SPECIALIZED TECHNIQUES

Recombineering: Genetic Engineering in Bacteria Using Homologous Recombination

Over the past few years, in vivo technologies have emerged which, due to their efficiency and simplicity, already complement and may one day replace standard genetic engineering techniques. The bacterial chromosome and episomes can be engineered in vivo by homologous recombination using PCR products and synthetic oligonucleotides (oligos) as substrates. This is possible because bacteriophage-encoded recombination functions efficiently recombine sequences with homologies as short as 35 to 40 bases. This technology, termed recombineering, allows DNA sequences to be inserted or deleted without regard to presence or location of restriction sites.

To perform recombineering, a bacterial strain expressing a bacteriophage recombination system is required. The phage enzymes can be expressed from either their own promoter or from a heterologous regulated promoter. Expressing the genes from their endogenous phage promoter confers the advantage of tight regulation and coordinate expression, which results in higher recombination frequencies. This is an important advantage, since in many cases high recombination frequencies will be essential to obtaining a desired recombinant. The authors of this unit routinely use a defective prophage located on the *E. coli* chromosome, and have recently transferred the critical elements of this prophage to a number of different plasmids (Thomason et al., 2005; also see Commentary). In this prophage system, the phage recombination functions are under control of the bacteriophage temperature-sensitive *cI857* repressor. At low temperatures (30 to 34 C), the recombination genes are tightly repressed, but when the temperature of the bacterial culture is shifted to 42 C, they are expressed at high levels from the p_L promoter. In the plasmid construct of Datsenko and Wanner (2000), the recombination genes are located on a plasmid and expressed from the arabinose promoter. The Datsenko and Wanner plasmid and some of the authors' plasmid constructs have temperature-sensitive origins of DNA replication. The plasmid-based systems have the advantage of mobility—they can be transferred among different *E. coli* strains or to *Salmonella typhimurium* and possibly other gram-negative bacteria. However, using the prophage system located on the bacterial chromosome is more facile if the recombineering is targeted to a plasmid. After induction of the recombination functions, the modifying DNA, either a double-stranded (ds) PCR product or a synthetic single-stranded (ss) oligonucleotide (oligo), is introduced into the prophage-containing strain by electroporation. Recombinants are obtained either by selection or screening of the population of cells surviving electroporation. Once the desired construct is obtained, the prophage can be removed by another recombination. Alternatively, engineered alleles on the chromosome can be moved into a different host by P1 transduction. Plasmids with temperature-sensitive replication origins can be lost from the recombinant strain by growth at the appropriate temperature.

Preparation of electrocompetent cells that have expressed the recombineering functions from the promoter and their transformation with double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) is the first procedure described in this unit (see Basic Protocol 1). Support Protocol 1 describes a two-step method of making genetic alterations without leaving any unwanted changes. In the latter protocol, the first of the two series

Contributed by Lynn Thomason, Donald L. Court, Mikail Bubunenko, Nina Costantino, Helen Wilson, Simanti Datta, and Amos Oppenheim

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of steps is a recombineering reaction that replaces the sequence to be modified with an antibiotic-resistance cassette and a counter-selectable marker (e.g., *sacB*, which is toxic when cells are grown on medium containing sucrose; Gay et al., 1985). The second series of steps is a subsequent recombination that replaces the antibiotic cassette and the counter-selectable marker with the desired genetic alteration. Moreover, the same selection and counter-selection can 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 and moving it 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 Protocol 5 details modifications necessary when targeting multicopy plasmids with recombineering. Basic Protocol 2 describes the removal of the defective prophage. Finally, Alternate Protocols 1 and 2 present methods for recombineering with an intact prophage and for introducing mutations onto bacteriophage , respectively.

STRATEGIC PLANNING

Before attempting to modify the *E. coli* chromosome or a plasmid, the DNA sequence of the desired final construct should be determined. A DNA-analysis computer 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 designed final construct as electronic files facilitates the design of oligonucleotides to be used as primers for PCR or as ssDNA recombination substrates themselves. The computer-determined sequences also allow rapid design of primers to analyze and verify the potential recombinants. One must be aware of gene-regulation issues when designing the constructs. 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 of this unit 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 1.16.2. One must also be careful of possible polarity effects and avoid creating unwanted fusion proteins when generating recombinants.

The DNA substrate used to generate the recombinants depends on the desired change. For a sizeable insertion, such as a drug cassette, a PCR product is generated that contains 40 to 50 base pairs of flanking homology to the chromosomal or plasmid target at each end. This homology is provided at the 5 end of each synthetic primer. Following this region of chromosomal homology, 20 bases of homology to the drug cassette provides the primers to amplify the cassette sequence. Thus, two primers are designed that will each be 60 to 70 nucleotides (nt) long: the 5 ends provide homology to the targeted region and the 3 ends provide homology to the cassette (see Fig. 1.16.1). Careful primer design is crucial (see above). The efficiency of recombineering with dsDNA can approach 0.1% (Yu et al., 2000). If deletions, small substitutions, or base changes are desired, a synthetic single-stranded oligo of 70 to 100 nt can be used. The oligo should have 35 to 40 nt of complete homology flanking the alteration. Recombineering with ssDNA in wild-type *E. coli* containing the defective prophage gives efficiencies approaching 1% (Ellis et al., 2001), and if host mismatch repair is inactivated, either by mutation or by using an oligo that creates a C-C mismatch, a 20% to 25% recombination frequency is achievable (Costantino and Court, 2003). This extremely high frequency means that, for oligo recombination, it is possible to create recombinants without a selection and find them by screening. Since an oligonucleotide corresponding to the lagging strand of DNA replication is some 20-fold more efficient than its complement, it is worthwhile to determine the direction of replication through one's region of interest and use the oligo that corresponds to the lagging strand (Costantino and Court, 2003).

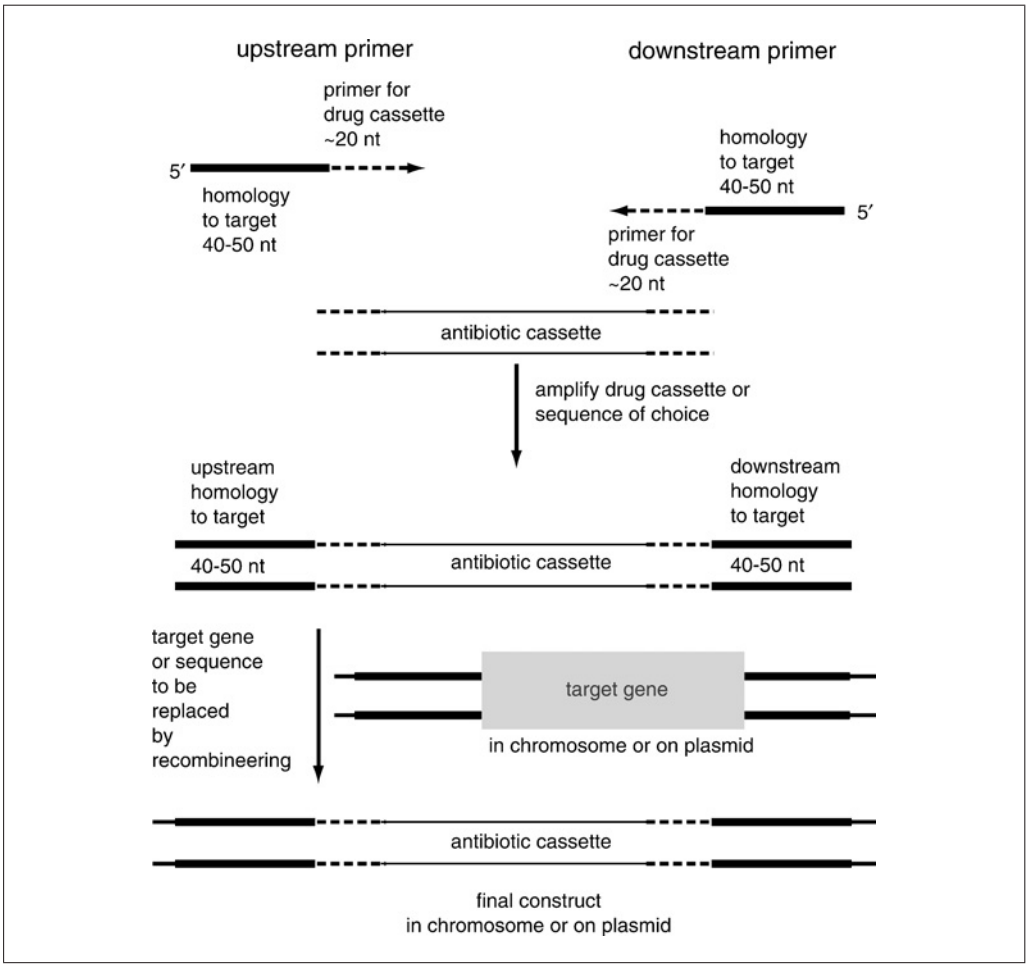

Figure 1.16.1 Targeting of an antibiotic cassette. Two primers with 5 homology to the target are used to PCR amplify the antibiotic cassette. The PCR product is introduced by electroporation into cells induced for the Red recombineering functions. The Red functions catalyze the insertion of the cassette at the target site, which may be on the bacterial chromosome or on a plasmid.

It is also possible to rescue or clone gene(s) from the bacterial chromosome onto a plasmid. To do this, create a linear PCR product of the plasmid using primers with homology flanking the target sequence, designed so that the sequence will be incorporated onto the circular plasmid in the appropriate orientation. The PCR-amplified linear plasmid will require an origin of DNA replication and a selective marker.

Multicopy plasmids can also be modified with recombineering, and changes occur at the same frequency as modifications of the *E. coli* chromosome (Thomason et al., 2007). This means that point mutations can be made at a frequency of 5% to 30% in the absence of mismatch repair. The frequency of insertion or removal of large DNA segments is much lower, however, and isolation of recombinant species will be problematic in the absence of a selection or screen. Designing the loss of a unique restriction site into the recombinant plasmid permits enrichment of the recombinant class by allowing destruction of the unmodified parental population by restriction digestion.

Some modification of Basic Protocol 1 is needed when targeting plasmids with recombineering, due to the fact that they exist in multiple copies in the cell. 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. This means that it is generally impossible to modify all copies of the episome of interest during a recombineering reaction, and thus cells containing

recombinant plasmids will also contain unmodified parental plasmid. The recombinant class of molecules can be separated from the unmodified class by plasmid DNA isolation and retransformation at a DNA concentration of less than one molecule per cell, which creates pure clonal populations of the recombinant plasmid species. Parental plasmids can also be eliminated by restriction digestion as mentioned above.

A complexity arising when plasmids are modified with recombineering is that circular multimeric plasmid species are formed during the recombination reaction. In the absence of linear substrate DNA, expression of the Red proteins 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 is added and modification of the target sequence occurs, circular multimeric species are formed. Multimer formation is thus a hallmark of a recombinant plasmid molecule (and could theoretically be used to identify recombinant species). It is important to begin recombineering with a monomer plasmid species and to do the reaction in a *recA* mutant bacterial strain defective in homologous recombination. Using a host defective in RecA-mediated recombination eliminates one route to plasmid-by-plasmid recombination.

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 bacteria. 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 be feasible.

BASIC PROTOCOL 1

MAKING ELECTROCOMPETENT CELLS AND TRANSFORMING WITH LINEAR DNA

This basic protocol describes making electrocompetent cells that are preinduced for the recombination functions and transforming them with the appropriate DNA to create the desired genetic change. As noted in Strategic Planning, the phage recombination functions are repressed by the phage temperature-sensitive *cI857* repressor, so that they are not expressed when the cells are grown at low temperature (30 to 34 C) but are highly expressed when the culture is shifted to 42 C. See Commentary for additional considerations before executing the procedure.

Materials

Purified PCR product or oligonuclotide primers with 40 to 50 bases of flanking homology on either side of desired change (also see *UNIT 15.1*)

Bacterial strain expressing the defective lambdoid prophage recombination system Red (Table 1.16.1; strains are available from the Court Laboratory; *court@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 µg/ml spectinomycin

30 to 32 C incubator

32 and 42 C shaking water baths

Table 1.16.1 Bacterial Strains Commonly Used for Recombineering

Strain	Genotype
DY329	W3110 *lacU169 nadA::Tn10 gal490 pgl* 8 *c*I*857 (cro bioA)* (TetR)
DY330	W3110 *lacU169gal490 pgl* 8 *c*I*857 (cro-bioA)*
DY331	W3110 *lacU169 srlA::Tn10 recA gal490 pgl* 8 *c*I*857 (cro-bioA)* (Tet^R)
DY378	W3110 *c*I*857 (cro-bioA)*
DY380[a]	*mcrA (mrr-hsdRMS-mcrBC)* 80d*lacZ* M15 *lacX74 deoR recA1 endA1 araD139 (ara, leu)7697 galU gal490 pgl* 8 *rpsL nupG (c*I*857ind1) {(cro-bioA)<>tetRA}* (Tet^R)
DY441	W3110 *gal490 pgl 8 c*I*857 (cro-bioA) int<>cat-sacB*
HME5	W3110 *lacU169 c*I*857 (cro-bioA)*
HME45[b]	W3110 *gal490 pgl* 8 *c*I*857 (cro-bioA)*
HME63	W3110 *lacU169 c*I*857 (cro-bioA) galKam mutS<>amp*
HME64	W3110 *lacU169 c*I*857 (cro-bioA) galKam uvrD<>kan*

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

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
0.1-cm electroporation cuvettes (Bio-Rad), chilled
Electroporator (e.g., Bio-Rad *E. coli* Pulser)

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*), examine it by agarose gel electrophoresis (*UNIT 2.5A*), and gel purify by isolating the desired band (*UNIT 2.6*) if unwanted products are obtained.

   If the DNA is 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 (*UNIT 2.1A*) or using a commercially available kit to remove salt.

   If a plasmid template is used to construct a PCR-amplified drug cassette, any intact circular plasmid remaining will transform the cells efficiently and give unwanted background. This 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. Always include a control reaction of uninduced cells transformed with the PCR product, to give a measure of any unwanted intact plasmid background.

Table 1.16.2 PCR Primers and Suggested Source of Template for Amplifying Drug Cassettes (from Yu et al., 2000)

Gene	Source	Primer sequence
Ampicillin	pBluescript SK(+) (Stratagene)	5 CATTCAAATATGTATCCGCTC 5 AGAGTTGGTAGCTCTTGATC
Tetracycline	*Tn10*	5 CAAGAGGGTCATTATATTTCG 5 ACTCGACATCTTGGTTACCG
Chloramphenicol	pPCR-Script Cam (Stratagene)	5 TGTGACGGAAGATCACTTCG 5 ACCAGCAATAGACATAAGCG
Kanamycin	*Tn5*	5 TATGGACAGCAAGCGAACCG 5 TCAGAAGAACTCGTCAAGAAG
Spectinomycin	DH5 PRO (Clontech)	5 ACCGTGGAAACGGATGAAGGC 5 AGGGCTTATTATGCACGCTTAA

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 at 30 to 32 C overnight.

   Most of the authors' strains containing the defective lambdoid prophage are W3110 derivatives; however, the prophage can be moved into other backgrounds. See Commentary for details. Plasmids expressing the recombination functions can be put into any strain of choice.

   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 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. One must make sure that one's dilution is at least 50-fold. Higher 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 from a plasmid, add antibiotic as appropriate to maintain selection during growth. If an alternate method of inducing the recombination functions is used (i.e., addition of arabinose), the inducer should be added to the medium. In this case remember to include an additional flask containing an uninduced culture as a negative control. Expression of the Red functions from the plasmids of Datsenko and Wanner (2000) is enhanced by use of 10 mM arabinose (for Ara$^+$ strains).

6. Place the flask in the 32 C shaking water bath and grow cells at 32 C with shaking for 2 hr.

   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 express the recombination functions well.

Induce recombination functions

7. Transfer half the culture to a 125-ml (baffled) Erlenmeyer flask and place that flask in 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.

The temperature shift is unnecessary when a chemical inducer like arabinose 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 another 30 ml ice-cold distilled water to each tube, seal, and gently invert to mix, again 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. Decant the 30-ml supernatant very carefully from the soft pellet in each tube and resuspend 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 resuspended cells to microcentrifuge tubes. Microcentrifuge 30 to 60 sec at maximum speed, 4 C. Carefully aspirate supernatant. In each of the tubes, resuspend the cell pellet in 200 µl 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 can be stored at 80 C after resuspending the cell pellet in 15% (v/v) glycerol. 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 electroporator other than Bio-Rad may work, but have not been tested in the authors' laboratory. The larger 0.2-cm cuvettes 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 10 to 100 ng of single-stranded oligonucleotide with 50 to 100 µl of the suspension of induced or uninduced cells (from step 12). Do the mixing and subsequent electroporation rapidly; 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 (for example, intact plasmid template from the PCR reaction will give rise to drug-resistant colonies here).

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

The time constant should be greater than 5 msec for optimal results. Low time constants indicate problems with the cells, the DNA, 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 sterile culture tubes and incubate the tubes with shaking at 30 to 34 C for 1 to 2 hr to allow expression of the antibiotic resistance gene.

Even if not selecting for drug resistance, it is still recommended that the cells outgrow to recover from the shock of electroporation. It has been observed in the authors' laboratory that omitting the outgrowth reduces the cell viability 10-fold.

An alternative, and in the author's experience more reliable, method for outgrowth is to spread appropriate dilutions of cells (see step 18 below) on a sterile 82-mm-diameter nitrocellulose filter atop a rich (LB) plate. Incubate this plate for 3 hr at 30 to 34 C. After the incubation, transfer the filter to the appropriate selective drug plate using sterile forceps. This method is preferable because the cells are less dense and more efficient outgrowth is achieved.

Determine cell titers

17. Make serial 1:10 dilutions of the electroporation mix through 10^{-6} using M9 medium or 1 TM buffer, dispensing 0.9 ml M9 or TM 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^{-5} and 10^{-6} dilutions on LB plates (rich plates without drug). Incubate the plates at 30 to 34 C for 1 to 2 days, 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 1:5 and 1:10 dilution.

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

For the no-DNA and uninduced controls, plate 200 µl directly on 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 above for chromosomal constructs. Minimal plates are used for selection based on prototrophy.

20. Incubate plates at the appropriate temperature (30 to 34 C).

At 30 C, colonies may take two days to come up on LB plates and 3 to 4 days on minimal plates. Candidates should be purified by streaking for single colonies 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 made. For an antibiotic cassette or other insertion, the primer pair used to amplify the insertion can also be used to confirm its presence. These primers will not determine whether the cassette has integrated at the desired location, however. The recombinant junctions can be confirmed with the help of two additional primers (all four should have compatible annealing temperatures) pointing outwards from the cassette; use one primer flanking the cassette and one internal cassette primer to amplify the unique junctions created by the recombination reaction. A 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.

MANIPULATING *cat-sacB* FOR COUNTER-SELECTION AND GENE REPLACEMENT

SUPPORT PROTOCOL 1

This protocol describes a two-step method to create precise genetic changes without otherwise altering the DNA sequence. First, *cat-sacB* (or another counter-selectable cassette) is placed on the DNA; this is then replaced with the desired alteration in a second recombineering event. The final construct will not have a drug marker.

Additional Materials *(also see Basic Protocol 1)*

Template for amplification of *cat-sacB*: bacterial strain DY441 (DY329 with a *cat-sacB* insertion on the *E. coli* chromosome) or the plasmid pEL04 (Lee et al., 2001). pEL04 has previously been called both pK04 and p*cat-sacB*. Both DY441 and pEL04 are available from the Court Laboratory; *court@ncifcrf.gov*.
*Spe*I and *Dpn*I restriction endonucleases (*UNIT 3.1*)
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
Invitrogen Platinum High Fidelity enzyme
LB-Cm plates: LB plates (*UNIT 1.1*) containing 10 μg/ml chloramphenicol
M63 minimal glycerol plates with sucrose (see recipe) or LB plates (*UNIT 1.1*) lacking NaCl but containing 6% (w/v) sucrose
Medium lacking carbon source: e.g., M9 medium (*UNIT 1.1*)
Thermal cycler (MJ Research PTC-100)

Amplify the cat-sacB element for recombineering

1. If using pEL04, completely cleave p*cat-sacB* with *Spe*I.

   This step is unnecessary if the chromosomal insertion of cat-sacB is used as template for PCR. Failure to digest when the plasmid is used as template will give a high background of intact plasmid transformants.

2. Amplify *cat-sacB*, either from the cleaved pEL04 or from DY441 with the Invitrogen Platinum High Fidelity enzyme and an MJ Research thermal cycler, using 50 pmol of each primer and the following cycling program:

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)

The PCR primers used to amplify the cat-sacB element are at the 3 end of chimeric primers that include 5 segments of bacterial target homology 40 to 50 nt in length. Hence each primer is 60 to 70 nt long.

Since the PCR product is greater than 3 kb, it can be difficult to amplify. The above conditions have been used successfully in the authors' laboratory.

3. If pEL04 was used as template, digest the completed PCR reaction with *Dpn*I to specifically eliminate the methylated plasmid template. Purify the PCR product to remove salt (*UNIT 2.1A*).

Perform electroporation and recombination to insert cat-sacB at the desired location

4. Insert the *cat-sacB* cassette into the chromosome as described above (see Basic Protocol 1, steps 4 to 21; also see Background Information for important tips) using the following techniques specific for the *cat-sacB* cassette.

   a. Select chloramphenicol-resistant (Cm^R) colonies and purify on LB-Cm plates to isolate single colonies.

   b. Test several Cm^R isolates for sensitivity to sucrose, either on minimal glycerol plates containing 5% sucrose or on LB plates lacking NaCl but containing 6% sucrose (Blomfield et al., 1991). Use the parental transformation strain as a sucrose-resistant 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 the PCR process generates mutations that inactivate sacB in a fraction of the clones.

Perform electroporation and recombination to replace cat-sacB with chosen allele

5. Use a confirmed Cm^R/sucrose-sensitive candidate from step 4 as the starting bacterial strain for a second round of recombineering by carrying out Basic Protocol 1, but suspend the electroporated cells at step 16 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) at 30 to 32 C for 4 hr to overnight for outgrowth following electroporation.

   The higher dilution promotes better cell recovery and allows complete segregation of recombinant chromosomes that no longer carry the cat-sacB cassette from nonrecombinant 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 sucrose sensitivity to the entire cell, thus preventing recovery of recombinants. This is generally true in counter-selection experiments and illustrates a common problem encountered with them.

6. Centrifuge cells 7 min at 4600 *g*, 4 C. Remove supernatant, then wash the cells twice, each time by resuspending in 1 ml minimal medium lacking a carbon source, such as M9, centrifuging again as before, and removing the supernatant. Resuspend and dilute the cells for plating, and spread appropriate dilutions of the cells on either

LB sucrose plates or minimal glycerol plates (if the defective prophage was moved to another strain, the metabolic requirements of that strain must be considered). In parallel, be sure to include a control of electroporated cells to which no PCR product was added, to determine the frequency of spontaneous sucrose-resistant mutants; these spontaneous mutants will retain the CmR cassette.

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 with the PCR product. The frequency of the latter is optimally 10- to 100-fold greater than that of spontaneous mutation.

7. Purify 10 sucrose-resistant colonies by streaking to isolate single colonies (*UNIT 1.3*) and then test for chloramphenicol sensitivity (Cm^S)—spontaneous mutants will be Cm^R while the recombinants will be Cm^S. Screen the Cm^S/sucrose-resistant colonies by PCR (see Basic Protocol 1, step 21), then sequence to confirm the presence of the desired change.

Screen a minimum of ten 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 a DNA sequence from the bacterial chromosome, either 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).

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 vector DNA by co-electroporation. Always remember to provide flanking homology so that the plasmid can recombine with the additional PCR product (it is easier to provide the homology on the short product to be cloned rather than on the vector).

Additional Materials (also see Basic Protocol 1)

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
Synthetic chimeric primers providing homology to sequence flanking gene of choice and to the plasmid sequence to be amplified

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. 1.16.2), since the plasmid template has the linear ends pointing toward each other. Both of the primers will have 50 nt of bacterial 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

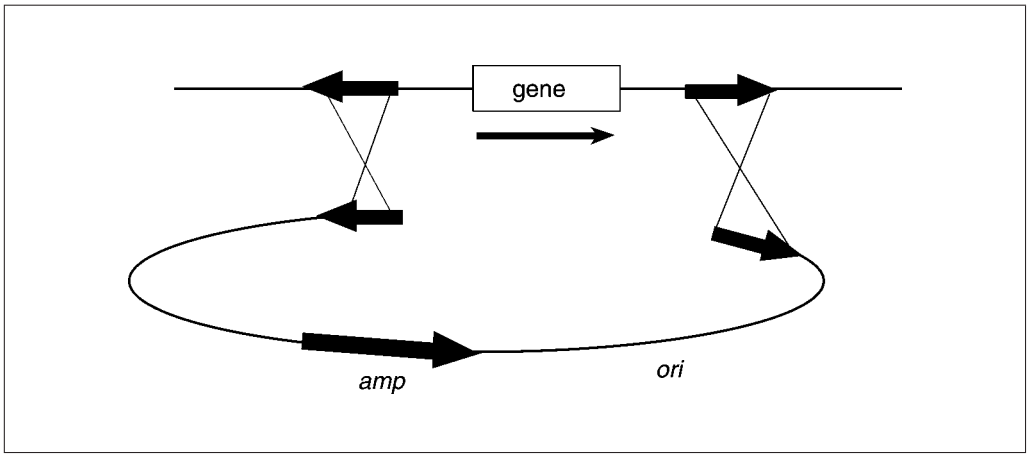

Figure 1.16.2 Cloning genes by gap repair of a plasmid. A linear plasmid with flanking homology to the target at the ends (indicated by dark arrows) is generated by PCR. The plasmid is introduced by electroporation into cells expressing the Red functions, which catalyze recombination of the vector with the target site, resulting in incorporation of the gene onto the plasmid.

that are homologous to the bacterial chromosome (e.g., lac); these can 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 will need to 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. Purify the PCR product to remove salt before proceeding.

Transform induced cells with the linear plasmid and select recombinants

3. 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. 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 DH5 or XL2 Blue, to generate pure clones.

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

   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 nonselectively. 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 religated vector is eliminated.

   Gap repair is less efficient than targeting to the chromosome. The maximal yield achieved in the authors' laboratory is 1000 recombinants/10^8 cells. 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.

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 nonselectively on LB. The authors have successfully used the nonradioactive Roche DIG (digoxigenin) system for colony hybridization 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 simply looking for colonies that grow more slowly than the majority class. Another useful method is the mismatch amplification mutation assay-PCR (MAMA-PCR; Cha et al., 1992; Swaminathan et al., 2001). MAMA-PCR is capable of identifying single base changes by screening colonies.

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 10^8, plate the cells nonselectively 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 4

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. If the oligonucleotide carrying the mutation to be introduced creates a C-C mismatch when paired at the target site, the recombination can be done in wild-type cells containing the defective prophage, since a C-C mismatch is not repaired. Otherwise, a bacterial strain with prophage and mutant for the mismatch repair system can be used. 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 *or*
oligonucleotide that creates C-C mismatch when annealed to target DNA strand: e.g., HME63 and/or HME64 (see Table 1.16.1)
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 oligo that creates a C-C mismatch 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.).

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 DH5 ; 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 step 14 of Basic Protocol 1, 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 step 15 of Basic Protocol 1. 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; let the culture 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 DH5 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-hour outgrowth, plate for single colonies on the appropriate media. Plate selectively to select recombinant colonies, or nonselectively on LB plates (plus

antibiotic for 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). Verify the presence of the desired modification on the monomer plasmid.

BASIC PROTOCOL 2

REMOVAL OF THE PROPHAGE BY RECOMBINEERING

Once the mutational changes are introduced, the defective 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.

Materials

Oligonucleotide primers for amplifying the bacterial *attB* site:
5 GAGGTACCAGGCGCGGTTTGATC 3
5 CTCCGGTCTTAATCGACAGCAAC 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 primers listed above, with an *E. coli* K12 strain lacking the prophage (e.g., W3110) as template (the PCR product is 2.5 kb), and the following program:

1 cycle:	2 min	94 C	(denaturation)
9 cycles:	15 sec	94 C	(denaturation)
	30 sec	65 C	(annealing)
	3 min	68 C	(extension)
19 cycles:	15 sec	94 C	(denaturation)
	30 sec	65 C	(annealing)
	3 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 touched with a sterile inoculating loop and mixed into the PCR reaction.

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

3. Wash the cells in minimal salts twice 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 2 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. As an additional confirmation that the prophage has been deleted, the same PCR primers can be used to confirm the presence of the 2.5-kb band in the purified strain.

RECOMBINEERING WITH AN INTACT PROPHAGE

ALTERNATE PROTOCOL 1

Basic Protocol 1 describes a method for recombineering using a defective lambdoid prophage. It is also possible to recombineer using an intact prophage (Court et al., 2003). If the phage of interest has a temperature-sensitive *cI857* repressor, 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 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.

Additional Materials *(also see Basic Protocol 1)*

Bacterial lysogen carrying the *cI857* bacteriophage of choice as a prophage
Chloroform
1× TM buffer (*APPENDIX 2*)

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

Additional reagents and equipment for plating phage to generate plaques (plaque purification; *UNIT 1.11*)

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. 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).

If the mutation of interest is targeted to the bacterial chromosome

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 hr at 30 to 32 C, then transfer the filter to the appropriate drug plate using sterile forceps.

The number of recombinants is generally less than 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 , 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

In this case, the 15-min induction time can be used.

3b. Dilute the electroporation mix into 5 ml LB medium and aerate by shaking in a shaking water bath 90 min at 39 C. Add 0.25 ml chloroform to complete cell lysis and release the phage particles. Dilute the lysate and plate for single plaques (see *UNIT 1.1*).

4b. Plaque-purify the positive candidates (*UNIT 1.11*). Resuspend 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). Resuspend 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 WITH THE DEFECTIVE PROPHAGE STRAINS

Sometimes it may be useful to target genetic changes to bacteriophage 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 follows. 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. 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 , 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)*

10% (w/v) maltose stock solution, filter sterilized
1 TM buffer (*APPENDIX 2*)
High-titer lysate of the bacteriophage to be engineered
PCR product with desired sequence changes and flanking homology to the target on the phage chromosome
Lambda plates and lambda top agar (see *UNIT 1.1*, adjust NaCl to 5 g per liter in both the plates and the top agar)
Chloroform
Appropriate bacterial indicator strain
Additional reagents and equipment for working with 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 the cells by centrifuging 7 min at 4600 *g*, 4 C. Resuspend pellet in 1 ml of 1 TM buffer. Infect the cells with the phage to be engineered at a multiplicity of 1 to 3 phages per cell.

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

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

   Phages other than may require different adsorption conditions.

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

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

5. Make 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 glucose plates

Per liter:
3 g KH_2PO_4
7 g K_2HPO_4
2 g $(NH_4)SO_4$
0.5 ml of 1 mg/ml $FeSO_4$
0.2% (v/v) glucose
0.001% (w/v) 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 up to several months at 4 C.

M63 minimal glycerol plates with sucrose

Per liter:
3 g KH_2PO_4
7 g K_2HPO_4
2 g $(NH_4)SO_4$
0.5 ml of 1 mg/ml $FeSO_4$

continued

0.2% (v/v) glycerol
5% (w/v) sucrose
0.001% (w/v) biotin
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 up to several months at 4 C.

COMMENTARY

Background Information

Bacteriophage encodes three genes important for recombineering. The *exo* and *bet* genes, respectively, encode a 5 to 3 double-strand exonuclease, Exo, and a single strand annealing protein, Beta, which together can recombine a double-stranded PCR product with short flanking homologies into the desired genetic target. The *gam* gene encodes a protein, Gam, that inhibits the RecBCD enzyme, which will otherwise degrade linear DNA introduced into the bacterial cell. Only the Beta single-strand annealing function is required to recombine single-stranded oligos containing the desired alterations. A cryptic lambdoid prophage, *rac*, is present in some strains of *E. coli*, and encodes RecE and RecT functions that are analogous to Exo and Beta, respectively. Unlike some other in vivo genetic engineering methods (Russell et al., 1989), recombineering does not require the host *recA* function. A strain mutant for *recA* provides more controllable recombination, since the strain is recombination proficient only when the phage functions are induced. A more detailed discussion of the molecular mechanism of recombineering can be found in Court et al. (2002).

Recently the Red genes have been moved to several plasmids (Table 1.16.3; Datta et al., 2006) having different DNA replication origins. 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 create the mutations in the prophage-containing strains and subsequently move them into a different background.

Critical Parameters and Troubleshooting

Induction times

When inducing the recombination functions from the defective prophage, the proper induction time is essential. Longer induction times will cause decreased cell viability, since the prophage Kil function is also induced by the temperature shift. The Gam function, necessary for efficient transformation of dsDNA, is also toxic to the cells (Sergueev et al., 2001).

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. Although one pioneering laboratory member reports that induced cells can be successfully stored on ice for several hours before electroporation, the authors do not recommend this procedure. As detailed in Basic Protocol 1, competent cells may be frozen in 15% glycerol, although their recombineering efficiency has been less than that of the freshly prepared cells.

Table 1.16.3 Plasmids Containing the Red System Under *cI857* Control

Plasmid designation	Plasmid origin	Approximate copy number/cell
pSIM5(Cm^R) pSIM6(Amp^R)	pSC101*ts*	16
pSIM7(Cm^R) pSIM8(Amp^R)	pBBR1	30-40
pSIM9(Cm^R)	pRK2*ts*	20-40

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, because a strain containing a drug cassette inserted by recombineering may be used later as a PCR template for that drug marker. Using a standard set of primers ensures that the homology used to amplify the drug cassette is always present, whatever strain is used for PCR amplification. Remember that it is best to have a promoter, the open reading frame, and a transcriptional terminator on each cassette. Standard priming sequences for the commonly used cassettes are listed in Table 1.16.2.

The cat-sacB element

The *cat-sacB* DNA segment described in Support Protocol 1 is amplified from plasmid pEL04, previously known as both pKO4 and p*cat-sacB*, or from the strain DY441, using colony PCR. If a different antibiotic resistance gene is required, the *cat* gene on p*cat-sacB* can be replaced with another drug marker. Use 50 bases of homology upstream of the 5 *cat* primer and downstream of the 3 *cat* primer to cross in the other marker. The sequence of pEL04 is available from the Court Laboratory (*court@ncifcrf.gov*). Since the *cat-sacB* PCR product is 3264 bp, it is not always easy to amplify. Optimized PCR conditions are detailed above, but other conditions and any other high-fidelity enzyme should also work. This 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. Please note that both primer sequences are different from those previously given in this protocol.

Detecting recombinants

If one has engineered a drug marker into the DNA, drug resistance will serve as the selection. The two-step *cat-sacB* replacement allows 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.

Mismatch repair minus conditions for oligonucleotide recombination

It has been reported (Costantino and Court, 2003) that extremely high levels of recombinants can be obtained when recombineering with oligos if the bacterial strain is mutant for the host methyl-directed mismatch repair system (the *mut HLS* system). Under these conditions 20% to 25% of the total viable cells are recombinant. Strains with the prophage and mutations in the mismatch repair system are available from the Court laboratory: these are HME63 and HME64 and contain a *mutS<>amp* and an *uvrD<>kan* mutation, respectively. Either mutation will eliminate mismatch repair; the latter strain can be used for engineering onto plasmids expressing ampicillin resistance. The same elevated level of recombination is obtained in mismatch repair proficient strains if the incoming oligo creates a C-C mismatch when annealed to the target DNA, since C-C mispairs are not subject to mismatch repair.

Counter-selections

The *cat-sacB* cassette is a counter-selection; it can be selected either for (by chloramphenicol resistance) or against (sucrose sensitivity). Several such counter-selections are available and may prove useful for particular situations; tetracycline resistance is another example (Bochner et al., 1980; Maloy and Nunn, 1981). If 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 nonrecombinants cells will grow; these nonrecombinants will be killed by the ampicillin (Murphy et al., 2000). Recombinant cells will not grow but will not be killed. 2-Deoxy-D-galactose is a toxic analog of galactose on which only mutants of galactokinase, the product of the *galK* gene, will grow (Alper and Ames, 1975). The *galK* selection has recently been adapted for use with BACs (Warming et al., 2005). Other counter-selections may be devised.

In vivo assembly with overlapping oligos

Complementary oligos will anneal in vivo when introduced by electroporation into cells

expressing Exo and Beta protein (Yu et al., 2003). Multiple overlapping oligos can be used to build moderately sized DNA products (100 to 150 bp) in an in vivo reaction similar to PCR assembly (Stemmer et al., 1995). By adding flanking homologies to chromosomal targets, these overlapping oligos are restored to linear dsDNA in vivo and recombined into the target.

Anticipated Results

As discussed above, ideally, transformation with dsDNA and ssDNA can approach frequencies of 0.1% and 25%, respectively. Because of this high efficiency, there should rarely if ever be experiments where no recombinants are found. 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). 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 are obtained in these control reactions. Most recombineering failures occur because the protocol is not executed carefully or properly.

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 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 creation of a C-C mispair 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 junction between two DNAs, which is not always possible with standard cloning.

The recombinant species can often be enriched for by destruction of the parental plasmid molecule. However, the formation of circular multimers during recombineering complicates isolation and analysis of recombinants, since only one of the target sites on a multimeric plasmid may be modified. Because of the propensity of recombinant plasmids to have multimerized, destroying parental plasmid will also result in loss of recombinant plasmids that are not modified at all target sites. It may be preferable to convert the plasmid population to 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 the circular multimer plasmid species arising during recombineering are not identical to the linear multimers observed by Cohen and Clark (1986) after expression of the Gam function. Plasmid multimers that form during recombineering are circular and are found in the recombinant plasmid population after oligo recombination when only the Beta protein 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 have been introduced into a recombineering-proficient *recA* mutant host strain.

*Engineering onto BAC***s**

Red recombineering has also been optimized for engineering bacterial artificial chromosomes (Copeland et al., 2001; Lee et al., 2001; Swaminathan and Sharan, 2004), 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. The recombineering plasmids listed in Table 1.16.3 may also be useful in manipulating BACs.

Mutating essential genes

Mutation of an essential gene usually results in cell death. Such a mutation 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.

Moving the prophage to a different background

If a different host is required for recombineering, the defective prophage can be moved by P1 transduction (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.

Time Considerations

The basic recombineering protocol can be executed in one day, with bacterial cultures started the evening before. The recombinants may take several days to grow on the selective plates, and it may take another day or so to confirm the recombinants.

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Contributed by Lynn Thomason, Donald L. Court, Mikail Bubunenko, Nina Costantino, Helen Wilson, Simanti Datta, and Amos Oppenheim
National Cancer Institute at Frederick
Frederick, Maryland