Chapter 21

Modifying Bacteriophage λ with Recombineering

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Abstract

Recombineering is a recently developed method of in vivo genetic engineering used in *Escherichia coli* and other Gram-negative bacteria. Recombineering can be used to create single-base changes, small and large deletions, and small insertions in phage λ as well as in bacterial chromosomes, plasmids, and bacterial artificial chromosomes (BACS). This technique uses the bacteriophage λ generalized recombination system, Red, to catalyze homologous recombination between linear DNA and a replicon using short homologies of 50 base pairs. With recombineering, single-stranded oligonucleotides or double-stranded PCR products can be used to directly modify the phage λ genome in vivo. It may also be possible to modify the genomes of other bacteriophages with recombineering.

Key words: Recombination, genetic engineering, bacteriophage λ, Red system, recombineering, mutation.

1 Introduction

Genome manipulation is an invaluable tool for the microbial geneticist. The ability to isolate mutations in specific genes has permitted analysis of microbial development and function. For example, in the study of bacteriophage λ, mutations in each morphogenetic gene enabled elucidation of the pathways for capsid *(1)* and tail assembly *(2)*. Classically, mutations in bacteriophages were often created by some form of global mutagenesis, such as UV irradiation or chemical treatment with a DNA-damaging agent (*see* **Chapter 17** for chemical mutagenesis protocols for bacteriophages). Genetic engineering allowed the creation of localized mutations by first cloning the region of interest into a plasmid and subjecting only that region to mutagenesis,

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and then re-introducing the altered DNA back into the host bacterium and ultimately into the phage genome.

Recently, a new in vivo technology for introducing genetic changes, namely recombineering *(3, 4, 5, 6)*, has been developed. In this method a specifically designed single- or double-stranded oligonucleotide is electroporated into a bacterial cell in such a way that it will recombine with an infecting phage. Recombineering has been facilitated by recent advances in genomic analysis, as it is only possible to recombineer an organism for which the DNA sequence is known. When used in combination with classical methods of screening and selection for the mutant genotypes, recombineering provides a powerful new tool to manipulate microbial genomes and episomes. The protocol described here is designed to enable modification of the bacteriophage λ genome, but we expect it to also work for at least some other bacteriophages.

Recombineering utilizes the bacteriophage λ-encoded generalized recombination functions, collectively termed Red to allow phage DNA to recombine with target DNA. As the 'target' is DNA that is electroporated into the cells before infection, this technique allows phage mutants to be created without having to make specific constructs for each desired mutation. The Red system can catalyze in vivo recombination using linear DNA as a substrate *(3, 4, 5, 6)*. Red consists of three λ proteins: Gam which inhibits the *E. coli* RecBCD enzyme, thus preventing degradation of double-strand DNA (dsDNA); Exo which is a 5'–3' dsDNA exonuclease; and Beta which is a single-strand DNA (ssDNA) annealing protein *(7)*. Together, Exo and Beta process and recombine the substrate DNA with the target DNA *(8)*.

Only one protein, Beta, is necessary for single-strand oligonucleotide (oligo) recombination *(4)*. Using single-strand oligos, point mutations can be made or corrected and deletions can be created. Heterologous insertions of double-stranded DNA (dsDNA) can also be made with recombineering; this reaction requires all three λ Red proteins *(3)*. Usually this dsDNA is provided in the form of a PCR product.

Whether the substrate for recombineering is an oligo or a dsDNA, the Red functions act at short regions of homology to recombine the substrate DNA with the target *(3, 4)*. We routinely use oligos which are 70 bases in length; these contain the alteration to be introduced at a central base(s). The dsDNA is usually in the form of a PCR product with 50 base pairs (bp) of homology to a target in the cell or to the phage chromosome at each end of the dsDNA. This homology is introduced during the PCR amplification and often flanks a drug cassette although other DNA elements can also be inserted (**Fig. 21.1**).

In vivo expression of the bacteriophage recombination system, Red, is required for recombineering. A λ lysogen with a

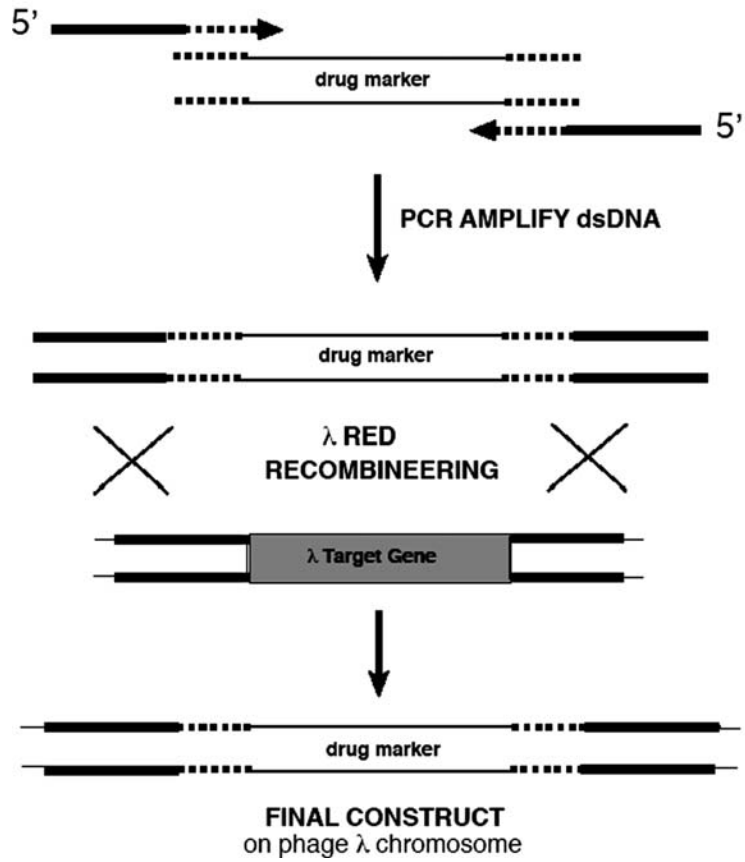

Fig. 21.1. Recombineering with dsDNA. Hybrid primers are used for PCR to amplify the DNA to be inserted, in this case a drug marker, which is targeted to the phage chromosome by recombineering. The heavy lines indicate the homology used for targeting the DNA; the dotted lines indicate the portion of primer used to amplify the DNA to be inserted.

defective prophage can be used to express Red from the strong λ p_L promoter *(3, 4)*, this mode of expression preserves the normal *red* gene regulation. The essential components of this prophage have been recently transferred to a number of different plasmids *(6, 9)*. Zhang et al., *(10)* and Datsenko and Wanner *(11)* have created plasmid constructs expressing the Red genes from the arabinose promoter, p_{BAD}. Some of the plasmids have temperature-sensitive (*ts)* origins of DNA replication *(9, 11)*, which permit them to be inactivated in the cell when they are no longer needed. Plasmids allow the Red functions to be readily transferred from one bacterial strain to another by transformation. This is especially convenient for introduction into *recA* mutant strains. The plasmid-borne prophage constructs *(9)* contain the *ts cI857* allele of the phage λ repressor, allowing Red expression to

be controlled by temperature, whether it is located on the *E. coli* chromosome or on a plasmid. At low temperatures (30–34°C), the Red recombination functions are strongly repressed, but when the temperature of the bacterial culture is shifted to 42°C, they are highly expressed. Very short times of expression (15 min) are required for fully active recombination.

Any phages to be modified can be supplied by the infection of a defective lysogen. Alternatively an intact λ *c*I857 prophage can be used for recombineering. In the latter situation the phage itself both supplies the Red functions and is targeted by the recombineering *(12)*. We have recently found that in many cases it is unnecessary to use a prophage-containing host when targeting the phage λ, since the infecting phage can itself supply the Red functions (unpublished data from this laboratory).

The desired DNA sequence of the final recombinant construct should be decided before attempting to modify the phage chromosome with recombineering. A computer program for DNA analysis is very helpful in this regard. Both the original phage genome and the modified genome should be maintained as electronic files; this facilitates the design of oligonucleotides for use as PCR primers or as ssDNA recombination substrates. Gene-regulation issues should be considered at the design stage. For example, when inserting a drug resistance gene, bringing in its promoter will help establish drug resistance, however, care must be taken to prevent transcription from this promoter from extending beyond the drug marker and thus affecting downstream genes. Yu et al., *(3)* describe drug cassettes containing a promoter, open reading frame, and a transcription terminator; primers for amplification of these cassettes are listed in **Table 21.1**. The computer file of the recombinant construct is also used to design primers needed to verify the recombinant candidates.

The desired change will determine the DNA substrate used for recombineering. For large heterologous insertions, such as a drug cassette, a pair of synthetic chimeric primers is used to amplify the desired PCR product. Built into the 5' end of each primer are 50 bases of homology to the phage chromosome which are required for targeting; these homologous bases are followed by around 20 bases used to prime and PCR amplify the drug cassette or other DNA region to be inserted. When modifying phage DNA to make deletions, small substitutions, or base changes a synthetic single-strand oligo of ~ 70–100 nt is used and 35–40 bases of complete homology to the phage should flank the alteration. Recombinant frequencies of several percent have been obtained for oligo recombination onto phage λ *(13)*. Frequencies for dsDNA are lower *(6)* and so a selection or screen should be applied.

Table 21.1
PCR primers and possible source of template for drug cassette amplification *(3)*

Gene	Source	Primer sequence
ampicillin	pBluescript SK(+) (Stratagene)	5' CATTCAAATATGTATCCGCTC
		5' AGAGTTGGTAGCTCTTGATC
chloramphenicol	pPCR-Script Cam (Stratagene)	5' TGTGACGGAAGATCACTTCG
		5' ACCAGCAATAGACATAAGCG
kanamycin	*Tn*5	5' TATGGACAGCAAGCGAACCG
		5' TCAGAAGAACTCGTCAAGAAG
spectinomycin	DH5αPRO(Clontech)	5'ACCGTGGAAACGGATGAAGGC
		5' AGGGCTTATTATGCACGCTTAA
tetracycline	*Tn10*	5' CAAGAGGGTCATTATATTTCG
		5' ACTCGACATCTTGGTTACCG

2 Materials

1. A bacterial strain expressing the λ Red recombination system (**Table 21.2**; request strains from Dr. Donald Court; *court@ncifcrf.gov*; **Note 1**).
2. Purified PCR product with 50 bases of flanking homology on both sides of the desired change, or an oligonucleotide primer ~70 nucleotides (nt) in length with the desired alteration centrally located.
3. High-titer lysate of the bacteriophage to be engineered *(14)*.
4. LB medium, tryptone plates and tryptone top agar *(14)*.
5. Electroporator (we use the Bio-Rad *E. coli* Pulser) and chilled 0.1 -cm electroporation cuvettes (Bio-Rad Laboratories; Hercules, CA; http://www.bio-rad.com/).
6. TM (10 mM Tris base, 10 mM $MgSO_4$, adjust pH to 7.4 with HCl) or TMG (10 mM Tris base, 10 mM $MgSO_4$, 0.01% gelatin, adjust pH to 7.4 with HCl).
7. Bacterial strains suitable for plating phage λ *(14)*.
8. Appropriate selective LB plates containing the concentrations of antibiotic required for drug resistance supplied by a single copy gene: 30 μg/ml ampicillin, 30 μg/ml kanamycin, 10 μg/ml chloramphenicol, 12.5 μg/ml tetracycline, and 50 μg/ml spectinomycin.
9. Sterile 82 -mm nitrocellulose filters if needed.

Table 21.2
Bacterial strains available for recombineering

Strain	Genotype
DY329	W3110 Δ*lacU169 nadA* :: *Tn*10 *gal490 pgl*Δ8 λ*c*I*857* Δ(*cro bioA*) (TetR)
DY330	W3110 Δ*lacU169gal490 pgl*Δ8 λ*c*I*857* Δ(*cro-bioA*)
DY331	W3110 Δ*lacU169* Δ(*srlA-recA*) *301* :: *Tn*10 *gal490 pgl*Δ8 λ*c*I857 Δ(*cro-bioA*) (Tet^R)
DY378	W3110 λ*c*I*857* Δ(*cro-bioA*)
DY380[1]	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*dlacZ*ΔM15 *lacX74 deoR recA*1 *endA*1 *araD*139 Δ(*ara*, *leu*) 7697 *galU gal490 pgl*Δ8 *rpsL nupG* λ(*c*I*857ind*1) Δ{(*cro-bioA*) <> *tetRA*}(Tet^R)
DY441	DY329 with *cat* − *sacB* inserted between *c*I*857* and *rexA* (Tet^R, Cm^R)
HME5	W3110 Δ*lacU169* λ*c*I*857* Δ(*cro-bioA*)
HME45[2]	W3110 *gal490 pgl*Δ8 λ*c*I*857* Δ(*cro-bioA*)
HME63	W3110 Δ*lacU169* λ*c*I*857* Δ(*cro-bioA*) *galKam mutS* <> *amp*
HME64	W3110 Δ*lacU169* λ*c*I*857* Δ(*cro-bioA*) *galKam uvrD* <> *kan*

[1] DH10B derivative (Invitrogen Corp.; Carlsbad, CA; http://www.invitrogen.com/)
[2] Gives less background on low concentrations of chloramphenicol than DY378.

3 Methods

The first method described is for modifying the DNA of an infecting phage. In this method, Red is expressed from a defective prophage, located either on the *E. coli* chromosome or on a plasmid, and the cells are infected with the phage to be altered. Variations of the procedure that are necessary when using an intact prophage are indicated in the Notes section. In the alternative method (described second), an intact λ prophage serves as both the source of Red and the target for recombineering. The general procedure is similar for both recombineering variations: cells are grown to exponential phase, the Red system is induced, then the cells are thoroughly washed and the appropriate substrate DNA carrying the desired genetic change is introduced by electroporation.

It is useful to try to design the construction so that the plating properties or plaque morphology of the modified phage differ from that of the parent phage and thus allowing visual screening. For example, sometimes a PCR product can introduce the desired mutation while at the same time correcting a known mutation on the phage. Amber or *ts* alleles are commonly available throughout the λ phage genome. Select for correction of the pre-existing mutation and screen among these recombinants for

the mutation of interest. If the alteration introduces or removes a restriction site, the region can be amplified with PCR and the product digested with the appropriate restriction enzyme to verify the change. When inserts or deletions are made, often plaque hybridization can also be used to identify recombinant phages if no selection or screen can be devised.

3.1 Preparation of 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 (**Note 2**). Primer sequences used to PCR amplify commonly used drug cassettes are listed in **Table 21.2**. Remember to add the targeting homology to the 5' ends of the oligos used to amplify PCR products.
2. Amplify the PCR product, examine it by gel electrophoresis and gel purify by isolating the desired band if unwanted products are obtained. Do not expose the PCR product to UV light during gel purification, since damaged DNA affects recombination frequency. Either use a non-UV based dye or if only one PCR product is obtained then just run 5 μl of product on a gel to check it is the correct size and then purify the remainder of the PCR product. Purify the PCR product by ethanol precipitation or using a commercially available kit to remove salt. It is better to avoid using a plasmid template to create a PCR-amplified drug cassette, since residual intact circular plasmid transforms the cells very efficiently and gives unwanted background that can obscure recombinant detection. If a plasmid template must be used, first linearize the plasmid with a restriction enzyme and digest the completed PCR with the restriction enzyme *Dpn*I before using it for electroporation. *Dpn*I will not cut the PCR product but will cleave DNA isolated from a Dam $^{+}$ host (**Note 3**).

3.2 Preparation of Bacterial Cultures

1. Inoculate a suitable bacterial strain (**Table 21.2** and **Note 4**) from a frozen glycerol stock or a single colony into 3–5 ml LB medium. Grow with aeration at 30 – 32°C overnight.
2. Equilibrate two shaking water baths to 30 –32 and 42°C, respectively. Add ~0.25 ml of the overnight culture to 20 ml of LB medium in a 125 ml (preferably baffled) Erlenmeyer flask (**Note 4**).
3. Place the flask in the 32°C shaking water bath and grow cells with shaking until the A600 is between 0.4 and 0.6 (**Note 5**).

3.3 Adsorption of Bacteriophage to be Modified (see Note 6 for a variation)

1. Harvest the cells by centrifuging for 7 min at 4600 × *g*, at 4°C.
2. Resuspend cell pellet in 1 ml TM buffer.
3. Infect the cells with the phage to be engineered at a multiplicity of infection of 1–3 phages per cell. The cells will be

at $\sim 1 - 2 \times 10^9$/ml. Let the phage adsorb to the cells for 15 min at room temperature (*see* **Note 7** for variation).

3.4 Induction of Recombination Functions

1. Transfer the infected cells to 5 ml of 42°C LB medium in a 50 ml Erlenmeyer flask and shake at 220 rpm for 15 min (*see* **Note 8** for variation) to induce the Red functions and allow the adsorbed phage to inject its DNA into the cells. While the cells are inducing at 42°C, fill an ice bucket with an ice-water slurry.
2. Immediately after the 42°C induction, rapidly cool the flask in the ice-water slurry with gentle swirling. Leave on ice for 5–10 min. While the cells are on ice, chill the necessary number of labeled 35- to 50-ml plastic centrifuge tubes in preparation for spinning in a pre-chilled (4°C) centrifuge.

3.5 Preparation of Electrocompetent Cells

1. Transfer the cultures to the appropriately labeled chilled 35- to 50-ml centrifuge tubes. Centrifuge for 7 min at 4600 × *g* at 4°C. Aspirate or decant supernatant.
2. Add 1 ml ice-cold distilled water to the cell pellet in the bottom of each tube and gently resuspend cells with a large bore pipette tip or by flicking the tube (do not vortex). Add 30 ml of ice-cold distilled water to each tube, seal, and gently invert to mix, again without vortexing (**Note 9**). Centrifuge tubes again as in step 1.
3. Decant the 30 ml supernatant very carefully so as not to lose or disturb the soft pellet in each tube (**Note 10)** and suspend each cell pellet gently in 1 ml ice-cold distilled water.
4. Transfer the suspended cells to cold microcentrifuge tubes. Centrifuge the cells in a microfuge 30–60 s at maximum speed at 4°C. Very carefully remove the supernatant again taking care not to lose the cells. In each of the tubes, suspend the cell pellet in 200 μl cold distilled water. There should be enough cells for four or five electroporations. If more electroporations are needed increase the number of starting cultures rather than changing the initial culture volume.

3.6 Introduction of DNA by Electroporation

1. Chill the required number of 0.1-cm electroporation cuvettes on ice. Turn on the electroporator and set to 1.80 kV (**Note 11**).
2. Mix 100–150 ng of salt-free PCR fragment or 10–100 ng of oligo with $50 - 100$ μl of the suspension of induced or uninduced cells in microcentrifuge tubes on ice. The mixing and subsequent electroporation steps should be done rapidly; do not leave the DNA-cell mixes on ice for extended periods. Be sure to include the following electroporation reactions and controls:

a. Phage-infected cells plus DNA. This reaction should yield the phage recombinants that you designed.
b. Phage-infected cells without DNA. This control will help to identify contamination in the phage stock, determine the reversion frequency of alleles being scored, and obtain some idea of the efficiency of the reaction.

3. Introduce the DNA into the cells by electroporation. The time constant should be greater than 5 ms for optimal results. Low time constants can indicate problems such as inadequate washing of the cells, impurities in the DNA, or technical problems with the electroporator.
4. Immediately after electroporation, add 1 ml LB medium to the cuvette using a micropipette with a 1000 μl pipette tip (*see* **Note 12** for variation).

3.7 Preparation of the Recombinant Phage Lysate

1. Add the 1ml LB-electroporation mix to 5 ml LB medium in a 50 ml baffled Erlenmeyer flask and aerate by shaking in a 39°C water bath for 90 min.
2. Add a few drops of chloroform and continue aeration a few minutes more to complete cell lysis and release the phage particles, then pellet the debris in a 4°C centrifuge at 8000 rpm for 10 min. Transfer the recombinant lysate into a storage tube.

3.8 Plating the Recombinant Lysate for Plaque Selection or Screening

1. Make 10-fold serial dilutions of the lysates in TM or TMG through 10^{-6} and spot 10 μl of each dilution on freshly poured lawns of a bacterial indicator permissive for phage growth using TB plates. Incubate plates at the appropriate temperature. Also use a selective indicator strain here if possible (**Note 13**). Store the diluted lysate at 4°C.
2. Analyze the spot plates, comparing plating properties of the recombinant lysate with those of the control lysate (no DNA added). Using the spot plates as a guide, plate from the appropriate dilution tube to obtain single plaques of the recombinant phage.
3. If you cannot select or screen visually for the recombinant plaques wanted, you may be able to use plaque hybridization *(15)* to detect them. Identification by plaque hybridization will require the presence of a short (≥15 bp) unique DNA sequence present in the recombinant and absent in the parent to be used as a target for the probe. Thus point mutations cannot be detected with plaque hybridization.

3.9 Recombinant Phage Analysis

1. Resuspend a purified candidate plaque in 50 μl of TM and use 20 μl of this suspension as template for a PCR (**Note 14**). Reserve the remainder of the plaque suspension to grow a stock of the correct isolate.
5. Prepare a lysate of the confirmed recombinant phage using standard methodologies *(14)* (**Note 15**).

4 Notes

1. Most of the strains containing the defective lambdoid prophage are W3110 derivatives; however the prophage can be moved into other backgrounds by P1 transduction *(3)*. Any strain of choice can be transformed with plasmids expressing the recombination functions *(9, 11)*. If the same complete prophage that provides Red is also to be engineered, it must have a functional integration and excision system, and the *exo*, *beta*, and *gam* genes with their regulatory circuitry must be intact. To recombineer using an intact phage, first construct and confirm the bacterial lysogen *(14)* with the *c*I857 phage of choice. We have recently found that *red* expression from an infecting phage is sufficient to catalyze recombineering, providing the Red functions are intact, and in some cases recombinant yields are better in the absence of the prophage. We recommend executing the entire procedure in parallel with two bacterial strains, one containing the defective prophage, and one that lacks it, such as C600, which is a good host for phage λ.
2. We routinely used salt-free oligos that are otherwise unpurified for many applications. However, the oligos are the greatest source of adventitious mutations that occur during recombineering *(13)* and for some applications Polyacrylamide Gel Electrophoresis (PAGE) purification may be helpful.
3. If the recombinant from a dsDNA (e.g., PCR product) recombination is selected as a lysogen, and the PCR product was amplified from a plasmid template, it is important to include a control reaction of un-induced cells transformed with the PCR product; drug resistant colonies appearing in the control reaction demonstrate that intact plasmid was present in the transformed DNA.
4. When subculturing the cells, the dilution should be at least 70-fold. If you are expressing the Red system from a plasmid, add the appropriate antibiotic to maintain selection during growth. If arabinose or some other inducer of the recombination functions is used, be sure to add it to the medium. 10 mM arabinose (for Ara^+ strains) induces expression of the λ Red functions from the plasmids of Datsenko and Wanner *(11)*. Also include an additional flask of an un-induced culture as a negative control. The temperature shift is unnecessary when arabinose or another chemical is used for Red induction.
5. Usually the cells will grow to the correct optical density in about 2 h. Don't let them enter early stationary phase ($>0.7-0.8$ OD_{600}), since this will result in poor expression of the Red functions.

6. If a lysogen is with an intact prophage that will both supply Red and be targeted by recombineering, skip this step and proceed directly to step 3.4.
7. Adsorption conditions may vary for phages other than λ. For example, a nonlambdoid phage such as T4 may require adsorption on ice.
8. The 15 min induction time will result in expression of the phage replication functions and resultant cell killing. Thus, for an intact prophage, the full 15 min induction time should only be used only when phages are being induced and a recombinant lysate prepared. If you desire to retain the targeted phage as a prophage and plate the recombinant bacterial colonies (selecting, for example, insertion of a drug resistance cassette onto the prophage), reduce the induction time to 4–5 min. Because the level of Red expression will be lower with the shorter heat pulse, recombinant frequencies will be correspondingly reduced.
9. Throughout the procedure, resuspend the cells gently and without vortexing. The washing steps remove any chemical added to induce the Red system.
10. Remove tubes from the centrifuge promptly after the distilled water wash. The pellet is very soft and care should be taken not to dislodge it, especially when processing multiple tubes.
11. Electroporation conditions are crucial and although other brands of cuvettes and electroporator may work, we have not confirmed this. 0.2 cm cuvettes will require different electroporation conditions and standardization to obtain optimal recombination frequencies by empirical methods (see the instruction manual for your electroporator).
12. When recombinant lysogens of a complete prophage are selected (such as by drug resistance), the yield will be very low, thus the entire contents of a single electroporation mix are spread on one Petri plate as described below. Lining the plate with an 82-mm diameter sterile nitrocellulose filter allows both the nonselective outgrowth and the selection to be done on Petri plates. Carefully lay a sterile 82-mm diameter nitrocellulose filter atop a rich nonselective (i.e., LB) plate with sterile forceps. Immediately after electroporation, add ~ 0.3 ml LB to the cells in the cuvette and spread them on the filter. Incubate the plates for 3 h or more at $30 - 32°C$; then transfer the filter to the appropriate drug plate, again 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 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.

13. If recombinant phages can be selected from the lysates by growth under some condition, spot the same dilutions on the selective indicator strain. Several bacterial strains and other genetic tricks are very useful for selecting certain phage λ genotypes *(14)*. For example, strains with the appropriate suppressor tRNA will allow a phage with an amber mutation in an essential gene to grow, while the isogenic non-suppressing strain will prevent growth. If the recombineering repairs such an amber mutation, the recombinant can be selected for directly by plating on the non-suppressing strain. If an amber mutation is introduced into an essential gene, the double-layer technique *(16)* can be used to identify phages with the amber by plaque morphology. Another example of a useful selective strain is a bacterial host mutant for *recA* function, since λ phages doubly mutant for both the *red* and *gam* genes it will not form plaques on this host. Conversely, a P2 lysogen is restrictive for wild- type λ and will plate only *red gam* mutant phages. A *ts* mutation can be selected against by plating at the appropriate temperature.
14. The genetic alteration created by recombineering determines the PCR primers needed to confirm recombinants. If a heterologous DNA such as a drug marker is inserted, design four primers, two flanking the insert and two pointing outwards from within the cassette, all with compatible annealing temperatures. Flanking primers are paired with the internal cassette primers to amplify the two junctions at the insertion site. The outside primer pair, which hybridizes to the external flanking sequences rather than to the insert itself, can also be used to demonstrate loss of the target sequences and presence of the insertion, as long as the relative sizes of the two possible PCR products differ. If a single base change alters a restriction site, recombinants bearing the change can be identified by generation of a PCR product encompassing the region and digestion of that product with the relevant restriction enzyme, followed by agarose gel electrophoresis.
15. Unwanted mutations can be introduced during the chemical synthesis of the oligo or primer population *(13)*; therefore, it is important to confirm the final construct by sequence analysis, especially the regions derived from the original oligos or primers.

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