).

   Gel purifying the DNA is not recommended. If it is essential that the DNA be gel purified, avoid exposing it to ultraviolet light, which will damage it and result in lower recombination frequencies.

3. Clean up the PCR product by ethanol precipitation or using a commercially available kit (e.g., Qiagen) to remove salt (*UNIT 2.1A*).

   A strain designated T-SACK available from the Court laboratory facilitates construction of tetracycline, kanamycin, ampicillin, and chloramphenicol cassettes as well as the TetA-SacB dual cassette. From this strain, custom cassettes can be amplified using colony PCR. In colony PCR, a PCR reaction is prepared without allowing any volume for the template DNA; a fresh colony of the appropriate E. coli K-12 strain is then touched with a sterile inoculating loop and mixed into the PCR reaction. Too many cells will inhibit the PCR reaction. Use of this strain to make cassettes rather than a plasmid template is encouraged, since if a plasmid template is used to construct a PCR-amplified drug cassette, any intact circular plasmid remaining will transform the cells extremely efficiently and give unwanted background, hindering recombinant identification. Such background can be minimized by using a linear plasmid template for the PCR and by digesting the completed PCR reaction with DpnI before using it for electroporation; however, using the bacterial template is still recommended. If linear plasmid templates are used, always use as little as possible and include a control reaction of uninduced cells transformed with the PCR product to give a measure of any unwanted intact plasmid background.

Prepare bacterial cultures

4. Inoculate the suitable bacterial strain (Table 1.16.1) from frozen glycerol stock or a single colony into 3 to 5 ml LB medium. Shake the culture overnight at 30° to 32°C.

   Most of the authors' strains containing the defective lambdoid prophage are W3110 derivatives; however, the prophage can be moved into other backgrounds by P1 transduction. See Commentary for details. Similarly, plasmids expressing the recombination

functions can be put into any strain of choice. Add antibiotic as appropriate to maintain plasmid selection during growth.

Either 30° or 32°C is acceptable for the low temperature throughout the procedure, since either temperature allows good repression by the CI857 repressor. The repression is tighter at 30°C but the cultures will grow more rapidly at 32°C.

5. Add ~0.5 ml of the overnight culture to 35 ml of LB medium in a 250-ml (baffled) Erlenmeyer flask.

   This is a 70-fold dilution. Ensure that the dilution is at least 70-fold. Higher (more dilute) dilutions will also work, but the cells will take longer to grow to the appropriate density. If targeting the recombineering to a plasmid, or expressing the recombineering proteins from a plasmid, do not add antibiotic for plasmid maintenance when sub-culturing cells. It has been observed that antibiotics may inhibit the recombination. If an alternative to temperature shift is used to induce the recombination functions (i.e., addition of arabinose or IPTG), the cells are grown at 37°C and inducer should be added to the medium. Expression of the λ Red functions from the plasmids of Datsenko and Wanner (2000) is enhanced by use of 10 mM arabinose (for Ara^+ strains) (see Datta et al., 2006). If using an inducer, remember to include an additional flask containing an uninduced culture as a negative control.

6. Place the flask into a 32°C shaking water bath and grow cells for ~2 hr at 32°C with shaking.

   The time will vary with different strains and dilutions. The cells are ready when the A_{600} is between 0.4 and 0.6. It is important not to over grow the cells, since stationary phase cells do not work well for recombineering with the Red system, which benefits from active replication forks.

Induce recombination functions

7. Transfer half of the culture to a 125-ml (baffled) Erlenmeyer flask and place flask into the 42°C water bath. Shake 15 min at 220 rpm to induce. Leave the remainder of the culture at 32°C (this will be used as the uninduced control that lacks recombination activity). While the cells are inducing, fill an ice bucket with an ice-water slurry.

8. Immediately after inducing for 15 min at 42°C, rapidly cool the flask in the ice-water slurry with gentle swirling. Leave on ice for ≥5 min. Follow the same cooling protocol with the uninduced 32°C culture. While the cells are on ice, precool the centrifuge to 4°C and chill the necessary number of 35- to 50-ml plastic centrifuge tubes, labeled for induced and uninduced cells.

   As mentioned above, a temperature shift is unnecessary when a chemical inducer like arabinose or IPTG is used.

Make electrocompetent cells

9. Transfer both the induced and uninduced cultures to the appropriately labeled chilled 35- to 50-ml centrifuge tubes. Centrifuge 7 min at 4600 × *g* (6700 rpm in a Sorvall SA-600 rotor), 4°C. Aspirate or pour off supernatant.

10. Add 1 ml ice-cold distilled water to the cell pellet in the bottom of each tube and gently resuspend cells with a large pipet tip (do not vortex). Add an additional 30 ml ice-cold distilled water to each tube, seal, and gently invert to mix (without vortexing). Centrifuge tubes again as in step 9.

    All subsequent resuspensions of cells through step 16 should be done gently and without vortexing. Preparation of the cells for electroporation washes out any added chemical inducing agent.

11. Very carefully decant the supernatant from the soft pellet in each tube and suspend each cell pellet in 1 ml ice-cold distilled water.

Remove tubes from the centrifuge promptly. The pellet is very soft and care should be taken not to dislodge it, especially when processing multiple tubes.

12. Transfer suspended cells to 1.5-ml microcentrifuge tubes. Microcentrifuge tubes for 30 to 60 sec at maximum speed, 4°C. Carefully aspirate off the supernatant. In each of the tubes, resuspend the cell pellet in 200 µl ice-cold distilled water, which will provide enough material for four or five electroporations.

    For routine procedures when optimal recombination frequency is not necessary, e.g., when selection is used to find recombinants, electrocompetent cells induced for the recombination functions can be used later after storage of the cell pellet, suspended in 15% (v/v) glycerol at –80°C. However, for highest efficiency, use freshly processed cells.

Introduce DNA by electroporation

13. Chill the desired number of 0.1-cm electroporation cuvettes on ice. Turn on the electroporator and set to 1.80 kV.

    Brands of cuvettes and electroporators other than that available from Bio-Rad may work, but have not been tested in the authors' laboratory. Larger 0.2-cm cuvettes have not been used successfully, and they may require different electroporation conditions (consult electroporator instruction manual) and standardization to obtain optimal recombination frequencies.

14. In microcentrifuge tubes on ice, mix 100 to 150 ng of salt-free PCR fragment (from step 3) or 1 pmol of single-stranded oligonucleotide with 50 to 100 µl of the induced or uninduced cell suspensions (from step 12). Rapidly mix and perform subsequent electroporation; do not leave the DNA/cell mixes on ice for extended periods. Be sure to include the following electroporation reactions and controls:

    a. Induced cells plus DNA.

       This is the culture that should yield the designed recombinants.

    b. Induced cells without DNA.

       This is a control to identify contamination, determine the reversion frequency, and obtain some idea of the efficiency of the selection.

    c. Uninduced cells plus DNA.

    This control tells whether there is some contaminating factor in the DNA that is contributing to the selected colonies (e.g., intact plasmid template from the PCR reaction will give rise to drug-resistant colonies here). If the recombination functions are partially constitutive, this control will give colonies.

15. Introduce the DNA into the cells by electroporation (*UNIT 1.8*).

    The time constant should be >5 msec for optimal results. Low time constants indicate problems with the cells, the DNA, the electroporation cuvettes, or even the equipment.

16. Immediately after electroporation, add 1 ml LB medium to the cuvette using a micropipettor with a 1000-µl pipet tip. If transforming with a drug cassette, transfer the electroporation mix to 15-ml sterile culture tubes and incubate tubes >2 hr with shaking at 30° to 32°C to allow expression of the antibiotic-resistance gene.

    If not selecting for drug resistance, it is still recommended that the cells be allowed to recover in broth from the shock of electroporation for at least 30 min. It has been observed in the authors' laboratory that omitting this recovery period greatly reduces the cell viability.

    An alternative, and in the author's experience more reliable, method for outgrowth is as follows. After the 30-min recovery period, spread appropriate dilutions of cells (see step 18 below) onto a sterile 82-mm diameter nitrocellulose filter atop a rich LB plate. Incubate this plate for 3 to 4 hr at 30° to 32°C. After the incubation, transfer the filter

to the appropriate selective drug plate using sterile forceps. This method is preferred because all of the recombinants are independent, the cells are less dense, and more efficient outgrowth is achieved.

Determine cell titers

17. Prepare serial 1:10 dilutions of the electroporation mix through 10^{-6} using M9 medium or 1× TM buffer, dispensing 0.9 ml of M9 or TM buffer and 0.1 ml of the cell suspension per tube.

    The dilutions can be made in rich medium if a selection for antibiotic resistance is applied.

18. To determine total viable cell count, spread 100 µl of 10^{-4}, 10^{-5}, and 10^{-6} dilutions on LB plates (rich plates without drug). Incubate plates 1 to 2 days at 30° to 32°C, depending on the growth requirements of the recipient strain.

19. To determine recombinant cell count, plate cells on selective plates as follows depending on the anticipated recombinant frequency.

    a. If efficient recombination is expected, spread both 10 and 100 µl of the 10^{-1} and 10^{-2} dilutions.

    b. If low numbers of recombinants are expected, spread 100 µl each of a 1:10 dilution and the undiluted culture. Pellet the remaining cells, suspend in 100 µl and plate.

    The authors routinely obtain ten-fold or higher recombinant yields with the prophage system than with the Datsenko and Wanner pBAD arabinose inducible system.

    c. For the no-DNA and uninduced controls, plate 200 µl directly onto selective plates.

    Since targeting to the chromosome results in a lower copy number of the drug cassette than is present with a multicopy plasmid, antibiotic concentrations must be adjusted accordingly. The authors routinely use the drug concentrations recommended in the materials list for single copy chromosomal constructs. Selections based on nutritional prototrophy require appropriate minimal plates.

20. Incubate plates at the appropriate temperature (30° to 32°C).

    At 30°C, colonies may take 2 days to develop on LB plates and 3 to 4 days on minimal plates. Candidates should be purified by streaking for single colonies on the selective plates and retested for the appropriate phenotype.

Analyze recombinants

21. Once recombinant clones are identified, confirm the presence of the desired mutation(s) by PCR analysis (*UNIT 15.1*) followed by DNA sequencing (Chapter 7) or restriction digestion analysis (*UNIT 3.1*), if appropriate.

    The design of the PCR primers depends on the changes to be made (see Strategic Planning). The recombinant junctions should be confirmed with two flanking primers and two additional primers pointing outwards from the cassette (all four should have compatible annealing temperatures); use one primer flanking the cassette and one internal cassette primer to amplify the unique junctions created by the recombination reaction. The primer pair that hybridizes to the external flanking sequences on each side rather than the insertion itself can also be used to demonstrate loss of the target sequences and presence of the insertion. Unwanted mutations can be introduced by heterologies (variations) in the synthetic primer population (Oppenheim et al., 2004); therefore, it is important to confirm the final construct by sequence analysis, especially the regions derived from the original primers.

SELECTION/COUNTER-SELECTIONS FOR GENE REPLACEMENT

SUPPORT PROTOCOL 1

This protocol describes several variations on two-step methods to create precise genetic changes without otherwise altering the DNA sequence. First, the selectable/counter-selectable cassette of choice is placed in the stretch of DNA to be altered; second, this cassette is replaced with the desired alteration in a second recombineering event. The final construct will have neither the cassette nor a drug marker. All these procedures require special media for the counter-selection step. The flow chart in Fig. 1.16.5 defines the

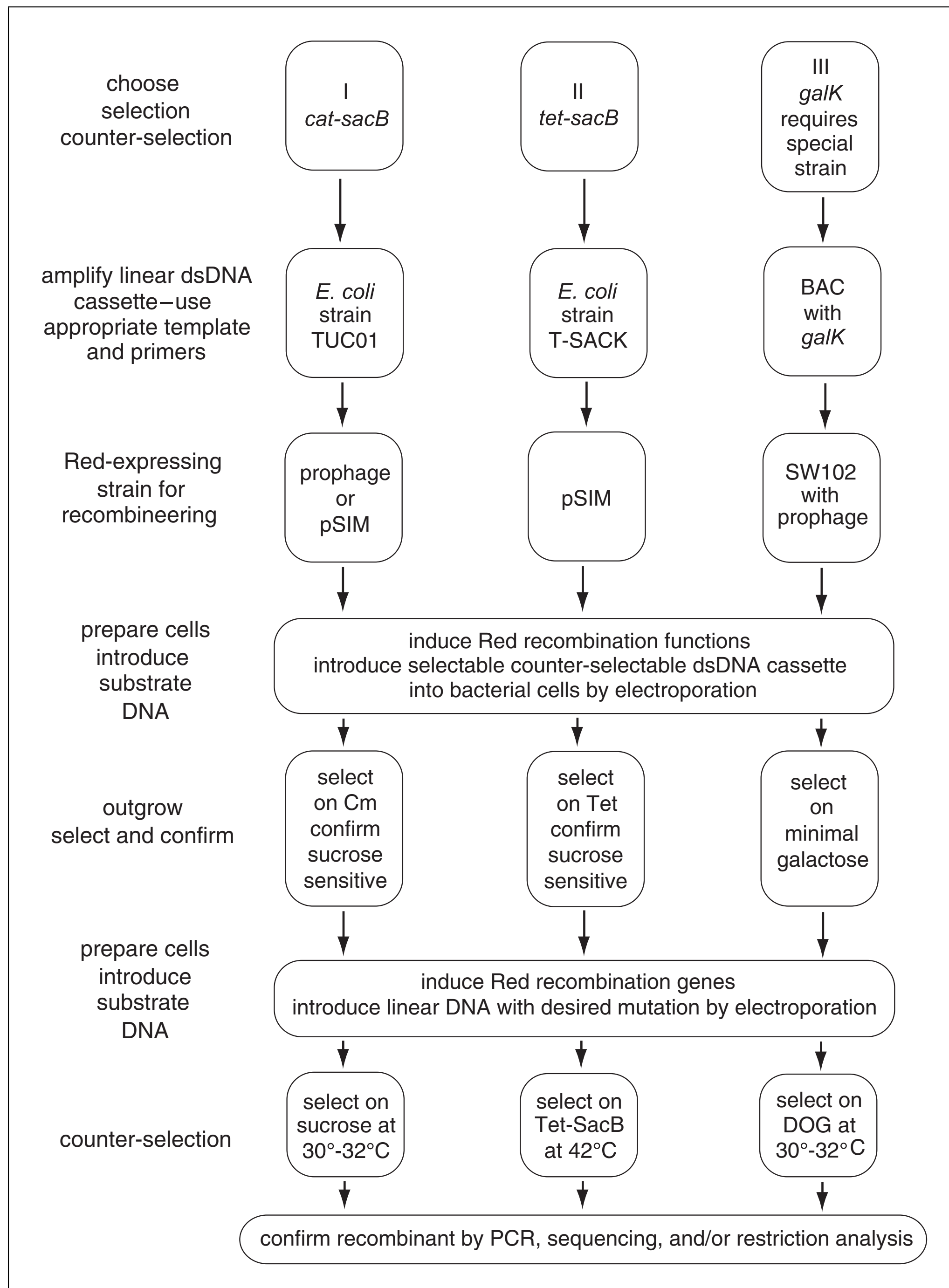

Figure 1.16.5 A flow chart for selection/counter-selections. Three procedures are provided, two using the *sacB* counter-selectable gene linked to either *cat* or *tetA*, and one using *galK* designed specifically for BAC modification.

three types of selection counter-selections for which protocols are given. Other possible selection/counter-selections are described in the Commentary.

Additional Materials *(also see Basic Protocol 1)*

Bacterial strain for specific selection/counter-selection:

cat-sacB: any recombineering strain can be used
tetA-sacB: the Red functions should be expressed from a heat curable plasmid rather than from a prophage
galK: use strain SW102, available from Frederick National Laboratory for Cancer Research: *http://ncifrederick.cancer.gov/research/brb/recombineeringInformation.aspx*

Appropriate template for amplification of selectable/counter-selectable cassette:

cat-sacB: use bacterial strain TUC01 (DY329 with a *cat-sacB* insertion on the *E. coli* chromosome), available from the Court Laboratory Website: *http://redrecombineering.ncifcrf.gov/*
tetA-sacB: *E. coli* strain T-SACK, available from the Court Laboratory
galK: the *galK* expression cassette has been cloned onto BAC pBeloBAC11, also available from Frederick National Laboratory for Cancer Research

Hybrid primers to amplify cassettes—all hybrid primers should have 50 bases of 5′ homology for targeting to the desired location:

cat-sacB
Primer L *sacB*: 5′-homology sequence-ATC AAA GGG AAA ACT GTC CAT AT-3′
Primer R *cat*: 5′-homology sequence-TGT GAC GGA AGA TCA CTT CG-3′
tetA-sacB
Primer L *sacB*: same as for *cat-sacB*
Primer R *tetA*: 5′-homology sequence- TCC TAA TTT TTG TTG ACA CTC TA-3′
galK
GalK forward primer: 5′-homology sequence-CCT GTT GAC AAT TAA TCA TCG GCA-3′
GalK reverse primer: 5′-homology sequence-TCA GCA CTG TCC TGC TCC TT-3′

Invitrogen Platinum High Fidelity enzyme
Selective media for inserting and removing cassettes:

cat-sacB selection, use LB-Cm plates: LB plates (*UNIT 1.1*) containing 10 μg/ml chloramphenicol; for counter-selection, use LB plates (*UNIT 1.1*) lacking NaCl but containing 6% (w/v) sucrose
tetA-sacB selection, use LB-Tet plates: LB plates (*UNIT 1.1*) containing 12.5 μg/ml tetracycline; for counter-selection, use Tet/SacB plates (see recipe)
galK selection, use M63 minimal D-galactose plates containing biotin and leucine, with chloramphenicol for BAC selection (see recipe); for screening, use MacConkey galactose plates (see recipe); for counter-selection, use M63 minimal glycerol with 2-deoxygalactose (DOG), biotin and leucine, and chloramphenicol for BAC selection (see recipe)

Thermal cycler (MJ Research PTC-100)

Amplify the selectable/counter-selectable element for recombineering

1. Amplify the cassette from the appropriate template with the Invitrogen Platinum High Fidelity enzyme and an MJ Research thermal cycler, using 50 pmol of each primer and one of the following cycling programs:

For *cat-sacB* or *tet-sacB* cycling (~3.5-kb product):

1 cycle:	2 min	94°C (denaturation)
9 cycles:	15 sec	94°C (denaturation)
	30 sec	55°C (annealing)
	3.5 min	68°C (extension)
19 cycles:	15 sec	94°C (denaturation)
	30 sec	55°C (annealing)
	3.5 min (adding 5 sec/cycle)	68°C (extension)
1 cycle:	7 min	68°C (extension)
1 cycle:	indefinite	4°C (hold).

For *galK* cycling (~1.3-kb product):

1 cycle:	2 min	94°C (denaturation)
30 cycles:	15 sec	94°C (denaturation)
	30 sec	60°C (annealing)
	1.5 min	68°C (extension)
1 cycle:	7 min	68°C (extension)
1 cycle:	indefinite	4°C (hold).

The PCR primers used to amplify the selection/counter-selection elements are at the 3′ end of chimeric primers that include 5′ segments of bacterial target homology ~50 nt in length. Hence, each primer is ~70-nt long. Since the PCR products for cat-sacB and tetA-sacB are ~3.5 kb, they can be difficult to amplify. The above conditions are used successfully in the authors' laboratory.

2. Visualize the PCR product on an agarose gel. Purify the PCR product to remove salt (*UNIT 2.1A*).

Perform electroporation and recombination to insert the element at the desired location

3. Insert the selectable/counter-selectable cassette into the chromosome as described in Basic Protocol 1, steps 4 to 21 (also see Background Information for important tips) using the following techniques specific for each cassette.

   a. Selection for *cat-sacB* or *tetA-sacB*: select drug-resistant colonies, Cm^R for *cat-sacB* or Tc^R for *tetA-sacB*, and purify on LB-plates containing drug to isolate single colonies. For *galK*: select $GalK^+$ chloramphenicol-resistant (Cm^R) colonies on the minimal galactose plates containing supplements and purify on the same plates to isolate single colonies.

   The em7 promoter that is amplified along with the galK gene results in constitutive expression of galK. Since bacteria grow more slowly on minimal medium, it will take several days for colonies to grow. Select on plates with chloramphenicol to ensure maintenance of the BAC.

   b. Confirmation for *cat-sacB* or *tetA-sacB*: test several drug resistant isolates for sensitivity to sucrose on LB plates lacking NaCl but containing 6% sucrose (Blomfield et al., 1991). Use the targeted strain lacking a *sacB* insertion as a sucrose-resistant control, and use TUC01 or T-SACK as a Suc^S control. Determine that the insertion is correct before proceeding with the next step of the procedure.

   The sucrose-resistant parent serves as a control for growth in the presence of sucrose. Sucrose sensitivity needs to be tested because in a fraction of the clones the PCR process generates mutations that inactivate sacB. Occasionally, one orientation of the dual cassette does not give good expression of the sacB cassette. In this case, all drug-resistant colonies

will also be Suc^R. If so, then different homologies can be designed to either invert the cassette or move it.

For *galK*: Purify candidate colonies on MacConkey galactose indicator plates to verify that they have become Gal$^+$. Include SW102 containing the BAC as a negative control. Colonies should grow overnight on this indicator medium and positive clones will be bright pink or red. Be careful not to lose the BAC. SW102 will not turn red on this indicator.

Perform electroporation and recombination to replace the selectable/counter-selectable cassette with chosen allele

4. Use a confirmed candidate from step 3 as the starting bacterial strain for a second round of recombineering by carrying out Basic Protocol 1, but at step 16, suspend the electroporated cells in a final volume of 10 ml instead of 1 ml of LB medium, and incubate with aeration (applied via shaking in a shaking water bath) for 4 hr to overnight at 30° to 32°C for outgrowth following electroporation.

   The higher dilution promotes better cell recovery and allows complete segregation of recombinant chromosomes that no longer carry the counter-selectable cassette from non-recombinant sister chromosomes that still contain it. If outgrowth is inadequate and sister chromosomes are not fully segregated, the presence of the cassette on one chromosome will confer sensitivity to the entire cell, thus preventing recovery of recombinants. This is generally true in counter-selection experiments and illustrates a common problem encountered when using them.

5. Centrifuge cells 7 min at 4600 × *g*, 4°C. Remove supernatant, then wash the cells two times, each time by suspending in 1 ml minimal medium lacking a carbon source, such as M9, centrifuging again as before, and removing the supernatant. Suspend and dilute the cells for plating, and spread appropriate dilutions of the cells on the appropriate solid medium as described below. Be sure to also similarly dilute and plate the cells without added DNA for comparison of titers.

   a. For *cat-sacB*: plate 100 µl of a 10^{-1}, 10^{-2}, and 10^{-3} dilutions on LB-sucrose plates at 30° to 32°C.

      The frequency of spontaneous sucrose-resistant cells is normally ~1 in 10^4. Thus, in the recombination experiment, the sucrose-resistant colonies that arise are of two types, spontaneous mutants like those found in the control, and deletions caused by replacing cat-sacB by recombination. The frequency of the latter is optimally 10- to 100-fold greater than that of spontaneous mutation. However, colonies should be analyzed even when no increase in titer with respect to the control without DNA is observed, since correct recombinants will usually be found.

   b. For *tetA-sacB*: plate 100 µl of the undiluted culture and the 10^{-1} and 10^{-2} dilutions on Tet/SacB plates at 42°C.

      The tetA counter-selection works best at 42°C. The tetA-sacB counter-selection gives much lower background than either of the other counter-selections, since two independent counter-selections are being applied.

   c. For *galK*: use minimal glycerol plates containing DOG and supplements at 30° to 32°C.

      Like sucrose-resistance, the frequency of DOG-resistance is ~1 in 10^4, and the comment above also applies to DOG-resistance. In parallel, be sure to include a control of electroporated cells to which no PCR product was added, to determine the frequency of spontaneous DOG-resistant mutants.

6. Purify ~12 colonies by streaking to isolate single colonies (*UNIT 1.3*) on the appropriate medium and test as follows:

a. For *cat-sacB* and *tetA-sacB*: purify on sucrose plates and test for drug sensitivity by patching to a drug plate.

Spontaneous mutants will be drug resistant while the recombinants will be sensitive.

b. For *galK*: purify on MacConkey galactose plates—recombinants will be white, i.e., GalK^{-}.

7. Screen the colonies by PCR (see Basic Protocol 1, step 21), then sequence to confirm the presence of the desired change.

Screen a minimum of twelve colonies. If high-efficiency recombination is not achieved, more colonies will need to be screened. Note that the frequency of spontaneous mutants remains relatively constant and provides an internal control for determining the efficiency of the recombination.

RETRIEVAL OF ALLELES ONTO A PLASMID BY GAP REPAIR

SUPPORT PROTOCOL 2

Often it is desirable to retrieve (i.e., rescue) DNA sequence from the bacterial chromosome onto a plasmid. For example, a researcher might want to do this to clone and amplify a gene, to express a gene under a given promoter, or to create a gene or operon fusion. To do this with recombineering, a PCR product with homology to the target at the ends is made from a linearized plasmid DNA and introduced into cells expressing the Red system. This homology will allow recombination with the sequence to be retrieved, yielding a circular plasmid containing the sequence. It is important to linearize the plasmid DNA used as template. This retrieval method works with ColEI and p15A (pACYC) replicons, but not with pSC101 (Lee et al., 2001). Retrieval is illustrated in Figure 1.16.4.

If the desired gene is not present on the chromosome, it can be provided as a PCR product and introduced into the cells along with the PCR-amplified linear vector DNA by co-electroporation. It has been reported (Fu et al., 2012) and confirmed by the Court laboratory that the RecET system (including the full-length RecE protein) promotes this reaction at a 100-fold higher level than does the λ Red system. Always provide flanking homology so that the linear plasmid fragment can recombine with the additional PCR product (it may be easier to provide the homology on the product to be cloned rather than on the vector).

Additional Materials *(also see Basic Protocol 1)*

Synthetic chimeric primers providing homology to sequence flanking gene of choice and to the plasmid sequence to be amplified
Plasmid onto which sequence of choice is to be rescued
Restriction enzyme(s) (*UNIT 3.1*) that do not cut within plasmid region to be amplified
Bacterial strain SIMD63 or other RecET expression system (with full length *recE*) for recombining two PCR products

Amplify linear plasmid PCR product with homology to the target

1. Design primers with homology that flanks the desired target.

Drawing a sketch of the plasmid as a gapped circle interacting with the target sequence will help one visualize the recombination reaction (see Fig. 1.16.4). Both of the primers will have ∼50 nt of sequence homology at the 5′ ends linked to 3′ plasmid sequence. It is not necessary to amplify the entire plasmid. Any portion can be amplified as long as the minimal requirements of a selectable marker and an origin of DNA replication are met. If the DNA to be retrieved is adjacent to an antibiotic-resistance gene, only a plasmid replication origin need be amplified; the origin can be used to retrieve both the desired sequence and the nearby drug marker. Avoid having other regions of the plasmid

that are homologous to the bacterial chromosome (e.g., lac); these may lead to unwanted rearrangements.

2. To minimize background, digest the plasmid with one or more restriction enzyme(s) that do not cut within the region to be amplified. Amplify the linear plasmid by PCR using the primers (reaction conditions must be established empirically).

   The amplified product will be a linear-gapped plasmid with flanking homology to either side of the allele to be rescued from the chromosome. Use the least amount of plasmid DNA possible for the PCR template, to minimize the background of false positives. Digestion of the completed PCR reaction mix with DpnI will help remove the template plasmid.

3. Purify the PCR product to remove salt before proceeding.

Transform induced cells with linear plasmid and select recombinants

4a. Introduce the linear plasmid PCR product into the strain (see Basic Protocol 1, steps 4 to 21). If necessary, also add the PCR product to be co-electroporated, in this case, be sure to use the RecET system; the bacterial strain SIMD63 has this system on the defective prophage under temperature control. Select for the marker on the plasmid, and transform the uninduced cells with the linear plasmid PCR product, to determine the background of intact plasmid present. Purify candidate colonies and screen them with PCR. Isolate the recombinant plasmids and use them to retransform a standard cloning strain such as DH5α or XL-2 Blue, to generate pure clones.

   Transformation into a recA mutant host ensures that the newly engineered plasmid does not undergo additional rearrangement.

4b. Alternatively, if the DNA of interest is linked to a drug marker and it is retrieved with a replication origin lacking a drug marker, dilute the electroporation mix into 10 ml LB medium and grow the culture overnight non-selectively. The next day, isolate plasmid DNA and transform into a high-efficiency cloning strain, selecting for the drug resistance of the rescued marker. The transformation should be done with a low concentration of DNA to minimize uptake of multiple plasmids into the same cell. The advantage of using only the plasmid origin for retrieval is that any possible background of circular vector is eliminated.

   The RecET system is able to recombine two linear DNA molecules, e.g., to form an intact plasmid, with a frequency of >10^4 to 10^5 per 10^8 viable cells. This type of linear by linear recombination is less efficient when performed with the Red system, where the maximal frequency achieved with Red in the authors' laboratory is ~10^3 recombinants per 10^8 viable cells. (This may be because λ Exo degrades one of the two complementary DNA chains of a dsDNA completely while RecE may not). A possible side reaction is joining of the plasmid ends without incorporation of the chromosomal marker. This is caused by small (>5 base) repeats near the linear ends (Zhang et al., 2000); it is likely that the Red functions facilitate the short repeat recombination that generates this background. The short repeat recombination can be reduced or eliminated by designing primers that are free of such repeated sequences.

SUPPORT PROTOCOL 3

SCREENING FOR OLIGO RECOMBINANTS BY PCR

Point mutations and small sequence changes can be created with oligo recombination at high efficiency in a methyl-directed mismatch repair proficient host. The method works because the user designs oligos with mispairs that are refractory to this repair system (Sawitzke et al., 2011). A procedure for creating and identifying such recombinants is given below and how it works is described in more detail in the Commentary.

Additional Materials *(also see Basic Protocol 1)*

70-mer oligo containing desired mutation

1. Obtain a 70-mer oligo containing the desired mutation centered in the oligo. Add additional base changes adjacent to the desired change to create a 5-base alteration, or add 4 to 5 wobble changes (i.e., alter the third base in 4 to 5 adjacent codons), either flanking or to one side of the desired mutation.

   The clustered bases can be changed to anything as long as they differ from the original sequence. The wobble changes should still code for the original amino acids. The mismatch repair system does not act efficiently on either distribution of mismatches so both these types of oligos will generally give efficient recombination in a Mut$^+$ host.

2. Perform Basic Protocol 1, steps 1 through 16, however, after recombineering, allow cells to recover for only 30 min.

3. Prepare 1:10 serial dilutions of the culture in M9 buffer out to 10^{-6}.

4. Plate the diluted cells non-selectively on LB-agar plates at 32°C to obtain single colonies, i.e., plate 100 µl of 10^{-4}, 10^{-5}, and 10^{-6} dilutions and plate several plates of each dilution.

5. To detect the recombinant, test 25 single colonies from step 4 using colony PCR and a pair of short (~20 to 24 base) primers designed to amplify a small PCR product (~200 to 500 bp in length) with one of the primers having a 3′ end consisting of the bases that were altered.

   The latter primer will prime only the mutant sequence. The desired T_m of this primer pair should be ~64°C. Make a grid of tested colonies, since the positive isolates will be returned to. Generally, it has been found that 1/2 to 1/10 of the total colonies contain recombinants. These colonies are genetically mixed, containing both recombinant and parental cells in a single colony.

6. Once a colony with the desired change has been identified, further purify this mixed colony to isolate genetically clonal single colonies from it, either by streaking for single colonies or by suspending the colony in broth, diluting, and again plating for single colonies. From this second round of plating, test 20 to 25 colonies using PCR and the same primer pair.

   If the mutation will give a slow growth phenotype, it is preferable to dilute and plate the positive colony rather than to simply streak it.

   It is critical to keep track of candidate colonies by returning to positive isolates. In this second round, it has been found that at least 1/10 of the colonies have the desired mutation.

7. Confirm the desired mutation by sequencing.

   Once the purified colony with the change has been identified, it is possible to do a second round of recombineering with another oligo that leaves only the desired mutation, restoring all the other changes back to wild type. As before, this will be a 4 to 5 base change, thus a high-efficiency event, so a mutation in mutS will not be necessary. Again, use PCR to detect the changes, with a primer designed to specifically target the desired sequence. As before, two rounds of PCR will be necessary—first screening unpurified colonies plated non-selectively after a 30-min recovery period, followed by a second round of screening for purified positive colonies.

   There are rare instances when this method does not work well; in this case, a strain defective for MMR (i.e., containing a mutS or uvrD mutation, see Table 1.16.1) should be used.

SUPPORT PROTOCOL 4

OTHER METHODS OF SCREENING FOR UNSELECTED RECOMBINANTS

When recombinant frequencies approach 1/1000, direct screening can often be used to find recombinant colonies from total viable cells plated out non-selectively on LB. The authors have successfully used the nonradioactive Roche DIG (digoxigenin) system for colony hybridization (UNIT 6.3) to detect the recombinant bacterial colonies (Thomason, unpub. observ.). For this method to be feasible, the sequence inserted by recombineering must be unique to the recombinant and absent in the starting strain. The authors have used a 21-nt long DIG-labeled oligonucleotide probe to detect insertion of the same sequence. For larger insertions or fusion proteins such as GFP derivatives, a labeled PCR product or gel-purified fragment could also be used as a probe. Both oligo probes and larger DNA fragments could be radioactively labeled with ^{32}P.

The authors have also successfully isolated unselected recombinants when the genetic change confers a slow growth phenotype by looking for colonies that grow more slowly than the majority class. This screening method has been used to isolate *recA* mutations.

Additional Materials *(also see Basic Protocol 1)*

Flanking primers for PCR analysis of mutation of interest (also see UNIT 15.1)

Additional reagents and equipment for colony hybridization (e.g., UNIT 6.6)

1. Introduce the genetic change of interest (see Basic Protocol 1, steps 1 to 18).

   Possible changes include small in-frame deletions or a protein tag. For subtle changes, it is helpful to engineer a restriction-site change that can be detected in a PCR product. For detection by hybridization, confirm beforehand that the sequence does not exist in the parental bacterial strain—e.g., using a BLAST search (UNIT 19.3; Altschul et al., 1990).

2. Assuming an expected viable cell count in the transformation mix of $\sim 10^8$, after a 30-min outgrowth, plate the cells non-selectively on rich plates so that the expected number of colonies per plate is ~500.

   Six plates should be adequate if the recombination is efficient. More crowded plates will mean that fewer plates must be screened, but if the plates are too crowded it will be more difficult to locate positive clones.

3. Screen for recombinants by performing colony hybridization using established procedures (e.g., UNIT 6.6). If using a nonradioactive labeling method, follow the conditions suggested by the manufacturer.

   The required length of oligonucleotide probes will depend on the sensitivity of the system.

   The authors have detected positives colonies at frequencies as low as 5×10^{-2} to 1×10^{-3}.

4. Streak positive candidates to obtain pure clones and retest.

   Since the recombination only alters one of several copies of the chromosome existing in a single cell, the colony from that cell will be heterozygous for the allele. Thus, on re-streaking, some fraction of the colonies will not give a positive signal. The signal can again be detected by colony hybridization. If a new restriction site has been designed into the construct, perform PCR and cut the product with the appropriate restriction enzyme to detect the recombinant (also see UNIT 3.1).

SUPPORT PROTOCOL 5

MODIFYING MULTICOPY PLASMIDS WITH RECOMBINEERING

Multicopy plasmids can be modified by recombineering with efficiencies similar to those obtained when targeting the *E. coli* chromosome. This protocol optimizes the basic procedure to deal with additional complexities arising when plasmids are targeted (see Commentary). Addition of both the plasmid and the linear substrate DNA, either

double- or single-stranded, by co-electroporation allows better control over the initial ratio of plasmid molecules to number of cells and minimizes opportunity for plasmid multimer formation. After recombineering, the recombinant species will be contaminated with unmodified parental plasmid and the two species must be separated.

Additional Materials *(also see Basic Protocol 1)*

Plasmid to be modified: monomer species, freshly isolated from *recA* mutant host such as DH5α; determine plasmid DNA concentration by A_{260}
recA mutant bacterial strain expressing Red functions (e.g., DY331 or DY380; see Table 1.16.1)
Appropriate selective plates, if needed (when selecting for drug resistance, use drug concentrations appropriate for the multicopy plasmid used)

Additional reagents and equipment for plasmid miniprep (*UNIT 1.6*) and agarose gel electrophoresis (*UNIT 2.5A*)

1. Follow Basic Protocol 1, steps 1 to 13, using a *recA* mutant strain containing the Red functions.

2. In Basic Protocol 1, step 14, mix the plasmid DNA and the linear DNA, either single-stranded or double-stranded, with the cells. The DNAs will be introduced into the cells in one electroporation reaction. Include the following electroporation reactions and controls:

   a. Induced cells plus plasmid and modifying DNA.

   This is the culture that should yield the recombinant plasmids.

   b. Induced cells without plasmid or linear DNA.

   This is a control to identify contamination in the bacterial culture.

   c. Induced cells plus plasmid only.

   This control will provide a measure of the plasmid transformation efficiency and serve as a negative control for recombinant selection or screening.

   d. Uninduced cells plus plasmid and linear DNA.

   This control will confirm that the "recombinants" are dependent on expression of the Red system.

   If the plasmid is introduced by co-electroporation with the linear substrate, enough molecules of plasmid must be added to obtain a high transformation frequency, since recombineering will occur only in cells receiving the plasmid. In most cases, ~10 ng of plasmid DNA per electroporation is sufficient, but it may be necessary to determine empirically the appropriate amount of plasmid DNA to add, since the transformation efficiency of the plasmid being used may differ. Ideally, each cell should receive about one plasmid molecule.

   If the plasmid is already resident in the cell, add only the modifying linear DNA at this step.

3. Perform electroporation as in Basic Protocol 1, step 15. After electroporation, follow step 16 for outgrowth, using the longer 2-hr time.

4. Add 9 ml LB medium and the appropriate amount of antibiotic for plasmid selection; allow the culture to grow overnight at 30° to 32°C.

5. The following day, isolate plasmid DNA from the culture with a standard miniprep procedure (*UNIT 1.6*).

   If the recombinant has been designed to remove a unique restriction site, digest several microliters of the DNA with this enzyme. This will help to eliminate the inevitable background of unmodified parental plasmid, thus enriching for the recombinant population.

6. Transform the plasmid miniprep DNA or the clean restriction digest into a standard *recA* cloning strain such as DH5α at a low DNA concentration (less than one DNA molecule/cell).

   Electroporation is highly recommended, since it is much more efficient than chemical transformation.

7. After a 2-hr outgrowth, plate for single colonies on the appropriate medium. Plate selectively to select recombinant colonies, or non-selectively on LB plates (maintaining plasmid selection) and screen colonies for the desired phenotype. Isolate the recombinant plasmid DNA from about ten candidate colonies and screen for a monomer species by visualization with agarose gel electrophoresis (*UNIT 2.5A*).

   Possible methods of screening include restriction digestion, identification of plasmids with altered size as assayed by migration on agarose gels, sequencing, and PCR analysis (i.e., MAMA-PCR; Cha et al., 1992).

8. If a multimeric plasmid that contains the desired modification has been identified, convert it to a monomer species by first digesting it with a unique restriction enzyme and subsequently ligating under conditions favoring an intramolecular reaction (i.e., a low DNA concentration) (*UNIT 3.14*). Re-transform and verify the presence of the desired modification on the monomer plasmid.

SUPPORT PROTOCOL 6

SCREENING FOR UNSELECTED PLASMID RECOMBINANTS

With the extremely high oligonucleotide recombination frequencies obtainable in the absence of mismatch repair (20% to 25% of total viable cells), direct sequencing of unselected plasmid clones can be used to find recombinants. It is helpful if the mutation to be inserted creates a restriction site change that can be monitored by digestion of a PCR-amplified fragment covering the region of interest.

Additional Materials *(also see Basic Protocol 1)*

Cells expressing Red but mutant for host mismatch repair system: e.g., HME63, HME64, and/or HME68 (see Table 1.16.1) *or* cells expressing Red and competent for host mismatch repair and an oligonucleotide that creates mismatches refractory to mismatch repair when annealed to target DNA strand

High-efficiency cloning strain (lacking the Red system)

1. Perform Basic Protocol 1, introducing both the plasmid and the oligonucleotide by co-electroporation into cells mutant for the host mismatch repair system, or using an oligonucleotide that escapes mismatch repair when annealed to the target DNA strand.

2. After the outgrowth, dilute the electroporation mix into 10 ml broth containing the appropriate antibiotic for plasmid selection and grow the culture overnight.

3. Isolate plasmid DNA from this culture.

4. Using a low DNA concentration, transform the engineered plasmid into a high-efficiency cloning strain (lacking the Red system), selecting for drug resistance. Purify colonies.

   Use a low DNA concentration to minimize uptake of more than one molecule/cell.

5. Screen to find the mutation, either by direct sequencing or, if there is a restriction site change, by amplifying a fragment with PCR and digesting it with the appropriate enzyme(s).

   The authors recommend looking at 25 to 50 colonies. This procedure has been successfully used for plasmid mutagenesis (Thomason, unpub. observ.).

REMOVAL OF THE PROPHAGE BY RECOMBINEERING

BASIC PROTOCOL 2

Once the mutational changes are introduced, the defective λ prophage can be removed if necessary. This is done by another Red-mediated recombination reaction. Alternatively, the prophage can be removed by P1 transduction. In both cases, the desired recombinant can be selected on minimal plates since it will grow in the absence of biotin. Be sure to provide supplements for any auxotrophies in the specific strain used.

Materials

Oligonucleotide primers for amplifying the bacterial *attB* site:

5′ GAG GTA CCA GGC GCG GTT TGA TC 3′
5′ GTT GCC GAT GTG CGC GTA CTG 3′

E. coli K12 strain lacking the prophage (e.g., W3110), but containing the *attB* and biotin (*bio*) genes
M63 minimal glucose plates (see recipe) with and without biotin

Additional reagents and equipment for recombineering (see Basic Protocol 1)

1. Amplify the bacterial *attB* site by PCR using 50 pmol of each of the oligonucleotide primers with an *E. coli* K12 strain lacking the prophage (e.g., MG1655 or W3110) as template (the PCR product is ∼1.0 kb), and the following program:

1 cycle:	2 min	94°C (denaturation)
9 cycles:	15 sec	94°C (denaturation)
	30 sec	60°C (annealing)
	1 min	68°C (extension)
19 cycles:	15 sec	94°C (denaturation)
	30 sec	60°C (annealing)
	1 min (adding 5 sec/cycle)	68°C (extension)
1 cycle:	7 min	68°C (extension)
1 cycle:	indefinite	4°C (hold).

The technique used here is colony PCR in which the PCR reaction is prepared without allowing any volume for the template DNA; a fresh colony of the E. coli K12 strain is then picked with a sterile inoculating loop and mixed into the PCR reaction.

2. Delete the prophage region by recombineering (see Basic Protocol 1, steps 1 to 18) using the *attB* PCR product for recombination.

3. Wash cells in minimal salts two times and resuspend them in the same medium for plating. Select for the desired recombinant on minimal glucose plates lacking biotin but containing vitamin B1 by incubating 4 hr at 32°C then shifting to 42°C until colonies appear.

LB has trace amounts of biotin, enough to complement a bio mutant for growth; therefore, the cells must be washed free of LB for this selection to be successful. Those cells in which the prophage has been deleted will be bio^{+}, since the PCR product brings in a wild-type (wt) biotin gene, and will grow at 42°C, since the removal of the prophage makes the cells temperature resistant. As a control, confirm that the prophage-containing strain, subjected to the same washes, does not plate on minimal glucose plates lacking biotin, but does plate when biotin is present.

The 32°C incubation allows time for the recombinant chromosomes to segregate away from those still containing the prophage, which expresses a killing function at high temperature (see Commentary). Streak the recombinant colonies to purify them. The same PCR primers can be used to confirm the presence of the 2.5-kb band in the purified strain, as an additional confirmation that the prophage has been deleted.

BASIC PROTOCOL 3

CURING RECOMBINEERING PLASMIDS CONTAINING A TEMPERATURE-SENSITIVE REPLICATION FUNCTION

A temperature-sensitive plasmid used to express the recombineering functions can be eliminated from the strain by propagation at high temperature after the recombinant has been confirmed.

Materials

Overnight culture of confirmed recombinant strain
Rich broth

50- or 125-ml baffled Erlenmeyer flask
37° to 42°C shaking water bath

Additional reagents and equipment for recombineering (see Basic Protocol 1)

1. Grow a fresh overnight culture of the confirmed recombinant strain.

   It is unnecessary to add drug to maintain plasmid selection.

2. Dilute the strain 1000-fold into 5 or 10 ml rich broth in a baffled Erlenmeyer flask. Allow the strain to grow at least 4 to 5 hr or to saturation in a shaking 37° to 42°C water bath.

   The temperature-sensitive replication protein encoded by the plasmid will be inactive at this temperature range. The high dilution and long growth are necessary to allow adequate cellular duplication for plasmid-free cells to predominate in the culture. An air shaker may not allow sufficient heat transfer to cure the plasmid efficiently.

3. Follow Basic Protocol 1, steps 17 and 18, to plate for total viable cells and obtain single colonies.

4. From the most dilute plate, patch ~20 colonies onto both a drug plate (of the resistance encoded by the plasmid) and a non-selective rich plate to find a clone that has lost the plasmid.

   Most, if not all, of these colonies should have lost the recombineering plasmid.

ALTERNATE PROTOCOL 1

RECOMBINEERING WITH AN INTACT λ PROPHAGE

Basic Protocol 1 describes a method for recombineering using a defective lambdoid prophage. It is also possible to recombineer using an intact λ prophage (Court et al., 2003). If the phage of interest has a temperature-sensitive *c*I*857* repressor gene, intact *exo*, *beta*, and *gam* genes, and is able to lysogenize the host, it can exist in the prophage state and can be induced by high temperature to express the recombination functions. Such a phage will provide the necessary functions for recombineering; it can also itself serve as a target for such engineering. Since the recombineering efficiency is lower with this method than with the defective prophage, a selection for the recombinants should be applied. To recombineer using an intact phage, construct and confirm a phage λ bacterial lysogen (Arber et al., 1983) with the phage of choice. Once the phage exists in the prophage state, it can be induced by a temperature shift, as described below, and the PCR product or oligo containing the desired genetic change can be introduced by electroporation. The procedure will vary slightly depending on whether the desired target is the bacterial genome or the prophage itself, and for the latter, whether a λ lysogen or a stock of modified phage is desired.

Additional Materials *(also see Basic Protocol 1)*

Bacterial lysogen carrying the λ *c*I857 bacteriophage of choice as a prophage
Chloroform
1× TM buffer (*APPENDIX 2*)

82-mm nitrocellulose filters, sterile
Sterile forceps
39°C shaking water bath

Additional reagents and equipment for plating λ phage to generate plaques (plaque purification; *UNIT 1.11*), PCR (*UNIT 15.1*), and DNA sequencing (Chapter 7)

1. Grow the host bacterial strain (lysogen) to mid-log phase at 32°C (see Basic Protocol 1, step 6).

2. Induce recombination functions, electroporate cells, and plate to determine viable cell counts (see Basic Protocol 1, steps 7 to 18, but reduce the induction time to 4 to 5 min in step 7).

   The induction time must be shorter to prevent lytic phage replication and resultant cell killing. The shorter induction time means that lower levels of the recombination functions are produced. If a modified prophage or a modified host genome still carrying the prophage is desired, use the shorter induction time (see below). The only situation in which the 15-min induction time should be used is when changes are being targeted to the bacteriophage chromosome itself (steps 3b and 4a) and a phage lysate is desired.

If the mutation of interest is targeted to the bacterial chromosome or to modify the prophage and maintain the lysogenic state

3a. Select for recombinants by plating an entire electroporation mix on one selective plate (because recombinant levels are reduced by the lower induction time). Use a sterile 82-mm diameter nitrocellulose filter atop a rich (LB) plate and incubate 3 to 4 hr at 30° to 32°C, then transfer the filter to the appropriate drug plate using sterile forceps.

   The number of recombinants is generally <500 per electroporation mix. Usually, approximately half of the surviving cells will have spontaneously lost the prophage. It is possible to screen for non-lysogens by testing candidate colonies for their ability to plate λ, since the prophage renders the cells immune to phage infection; the cured cells will also be viable at 42°C while those containing the prophage will not (see Commentary).

4a. Use PCR (*UNIT 15.1*) and subsequent DNA sequencing (Chapter 7) to confirm the mutation.

If mutations have been targeted to the bacteriophage itself and the free phage is desired, the 15-min induction time can be used

3b. Dilute the electroporation mix into 10 ml LB medium and aerate by shaking 90 min in a 39°C shaking water bath. Add 0.25 ml chloroform to complete cell lysis and release the phage particles. Centrifuge the lysate to remove debris. Dilute the lysate and plate for single plaques (see *UNIT 1.1*). Identify positive plaques by the expected phenotype. To check by PCR, suspend a plaque in 50 μl of sterile water and use 20 μl of this suspension as template for a PCR reaction (*UNIT 15.1*; reduce the amount of water accordingly).

4b. Plaque-purify positive candidates (*UNIT 1.11*). Suspend another plaque from the same plate in 1 ml of 1× TM buffer to grow a stock. Reconfirm the mutation after growing the stock.

ALTERNATE PROTOCOL 2

TARGETING AN INFECTING PHAGE λ WITH THE DEFECTIVE PROPHAGE STRAINS

Occasionally, it may be useful to target genetic changes to bacteriophage λ derivatives. A strain carrying the Red system on the prophage or a plasmid can be infected with a phage and recombineering can then be targeted to the incoming phage chromosome. A procedure for this method is described. Ideally, the construction should be designed so that the plaque morphology of the recombinant phage will differ from that of the parent. For example, a PCR product able to both introduce the mutation of interest and correct a known mutation (such as an amber or temperature-sensitive allele) can be recombined onto a phage containing the known mutation to be corrected. In this case, the investigator would select for correction of the known mutation and screen among these recombinants for the mutation of interest. If no selection exists, plaque hybridization can sometimes be used to identify recombinant phages. While the authors have only tested this method for phage λ, theoretically, it may be possible to introduce genetic changes onto the chromosome of any phage able to propagate in the defective prophage host (Oppenheim et al., 2004).

Additional Materials *(also see Basic Protocol 1)*

Host strain with defective prophage
10% (w/v) maltose stock solution, filter sterilized
1× TM buffer (*APPENDIX 2*)
High-titer lysate of bacteriophage to be engineered
PCR product with desired sequence changes and flanking homology to the target on the phage chromosome
Chloroform
Lambda plates and lambda top agar (see *UNIT 1.1*, adjust NaCl to 5 g per liter in both plates and top agar)
Appropriate bacterial indicator strain

Additional reagents and equipment for working with λ bacteriophages (*UNITS 1.9-1.13*)

1. Grow host strain with defective prophage to mid-log phase at 32°C (see Basic Protocol 1, step 6), except supplement the LB medium with 0.4% maltose.

   Maltose induces the phage receptor on the bacterial cell surface, ensuring efficient adsorption of the phage to the host cells.

2. Harvest cells by centrifuging 7 min at 4600 × *g*, 4°C. Resuspend pellet in 1 ml of 1× TM buffer. Infect cells with the phage to be engineered at a multiplicity of one to three phages per cell.

   The cells will be at $\sim 1 \times 10^8$*/ml before concentration.* UNITS 1.9-1.13 *contain protocols for working with λ bacteriophages.*

3. Allow the phage to adsorb the cells for 15 min at room temperature.

   Phages other than λ may require different adsorption conditions.

4. Transfer infected cells to 5 ml of 42°C LB medium and shake vigorously for 15 min. At end of incubation, chill the culture rapidly on ice.

   This serves to both induce the Red functions and allow phage infection to proceed.

5. Prepare electrocompetent cells and introduce transforming DNA (i.e., introduce PCR product with mutation of interest; see Basic Protocol 1, steps 8 to 14).

6. Dilute electroporation mix into 5 ml warm LB medium and shake vigorously for 90 min at 42°C to allow the phage to complete a lytic cycle. Add 0.25 ml chloroform to completely lyse infected cells. Plate phage on lambda plates with lambda top agar (see *UNIT 1.11*) using the appropriate bacterial indicator strain as a host. Apply a selection, if possible, or plate non-selectively and screen for the desired mutation with plaque hybridization.

    Amber mutations can be specifically selected (Oppenheim et al., 2004).

REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

M63 minimal galactose plates

Per 1 liter, add:
3 g KH_2PO_4
7 g K_2HPO_4
2 g $(NH_4)SO_4$
0.5 ml 1 mg/ml $FeSO_4$
0.2% (v/v) galactose
0.001% (w/v) D-biotin
1 ml 1% (w/v) vitamin B1 (thiamine)
1 ml 1 M $MgSO_4$
Add chloramphenicol to 12.5 μg/ml for BAC retention
15 g agar
Pour 40 ml of the agar-containing medium per plate
Store plates for 2 months at 4°C

M63 minimal glucose plates

Per 1 liter, add:
3 g KH_2PO_4
7 g K_2HPO_4
2 g $(NH_4)SO_4$
0.5 ml 1 mg/ml $FeSO_4$
0.2% (v/v) glucose
0.001% (w/v) D-biotin (omit for control)
1 ml 1% (w/v) vitamin B1 (thiamine)
1 ml 1 M $MgSO_4$
15 g agar
Pour 40 ml of the agar-containing medium per plate
Store plates for 2 months at 4°C

M63 minimal glycerol plates with 2-deoxy-galactose (DOG)

Per 1 liter, add:
3 g KH_2PO_4
7 g K_2HPO_4
2 g $(NH_4)SO_4$
0.5 ml 1 mg/ml $FeSO_4$
0.2% (v/v) glycerol
0.2% (v/v) 2-deoxy-galactose (DOG)
0.001% (w/v) biotin
1 ml 1% (w/v) vitamin B1 (thiamine)
1 ml 1 M $MgSO_4$

continued

Add chloramphenicol to 12.5 μg/ml for BAC retention
15 g agar
Pour 40 ml of the agar-containing medium per plate
Store plates for 2 months at 4°C

MacConkey galactose indicator plates

Per 1 liter, add:
40 g Difco MacConkey agar base
1% galactose

Heat with frequent agitation and boil for 1 min to completely dissolve the powders. Autoclave mixture 15 min at 121°C. After cooling the solution to ~50°C, add chloramphenicol to 12.5 μg/ml for BAC retention. Store plates for 2 months at 4°C.

Tet/SacB counter-selection agar

Per 1 liter, add:
4 g tryptone
4 g yeast extract
15 g Difco agar
Mix the above three ingredients, add 400 ml dH_2O, and autoclave 15 min at 121°C
8 g NaCl
8 g $NaH_2PO_4 \cdot H_2O$
Mix the salts, add 400 ml dH_2O, and autoclave 15 min at 121°C
Autoclave 100 ml of a 60% sucrose stock solution in dH_2O separately.

After autoclaving the above three solutions separately, mix them together. Allow solution to cool to 55°C.

After cooling, add the following supplements:
0.5 ml of a 48 mg/ml fusaric acid stock solution in ethanol (store at −20°C in a lightproof container)
32 ml of a 25 mM $ZnCl_2$ stock (filter sterilize, do not autoclave)

Bring final volume to 1 liter with sterile dH_2O. Pour 40 ml agar solution into each 100-mm petri plate. After plates solidify, wrap in aluminum foil and store up to 2 months at 4°C.

COMMENTARY

Background Information

Bacteriophage λ encodes three genes important for recombineering. The *exo* and *bet* genes, respectively, encode a 5′ to 3′ double-stranded exonuclease, Exo, and a single-stranded annealing protein, Beta, which together can recombine a double-stranded PCR product with short flanking homologies into the desired genetic target. The λ *gam* gene encodes a protein, Gam, which inhibits the RecBCD enzyme that will otherwise degrade linear DNA introduced into the bacterial cell. Only the Beta single-stranded annealing function is required to recombine single-stranded oligos containing the desired alterations. Unlike some other in vivo genetic engineering methods (Russell et al., 1989), recombineering does not require the host *recA* function (Sawitzke et al., 2011). The ability to perform the recombination in a strain mutant for *recA* provides more controllable recombination, since the strain is recombination proficient only when the phage functions are induced. Although it is not completely understood how dsDNA recombineering works, there is evidence that it may proceed through a single-stranded intermediate (Maresca et al., 2010; Mosberg et al., 2010). An older, more detailed discussion of the molecular mechanism of Red-mediated recombineering can be found in Court et al. (2002).

While the Court laboratory routinely uses a defective prophage resident on the *E. coli* chromosome to express the recombineering functions, others have also moved the λ Red genes to several plasmids (Table 1.16.3; Datsenko

Table 1.16.3 PCR Primers and other Suggested Sources of Template for Amplifying Drug Cassettes (Modified from Yu et al., 2000)[a]

Gene	Source	Primer sequence
Ampicillin	SACK strain (*bla*)	5′ CATTCAAATATGTATCCGCTC
	pBluescript SK(+) (Stratagene)	
		5′ AGAGTTGGTAGCTCTTGATC
TetA only	T-SACK strain (Tn*10*)	5′ TCCTAATTTTTGTTGACACTCTA
		5′ ACTCGACATCTTGGTTACCG
Chloramphenicol	T-SACK strain (Cat)	5′ TGTGACGGAAGATCACTTCG
	pPCR-Script Cam (Stratagene)	
		5′ ACCAGCAATAGACATAAGCG
Kanamycin	T-SACK strain (Tn*5*)	5′ TATGGACAGCAAGCGAACCG
		5′ TCAGAAGAACTCGTCAAGAAG
Spectinomycin	DH5αPRO (Clontech)	5′ ACCGTGGAAACGGATGAAGGC
		5′ AGGGCTTATTATGCACGCTTAA

[a]It is preferable to use a single copy insertion on the *E. coli* chromosome as a template (i.e., the T-SACK strain) and the "colony PCR" procedure for amplification. Although some plasmid templates are suggested for convenience, use of a plasmid template is not recommended.

and Wanner 2000; Murphy and Campellone, 2003; Datta et al., 2006) having different DNA replication origins and drug markers. Here, the essential control elements of the prophage system are retained and recombination functions are induced by a temperature shift as in Basic Protocol 1. The plasmids are especially useful when one wants to create a mutation in a particular bacterial strain, rather than creating the mutations in the prophage-containing strains and subsequently moving them into a different background.

Some recombineering reactions can also be performed with proteins encoded by a cryptic lambdoid prophage, Rac. Rac, present in some strains of *E. coli*, but not others, codes for the RecE and RecT functions, which are largely analogous to Exo and Beta, respectively (Murphy, 2012). The two systems are not interchangeable, and should not be substituted for each other without careful consideration. In a side-by-side comparison, Red Beta gave 100-fold more oligo recombinants than did RecT (Datta et al., 2008). The entire Red system is also better than RecET for dsDNA recombination. (L. Thomason, unpub. observ.). On the other hand, the RecET system is superior to the Red system for recombining two linear DNA molecules to form an intact plasmid (Fu et al., 2012). While the wild-type RecE protein is 866 amino acids in length, the exonuclease domain is the last 260 amino acids, and some available expression systems for RecE express only this C-terminal domain (Zhang et al., 1998). The full-length RecE protein is necessary to achieve high levels of linear by linear DNA recombination (Fu et al., 2012).

Attributes of selection/counter-selection schemes

These positive-negative selections serve a special purpose. It is not generally necessary to use them when creating point mutations or changes of only a few bases, since this type of modification can be created with extremely high efficiency using Red-mediated oligonucleotide recombination and screened for by a PCR reaction with primers specific for the change (see Support Protocol 3). Selection/counter-selections are extremely useful, however, when it is necessary to either create a large deletion or insert a large heterologous piece of DNA without also leaving a selectable marker. In either of these cases, a selectable insertion can be made at the site to be modified in the first step, and the insertion replaced by the desired change in a subsequent counter-selectable step.

A number of selection/counter-selection systems exist; they can be classified according to different criteria. The most flexible ones do not require a special bacterial strain. These include, for example, systems with *sacB* linked to a co-selected drug marker. Protocols for two such systems, *cat-sacB* and *tetA-sacB* are included here. Both dual cassettes can be selected for by drug resistance and against by sucrose sensitivity. Of these, the

tetA-sacB counter-selection is unique and has lower background than *cat-sacB* since *tetA* can also be selected against (Bochner et al., 1980; Maloy and Nunn, 1981; Li et al., 2013). Another class of selection/counter-selections uses single genes that can be either selected for or against, for example, *tetA* and *galK*. Some of these selections/counter-selections require specialized strains with specific mutations; among these is the *galK* system, designed specifically for BAC modification and included in this unit. Other counter-selections that require specialized strains and have been used successfully by others include *rpsL*, *thyA*, and *tolC*. A promising new approach using a combination of recombineering and Cas9/CRISPR to reduce non-recombinant background has been reported by Jiang et al. (2013). Here, protocols for three selection counter-selections are provided in this unit and are described in detail below.

The cat-sacB and tetA-sacB dual cassettes

A versatile selection/counter-selection system in wide use is *cat-sacB*. The primary advantage of *cat-sacB* and similar *sacB* cassettes is that they do not require a special bacterial strain. Insertion of the dual cassette is by selection for drug resistance, i.e., Cm^R; the counter-selection is against the *sacB* gene on sucrose medium (Gay et al., 1985), as the levansucrase SacB converts sucrose to a toxic polymer, levan, which accumulates in the *E. coli* periplasm. Dual cassettes with *sacB* linked to a different drug may be created. However, a disadvantage of the *sacB* counter-selection is often a high background of sucrose-resistant cells. To lower this background, a *tetA-sacB* selection/counter-selection system has been recently developed. One of the earliest counter-selections reported used the *tetAR* genes of the *Tn*10 transposon (Bochner et al., 1980; Maloy and Nunn, 1981). TetR is a repressor of *tetA* and is inactivated by tetracycline. The TetA protein enables both a selection for Tet^R and counter-selection against it. The TetA protein inserts into the bacterial membrane where it serves as an efflux pump for tetracycline, thus allowing selection for tetracycline resistance. However, the TetA protein also causes the cells to become sensitive to lipophilic chelating agents, such as fusaric acid. Therefore, fusaric acid and similar chelating compounds can be used to select against tetracycline-resistant strains, as first reported by Bochner et al. (1980) and modified by Maloy and Nunn (1981). Separating *tetR* from *tetA* allows its constitutive expression and use as a selectable cassette. The combination of *tetA* and *sacB* allows selection for cassette insertion by Tet^R, and counter-selection against *tetA* and/or *sacB* (Li et al., 2013). The use of these cassettes is described in Support Protocol 1. The presence of TetA in the membrane enhances the counter-selection against SacB on sucrose plates.

galK

Another selection/counter-selection commonly used for BAC work utilizes GalK (Warming et al., 2005). To use this selection/counter-selection, a special bacterial strain, SW102, is required; this DH10B derivative contains the defective λ prophage and a precise deletion of the *galK* gene. For the *galK* system, a wild-type *galK* gene driven by a constitutive promoter is amplified by the PCR with chimeric primers and targeted to the region to be modified. The *galK* insertion is selected on minimal plates with galactose as the sole carbon source, supplemented with biotin and leucine to complement auxotrophies in SW102 and chloramphenicol (or other appropriate drug) for BAC maintenance. During the counter-selection step, the *galK* gene is replaced with the genetic modification of choice. Here selection is against the *galK* gene on plates containing a galactose analogue, 2-deoxy-galactose (DOG) (Alper and Ames, 1975). In the cell, the GalK enzyme phosphorylates DOG, resulting in the buildup of a toxic compound, 2-deoxy-galactose-1-phosphate. The primary advantage of the GalK selection/counter-selection is that *galK* is smaller than a dual cassette such as *cat-sacB*, thus easier to amplify. Strain SW102 and the p*galK* plasmid template for *galK* amplification are available from the Biological Resources Branch of the Frederick National Laboratory for Cancer Research (*http://ncifrederick.cancer.gov/RESEARCH/BRB/*).

rpsL

Yet another selection/counter-selection system utilizes the *rpsL* gene, encoding the S12 ribosomal subunit. Mutations exist in *rpsL* that make the strain resistant to streptomycin, and a few commonly used *E. coli* bacterial strains have this mutation, notably DH10B. The selection/counter-selection cassette contains a wild-type *rpsL* gene linked to a drug marker. When both *rpsL* alleles are expressed in the same cell, that cell becomes sensitive to streptomycin, since the wild-type gene is dominant. Therefore, once the dual cassette is targeted to a region, replacement of the cassette

with the DNA of choice will remove the wild-type copy of *rpsL* and restore Strep resistance. The *rpsL* gene has been linked to numerous selectable drug markers, including *neo*, *bla*, and *tetA* (Bird et al., 2011). A drawback to this system is that the cells necessarily have a mutant copy of the *rpsL* gene, which will also be a target for recombination with the dual cassette, thus some fraction of the recombination will occur there, restoring the wild-type allele. In *recA*$^+$ cells, the *rpsL*$^+$and *rpsL*^{Str} alleles can also recombine via the *E. coli* generalized recombination system; this is not an issue in the *recA* mutant DH10B. For RecA$^+$ strains, to circumvent adventitious recombination problems, an *rpsL* gene from *P. luminescens* has been substituted for *E. coli* wild-type *rpsL* (Bird et al., 2011). This gene is different enough in sequence from the *rpsL*^{Str} gene to prevent homologous recombination.

thyA

Like *galK*, the single gene *thyA* can also be used for counter-selection. The *thyA* gene encodes thymidylate synthase, which is necessary for conversion of dUMP to dTTP. A *thyA* mutant will not grow in minimal medium unless it is supplemented with thymine, thus the *thyA* cassette can be selected on minimal medium lacking thymine. Counter-selection against ThyA$^+$ is possible on minimal medium containing thymine and trimethoprim because the enzyme requires a cofactor, tetrahydrofolate (THF), which is converted to dihydrofolate (DHF) during the reaction. THF is regenerated from DHF by dihydrofolate reductase, which is inhibited by trimethoprim. Thus, when a functional *thyA* gene is present, the thymidylate synthase enzyme will utilize the THF, depleting it from the cell. Inhibition of dihydrofolate reductase by trimethoprim will prevent THF regeneration and thus cell growth, since THF is required for a number of cellular reactions. A *thyA* null strain containing the defective prophage, QW1, is available from the Huang laboratory (Wong et al., 2005).

tolC

A selection/counter-selection was developed for the channel protein TolC (DeVito, 2008). The killing function colicin E1 requires a functional TolC channel for access to the cytoplasmic membrane, thus *tolC* mutants are colicin-tolerant. For this system to work, the *tolC* gene was deleted from DY329 to make RS206, which became colicin-tolerant. A *tolC* cassette was targeted to a number of genes in this background, making cells sensitive to the colicin; the cassette was subsequently removed with either an ssDNA oligo or a dsDNA PCR, selecting in the presence of colicin E1. The major disadvantage of this particular selection/counter-selection is that colicin E1 is no longer commercially available.

Ampicillin enrichment

While removing a bacteriostatic drug marker (such as tetracycline), enrichment for recombinants is possible using ampicillin. Nonrecombinant bacteria will still express resistance to the *tet* marker encoded on the chromosome; when the electroporated culture is propagated in the presence of both tetracycline and ampicillin, only nonrecombinant cells will grow; these nonrecombinants will be killed by the ampicillin (Murphy et al., 2000). Recombinant cells will not divide because they lack the Tet^R marker, and because they do not divide, they are not killed by ampicillin.

Critical Parameters

Choice of recombineering system

Although this unit mentions various ways of expressing recombineering functions, the authors have the most experience with the Red-mediated recombineering that expresses the Red functions under the control of the λ P_L promoter. These constructs express recombineering functions either from a defective prophage on the *E. coli* chromosome or on low copy plasmids. A side-by-side comparison of this system with two other expression systems, the arabinose-inducible plasmid pDK119 (Datsenko and Wanner, 2000; Datta et al., 2006) and the IPTG-inducible plasmid pKM208 (Murphy and Campellone, 2003), has been done. It has been found that the former gives about ten-fold lower recombination efficiencies than the prophage, while pKM208 gives similar frequencies to the prophage but has elevated recombination levels even when uninduced. Other expression systems have not been carefully examined. For these reasons, the researcher should not expect to attain the frequencies reported here for the λ prophage when using other expression systems. If a different means of induction is chosen, be sure to make the modifications detailed in Basic Protocol 1.

Induction times

When inducing the recombination functions from the defective prophage, the proper induction time is essential. Longer induction times may cause decreased cell viability, since

the prophage Kil function is also induced by the temperature shift and will eventually kill the cells. The Gam function, necessary for efficient transformation of dsDNA, is also toxic to the cells (Sergueev et al., 2001). When using a chemical inducer such as arabinose or IPTG, the overnight culture can be diluted into broth supplemented with the inducer.

Storage of induced cells

It is also important to use the induced cells promptly, since the induced phage functions will decay over time, especially at 32°C. Induced cells can be successfully stored on ice for several hours before electroporation without a reduction in recombination efficiency. As detailed in Basic Protocol 1, competent cells may be frozen in 15% glycerol, although their recombineering efficiency has been about ten-fold less than that of the same cells freshly prepared.

Amplification of drug cassettes

It is strongly recommended that each drug cassette be amplified with only one standard set of primers. Confusion has arisen in the authors' laboratory when individuals have designed their own primers for the drug cassettes. Cassettes with different endpoints can cause differential expression and functions during the course of a construction project. Standard priming sequences for the commonly used cassettes are listed in Table 1.16.3, which amplify well in PCR reactions and give selectable recombinants.

Amplification of the cat-sacB and tetA-sacB elements

It is preferable to amplify the *cat-sacB* and *tetA-sacB* DNA cassettes described in Support Protocol 1 from the suggested bacterial strains using colony PCR as described in Basic Protocol 1. Since the PCR products are ~3.5 kb, they are not always easy to amplify. Optimized PCR conditions are detailed above, but other conditions and any other high-fidelity enzyme should also work. Getting the PCR to work is really an empirical problem. The biggest factors, if sufficient extension times and appropriate temperatures are used, are the PCR machine itself and the polymerase. Adjusting the Mg^{2+} concentration may also improve yield. There has been some confusion regarding the primers for amplification of the *cat-sacB* cassette. An optimal primer pair has been designed for amplification of this DNA, and it is strongly recommended that only this set of primers be used (Table 1.16.3).

Outgrowth procedures

The exact details of the outgrowth procedure will vary depending on the type of recombineering reaction performed and how the recombinants will be identified. The bacterial cells are fragile following electroporation and always require a 30-min minimal recovery period in LB at 30° to 32°C before plating. When screening for high-efficiency oligo recombinants, plate the cells non-selectively on LB-plates immediately after the 30-min recovery, before chromosome segregation occurs (Sawitzke et al., 2007). A longer outgrowth is needed to allow expression of selectable drug markers encoding antibiotic resistance. Routinely, at least 2 hr of recovery in broth (without drug) is allowed before plating the cells when selecting drug resistance. The counter-selection step of selection/counter-selections requires a still longer outgrowth of at least 3 hr, optimally, with a ten-fold dilution, to promote complete chromosome segregation. These can also be outgrown overnight and plated the next day.

Enhancing the efficiency of oligonucleotide recombination by using a host deficient in mismatch repair

When point mutations and small changes are desired, extremely high levels of recombinants can be obtained with oligo recombination if the bacterial strain is mutant for the host methyl-directed mismatch repair system (the *mutHLS* system; Costantino and Court, 2003). Under these conditions, ~20% to 50% of the total viable cells are recombinant. Strains containing the prophage and mutations in the mismatch repair system are available from the Court laboratory: HME63 and HME68 are mutant for the MutS protein that binds the mismatch, these contain a *mutS<>amp* and a *mutS<>cat* mutation, respectively; while HME64 is mutant for the UvrD helicase gene involved in removal of the unmethylated strand of mispaired DNA and contains *uvrD<>kan* (Costantino and Court, 2003). Any of these mutations will eliminate mismatch repair. The same elevated level of recombination can be obtained in strains proficient for mismatch repair when the incoming oligo creates configurations of mispairs that are not recognized by the wild-type mismatch repair system as described below.

Enhancing the efficiency of oligonucleotide recombination in a Mut$^+$ (mismatch repair-proficient) host

Extremely high levels of oligo recombination, equal to that obtained in the absence

of mismatch repair (i.e., approaching >50%), can be obtained in a Mut^+ host (i.e., a host that is wild type for all mismatch repair functions) host by modifications in oligo design (Sawitzke et al., 2011). For example, C-C mispairs are not subject to mismatch repair, but this is of limited usefulness. If the desired point mutation is embedded in a small (~4 to 5 base) cluster of bases that do not pair when the oligo is annealed to the complementary target sequence, the mispaired bubble will generally escape mismatch repair, thus the recombinant can be formed at high efficiency. A more general and very useful technique to abolish mismatch repair is to flank the desired change by four or five silent changes in adjacent wobble codons (i.e., third position changes); these wobble changes can also be all to one side of the desired change (Sawitzke et al., 2011). The wobble mispairs also generally prevent mismatch repair from acting on the recombinant, thus the modification will be created efficiently. Since this method does not result in amino acid changes in the wobble positions, it can be used to modify essential genes. Either type of change can be identified by a colony PCR screening method similar to the mismatch amplification mutation assay, MAMA-PCR (Cha et al., 1992; Swaminathan et al., 2001). For either of the two types of oligo design, if the newly created base changes that flank the desired modification are deemed undesirable, they can be removed by a second round of recombination using an oligo containing only the desired change. This will allow restoration of the original wobble bases while retaining the desired alteration. This procedure is detailed in Support Protocol 3. Note that occasionally recombinants are not found by this method, in which case an MMR deficient strain should be used.

Detecting recombinants

If a drug marker has been engineered into the DNA, drug resistance will serve as the selection. The two-step selection/counterselections allow precise modification without leaving an antibiotic-resistance cassette or other marker behind. Using colony hybridization with a labeled oligo probe has also been successful, although a unique sequence hybridizing only to the probe must be inserted (or a unique sequence must be created by a deletion) for this method to be an option. A PCR technique for detecting small changes created by oligos is described in Support Protocol 3.

Recombineering onto plasmids

When considering using recombineering to modify a plasmid, it is important to first determine whether it is the appropriate method to use. Recombineering targeted to a plasmid generally works as efficiently as when it is used to target the bacterial chromosome, and the same guidelines apply. This means that when a lagging-strand oligo is used for recombineering in the absence of mismatch repair, point mutations and modifications of a few bases can be made at a frequency that allows their isolation in the absence of selection. Mismatch repair can be eliminated either by use of the mismatch repair mutant strains or by careful design of mispaired bases when the oligo is annealed to the target. For insertion and deletion of larger segments of DNA, however, standard cloning techniques may be preferable, since in vitro cloning methods are more efficient than recombineering for these reactions. Recombineering-mediated insertion and deletion of larger pieces of DNA on plasmids occurs at a lower efficiency than creation of point mutations; thus a selection or way to separate recombinant plasmids from unmodified parental plasmids is required. If the goal is simply to insert or remove a segment of DNA and the exact junctions are not critical, in vitro cloning will often serve the purpose well. Recombineering, however, allows creation of a precise nucleotide sequence at the junctions between two DNAs, which is difficult with standard cloning.

The formation of circular plasmid multimers during recombineering complicates isolation and analysis of recombinants, since only one of the target sites on a multimeric plasmid is likely to be modified. Thus, although the recombinant species can often be enriched for by restriction endonuclease-mediated destruction of the parental plasmid molecules, destroying parental plasmid incorporated into these multimers will also result in loss of recombinant plasmids. It may be preferable to convert the plasmid population to a monomer first by digesting it with a restriction enzyme that cuts at a unique site in both parental and recombinant plasmids, then ligating under dilute conditions. The resulting monomer population can then be enriched for recombinants by digestion of the monomer parental molecules. It should be noted that these circular multimer plasmid species arising during recombineering are not identical to the linear multimers observed by Cohen and Clark (1986) after expression of the λ Gam function. Those linear multimers arise from Gam-mediated inhibition of the RecBCD

exonuclease, allowing the plasmid to replicate by the rolling circle mode of DNA replication. Plasmid multimers that form during recombineering are circular and are found in the recombinant plasmid population after oligo recombination when only the λ Beta protein (in the absence of Gam) has been used to catalyze the reaction (Thomason et al., 2007).

The plasmid DNA can be introduced into the cell at the same time as the linear substrate DNA (co-electroporation), or it can be resident in the cell prior to electroporation. Each option has advantages and disadvantages. Co-electroporation allows more control over the number of plasmids introduced per cell, but is less convenient if the plasmid is large (>10 kb). In the protocols described here, directions are given for co-electroporation. To target a resident plasmid, the episome should first be introduced into a recombineering-proficient *recA* mutant host strain.

Recombineering onto BACs

Red and RecET recombineering has also been optimized for engineering bacterial artificial chromosomes (BACs) (Muyrers et al., 1999; Copeland et al., 2001; Lee et al., 2001; Swaminathan and Sharan, 2004; Sharan et al., 2009), which are able to accommodate hundreds of kilobases of foreign DNA. DY380 is the bacterial host of choice for use with BACs; it is a DH10B derivative containing the defective prophage, as is SW102, a DY380 derivative that has been modified to enable the GalK selection/counter-selection. The recombineering plasmids listed in Table 1.16.3 may also be useful in manipulating BACs.

Undertaking in vivo assembly with overlapping oligos

Complementary oligos will anneal in vivo when introduced by electroporation into cells expressing Exo and Beta protein. Multiple overlapping oligos can be used to build moderately sized DNA products (100 to 150 bp) in an in vivo reaction (Yu et al., 2003) similar to PCR assembly (Stemmer et al., 1995). When flanking homologies to chromosomal targets are added, these overlapping oligos will assemble into a linear dsDNA in vivo and recombine into the target.

Mutating essential genes

It is possible to create point mutations in essential genes using oligo recombination and identifying them by a PCR screen as described above and detailed in Support Protocol 3. Deletion of an essential gene usually results in cell death. Such a deletion can be recovered in a diploid state; however, the cell will maintain a wild-type copy of the gene as well as a mutant version. This diploid state can usually be detected by PCR analysis (Bubuenenko et al., 2007).

Moving the defective prophage used for recombineering to a different background

If a different host is required for recombineering, the defective prophage can be moved by P1 transduction (*UNIT 1.17*; Miller, 1972; Yu et al., 2000). Grow a P1 lysate on DY329 and transduce the strain of choice. Select for tetracycline resistance and screen for temperature sensitivity or an inability to grow in the absence of biotin. Check the genotype of the strain first and supplement the minimal plates to complement any additional auxotrophies when performing the biotin screen.

Troubleshooting

Troubleshooting dsDNA recombination

A number of factors can account for problems in obtaining adequate recombination frequencies. It has been observed that the final recombination efficiency obtained results from the cumulative effect of a number of different factors, with each making an independent contribution. Thus, if one or more of these parameters are not optimal, the recombination frequency can be dramatically reduced. These parameters include the following. (1) Induction of the recombination proteins: To obtain good induction using a temperature shift, it is essential to use a 42°C water bath with aeration. Air shakers will not provide adequate heat exchange. Be sure to chill the cells thoroughly in an ice-water slurry after induction. (2) Too few viable cells: If recombinants are not obtained when inserting a drug marker, take the time to determine the titer of viable cells following outgrowth. There should be $\sim 10^8$ viable cells per milliliter in the culture. If there are far fewer (i.e., $<10^7$ cells/ml), recombinants may not be found. Take care when washing the cells with water, as the cell pellet is very soft and can easily be lost if the aqueous supernatant is not decanted promptly. Inadequate time for cell recovery after electroporation and before plating can also result in poor viability. (3) Inappropriate antibiotic concentration: When plating the cells on antibiotic plates, be sure to use the lower drug concentrations suggested for single-copy genes rather than the concentrations appropriate for multi-copy plasmids.

Applying double drug selections by the use of petri plates containing more than one antibiotic is not suggested. (4) Problems with DNA uptake. Electroporation is used to introduce the linear DNA substrates, thus problems with the electroporation apparatus can prevent recombinant formation. If this is a concern, test the efficiency of the electroporation device by introducing a super-coiled plasmid into the cells. Inadequate cleaning of the PCR product can result in a low time constant with consequent poor DNA uptake. Growing cells in minimal medium also causes poor DNA uptake (Sawitzke et al., 2011) for reasons that are not understood. (5) Non-optimal expression of Red functions. While convenient, the plasmids described in Datsenko and Wanner (2000) give about ten-fold fewer recombinants than does the prophage. A comparison of the two expression systems is given in Datta et al. (2006). (6) Too much NaCl in LB reduces cell viability. Use 5g/liter rather than 10 g/liter.

Troubleshooting oligo recombination

General comments in the previous section for dsDNA recombination also apply here. Many of the difficulties in obtaining high oligo recombination frequencies are discussed in Sawitzke et al., (2011). As well as the problems mentioned above, troubles specific to oligo recombination could be caused by the following. (1) Using an inappropriate oligo length—70-nt oligos are routinely used. (2) Attempting reactions other than creating point mutations or changes of a few bases (i.e., removing a large piece of DNA is not a highly efficient reaction). (3) Using a leading-strand oligo rather than a lagging-strand. (4) Using too little oligonucleotide, which allows subsequent degradation by ss-exonucleases. (5) Methyl-directed mismatch repair correction of heteroduplex recombination intermediates. (6) Long outgrowth following electroporation, which reduces the apparent frequency of recombination by about six- to ten-fold because it allows complete segregation of the recombinant bacterial chromosome away from its nonrecombinant siblings. In addition, if the recombinant confers a slow-growth phenotype, the mutant will be diluted further. (7) Use of a different recombinase. Datta et al. (2008) observed ~100-fold fewer recombinants for RecT-mediated oligo recombination than for that mediated by Beta.

Anticipated Results

As discussed above, when the prophage system is used to express the phage λ Red functions under optimized conditions, recombination with dsDNA containing a heterology usually has a frequency of 0.01%. Homeologous (partially homologous) dsDNA gives a somewhat higher frequency, and ssDNA can approach a frequency of 50%. However, in practice, recombineering frequency may be partially context dependent; certain areas of the chromosome appear "hotter" for recombination than others (Ellis et al., 2001). Because of this high efficiency, there should rarely, if ever, be experiments where no recombinants are found. No guarantee as to achievable recombination efficiency is provided when other expression systems or alternative methods are used. If recombinants are not obtained, check the construct design and redesign primers if necessary, as this is one reason for failure. If the recombineering reaction does not work, a control experiment using known strains and oligos or PCR products, as described in Yu et al. (2000), Ellis et al. (2001), and Costantino and Court (2003), is recommended to verify that the predicted number of recombinants is obtained in these control reactions. Most recombineering failures occur because the protocol is not executed carefully or properly, or the desired construction is lethal.

Time Considerations

Once the desired construct has been designed and the linear targeting DNA has been generated or procured, the basic recombineering protocol can be executed in 1 day, with bacterial cultures started the evening before. The recombinants may take 1 to several days to grow on the selective plates, and it may take another day or so to confirm them. Thus, the whole procedure can generally be completed in <1 week, although selection/counterselections may take slightly longer.

Literature Cited

Adhya, S. and Gottesman, M. 1978. Control of transcription termination. *Ann. Rev. Biochem.* 47:967-996.

Alper, M.D. and Ames, B.N. 1975. Positive selection of mutants with deletions of the *gal-chl* region of the *Salmonella* chromosome as a screening procedure for mutagens that cause deletions. *J. Bacteriol.* 121:259-266.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.

Arber, W., Enquist, L., Hohn, B., Murray, N.E., and Murray, K. 1983. Experimental methods for use with lambda. *In* Lambda II (R. Hendrix, J. Roberts, F. Stahl, and R. Weisberg, eds.) pp. 433-471. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Bird, A.W., Erler, A., Fu, J., Hériché, J-K., Maresca, M., Zhang, Y., Hyman, A.A., and Stewart. A.F. 2011. High-efficiency counterselection recombineering for site-directed mutagenesis in bacterial artificial chromosomes. *Nat. Methods* 9:103-109.

Blomfield, I.C., Vaughn, V., Rest, R.F., and Eisenstein, B.I. 1991. Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. *Mol. Microbiol.* 5:1447-1457.

Bochner, B.R., Huang, H.C., Schieven, G.L., and Ames, B.N. 1980. Positive selection for loss of tetracycline resistance. *J. Bacteriol.* 143:926-933.

Bubuenenko, M., Baker, T., and Court, D.L. 2007. Essentiality of ribosomal and transcription antitermination proteins analyzed by systematic gene replacement in *Escherichia coli*. *J. Bacteriol.* 189:2844-2853.

Carter, D.M. and Radding, C.M. 1971. The role of exonuclease and β protein of phage λ in genetic recombination. II. Substrate specificity and the mode of action of λ exonuclease. *J. Biol. Chem.* 246:2502-2512.

Cha, R.S., Zarbl, H., Keohavong, P., and Thilly, W.G. 1992. Mismatch amplification mutation assay (MAMA): Application to the c-H-ras gene. *PCR Methods Appl.* 2:14-20.

Cohen, A. and Clark, A. J. 1986. Synthesis of linear plasmid multimers in *Escherichia coli* K-12. *J. Bacteriol.* 167:327-335.

Copeland, N.G., Jenkins, N.A., and Court, D.L. 2001. Recombineering: A powerful new tool for mouse functional genomics. *Nat. Rev. Genet.* 2:769-779.

Costantino, N. and Court, D.L. 2003. Enhanced levels of lambda Red-mediated recombinants in mismatch repair mutants. *Proc. Natl. Acad. Sci. U.S.A.* 100:15748-15753.

Court, D.L., Sawitzke, J.A., and Thomason, L.C. 2002. Genetic engineering using homologous recombination. *Ann. Rev. Genet.* 36:361-388.

Court, D.L., Swaminathan, S., Yu, D., Wilson, H., Baker, T., Bubunenko, M., Sawitzke, J., and Sharan, S.K. 2003. Mini-lambda: A tractable system for chromosome and BAC engineering. *Gene* 315:63-69.

Datsenko, K.A. and Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* 97:6640-6645.

Datta, S., Costantino, N., and Court, D.L. 2006. A set of recombineering plasmids for Gram-negative bacteria. *Gene* 379:109-115.

Datta, S., Costantino, N., Zhou, X., and Court, D.L. 2008. Identification and analysis of recombineering functions from Gram-negative and Gram-positive bacteria and their phages. *Proc. Natl. Acad. Sci. U.S.A.* 105:1626-1631.

DeVito, J.A. 2008. Recombineering with *tolC* as a selectable/counter-selectable marker: Remodeling the rRNA operons of *Escherichia coli*. *Nucleic Acids Res.* 36:e4.

Ellis, H.M., Yu, D., DiTizio, T., and Court, D.L. 2001. High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 98:6742-6746.

Fu, J., Bian, X., Hu, S., Wang, H., Huang, F., Seibert, P., Plaza, A., Xia, L., Müller, R., Stewart, A.F., and Zhang, Y. 2012. Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. *Nat. Biotechnol.* 30:440-446.

Gay, P., Le Coq, D., Steinmetz, M., Berkelman, T., and Kado, C.I. 1985. Positive selection procedure for entrapment of insertion sequence elements in Gram-negative bacteria. *J. Bacteriol.* 164:918-921.

Hall, S.D., Kane, M.F., and Kolodner, R. 1993. Identification and characterization of the *Escherichia coli* RecT protein, a protein encoded by the *recE* region that promotes renaturation of homologous single-stranded DNA. *J. Bacteriol.* 175:277-287.

Jiang, W., Bikard, D., Cox, D., Zhang, F., and Marraffini, L.A. 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat. Biotechnol.* 31:233-239.

Joseph, J.W. and Kolodner, R. 1983. Exonuclease VIII of *Escherichia coli*. II. Mechanism of action. *J. Biol. Chem.* 258:10418-10424.

Karu, A.E., Sakaki, Y., Echols, H., and Linn, S. 1975. The γ protein specified by bacteriophage λ. Structure and inhibitory activity for the RecBC enzyme of *Escherichia coli*. *J. Biol. Chem.* 250:7377-7387.

Kulkarni, S.K. and Stahl, F.W. 1989. Interaction between the *sbcC* gene of *Escherichia coli* and the *gam* gene of phage λ. *Genetics* 123:249-253.

Lee, E.C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D.A., Court, D.L., Jenkins, N.A., and Copeland, N.G. 2001. A highly efficient *Escherichia coli*–based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 73:56-65.

Li, X., Thomason, L.C., Sawitzke, J.A., Costantino, N., and Court, D.L. 2013. Positive and negative selection using the *tetA-sacB* cassette: Recombineering and P1 transduction in *Escherichia coli*. *Nucleic Acids Res.* 41:e204.

Little, J.W. 1967. An exonuclease induced by bacteriophage λ. II. Nature of the enzymatic reaction. *J. Biol. Chem.* 242:679-686.

Maloy, S.R. and Nunn, W.D. 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. *J. Bacteriol.* 145:1110-1111.

Maresca, M., Erler, A., Fu, J., Friedrich, A., Zhang, Y., and Stewart, A.F. 2010. Single-strand heteroduplex intermediates in λ Red homologous recombination. *BMC Mol. Biol.* 11:54.

Miller, J.M. 1972. Generalized transduction: Use of P1 in strain construction. *In* Experiments

in Molecular Genetics (J.M. Miller, ed.) pp. 201-205. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mosberg, J.A., Lajoie, M.J., and Church, G.M. 2010. Lambda Red recombineering in *Escherichia coli* occurs through a fully single-stranded intermediate. *Genetics* 186: 791-799.

Muniyappa, K. and Radding, C.M. 1986. The homologous recombination system of phage λ. Pairing activities of β protein. *J. Biol. Chem.* 261:7472-7478.

Murphy, K.C. 2012. Phage recombinases and their applications. *Adv. Virus Res.* 83: 367-414.

Murphy, K.C. and Campellone, K.G. 2003. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli*. *BMC Mol. Biol.* 4:11.

Murphy, K.C., Campellone, K.G., and Poteete, A.R. 2000. PCR-mediated gene replacement in *Escherichia coli*. *Gene* 246:321-330.

Muyrers, J.P.P., Zhang, Y., Testa, G., and Stewart, A.F. 1999. Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res.* 27:1555-1557.

Noirot, P. and Kolodner, R.D. 1998. DNA strand invasion promoted by *Escherichia coli* RecT protein. *J. Biol. Chem.* 273:12274-12280.

Oppenheim, A.B., Rattray, A.J., Bubunenko, M., Thomason, L.C., and Court, D.L. 2004. In vivo recombineering of bacteriophage λ by PCR fragments and single-strand oligonucleotides. *Virology* 319:185-189.

Russell, C.B., Thaler, D.S., and Dahlquist, F.W. 1989. Chromosomal transformation of *Escherichia coli recD* strains with linearized plasmids. *J. Bacteriol.* 171:2609-2613.

Rybalchenko, N., Golub, E.I., Bi, B., and Radding C.M. 2004. Strand invasion promoted by recombination protein β of coliphage λ. *Proc. Natl. Acad. Sci. U.S.A.* 101:17056-17060.

Sawitzke, J.A., Thomason, L.C., Costantino, N., Bubunenko, M., Datta, S., and Court, D.L. 2007. Recombineering: In vivo genetic engineering in *E. coli*, *S. enterica*, and beyond. *Methods Enzymol.* 421:171-199.

Sawitzke, J.A., Costantino, N., Li, X.T., Thomason, L.C., Bubunenko, M., Court, C., and Court, D.L. 2011. Probing cellular processes with oligo-mediated recombination and using the knowledge gained to optimize recombineering. *J. Mol. Biol.* 18:45-59.

Schofield, M.J. and Hsieh, P. 2003. DNA mismatch repair: Molecular mechanisms and biological function. *Annu. Rev. Microbiol.* 57:579-608.

Sergueev, K., Yu, D., Austin, S., and Court, D. 2001. Cell toxicity caused by products of the *P*L operon of bacteriophage lambda. *Gene* 272:227-235.

Sharan, S., Thomason, L.C., Kuznetsov S.G., and Court, DL. 2009. Recombineering: A homologous recombination-based method of genetic engineering. *Nat. Protoc.* 4:206-223.

Stemmer, W.P., Crameri, A., Ha, K.D., Brennan, T.M., and Heyneker, H.L. 1995. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Gene* 164:49-53.

Swaminathan, S. and Sharan, S.K. 2004. Bacterial artificial chromosome engineering. *Methods Mol. Biol.* 256:89-106.

Swaminathan, S., Ellis, H.M., Waters, L.S., Yu, D., Lee, E-C., Court, D.L., and Sharan, S.K. 2001. Rapid engineering of bacterial artificial chromosomes using oligonucleotides. *Genesis* 29:14-21.

Thomason, L.C., Myers, R.S., Oppenheim, A., Costantino, N., Sawitzke, J.A., Datta, S., Bubunenko, M., and Court, D.L. 2005. Recombineering in prokaryotes. *In* Phages: Their Role in Bacterial Pathogenisis and Biotechnology (M.K. Waldor, D.I. Friedman, and S.L. Adhya, eds.) pp. 383-399. ASM Press, Herndon, Virginia.

Thomason, L.C., Costantino, N., Shaw, D.V., and Court, D.L. 2007. Multicopy plasmid modification with phage λ Red recombineering. *Plasmid* 58:148-158.

Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., and Court, D.L. 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 97:5978-5983.

Yu, D., Sawitzke, J.A., Ellis, H., and Court, D.L. 2003. Recombineering with overlapping single-stranded DNA oligonucleotides: Testing a recombination intermediate. *Proc. Natl. Acad. Sci. U.S.A.* 100:7207-7212.

Warming, S., Costantino, N., Court, D.L., Jenkins, N.A., and Copeland, N.G. 2005. Simple and highly efficient BAC recombineering using *galK* selection. *Nucleic Acids Res.* 33:e36.

Wong, Q.N., Ng, V.C., Lin, M.C., Kung, H.F., Chan, D., and Huang, J.D. 2005. Efficient and seamless DNA recombineering using a thymidylate synthase A selection system in *Escherichia coli*. *Nucleic Acids Res.* 33:e59.

Zhang, Y., Buchholz, F., Muyrers, J.P.P., and Stewart, A.F. 1998. A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat. Genet.* 20:123-128.

Zhang, Y., Muyrers, J.P., Testa, G., and Stewart, A.F. 2000. DNA cloning by homologous recombination in *Escherichia coli*. *Nat. Biotechnol.* 12:1314-1317.