Chapter 2

Targeted Chromosomal Gene Knockout Using PCR Fragments

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Abstract

The development of recombineering technology has converged to a point that virtually any type of genetic modification can be made in the *Escherichia coli* chromosome. The most straightforward modification is a chromosomal gene knockout, which is done by electroporation of a PCR fragment that contains a selectable drug marker flanked by 50 bp of target DNA. The phage λ Red recombination system expressed in vivo from a plasmid promotes deletion of the gene of interest at high efficiency. The combination of this technology with site-specific recombination systems of Cre and Flp has enabled genetic engineers to construct a variety of marked and precise gene knockouts in a variety of microbial chromosomes. The basic protocols for designing PCR substrates for recombineering, generating recombineering-proficient electrocompetent strains of *E. coli*, and for selection and verification of recombinant clones are described.

Key words: Recombineering, Lambda red, Gene replacement, Strain development, Electroporation, Phage lambda, Beta, Exo, Gam, PCR

1. Introduction

The precise deletion of a gene of interest in the *Escherichia coli* chromosome is a central step to understanding gene function or to remove undesirable byproducts for strain engineering purposes. Classically, this has been done by random mutagenesis, or by integrating nonreplicating plasmids containing an altered target gene, with the hope of being able to generate a resolution event that excises the wild-type copy leaving the modified (deleted) copy of the gene in the chromosome. These processes were often time consuming and/or unsuccessful at generating gene knockouts.

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In the last decade, a new approach has evolved that takes advantage of the recombination proficiency of the bacteriophage λ Red recombination system (identified by *re*combination *d*efective phage mutants) and of the *rac* prophage RecET system; the process has been referred to as "recombineering" (from *recombi-n*ational engin*eering*) (1–7). The key to successful use of this system is that the Red system consists of only two genes (*exo* and *beta*) that initiate a recombination event that requires only limited amounts of homology to the target gene (~40–50 bp). The λ Exo protein is a processive 5′–3′ dsDNA exonuclease that binds to dsDNA ends and degrades the 5′ strand at the site of entry, leaving 3′ ssDNA tails (8, 9). The λ Beta protein, which binds to the ssDNA generated by λ Exo, is a member of a class of proteins known as single-stranded DNA annealing proteins (SSAPs) that share a common ring-like quaternary structure, promote annealing of ssDNA in vitro, and stimulate DNA recombination events in vivo (10–15). The Red functions are assisted by the λ *gam* gene, which encodes an inhibitor of the host RecBCD enzyme, a destructive dsDNA exonuclease that would otherwise compete with the Red functions for dsDNA ends (16–18). In recombineering events, it is thought that the action of the Red genes in vivo produces either a long ssDNA intermediate bound by Beta, or a linear dsDNA molecule that has Beta bound to 3′ ssDNA overhangs on either end of the substrate (5, 19). In both models, the replication fork is the likely target for the Red-generated intermediates (19, 20). These interactions might occur via annealing of the ssDNA intermediate to the lagging strand template of a replication fork, or by consecutive interactions of each end of the dsDNA intermediate with two independent replication forks.

The procedure presented here describes a simple straightforward method for generating a gene knockout in *E. coli*. An *E. coli* strain of choice, containing a plasmid that overexpresses the λ *exo*, *beta*, and *gam* genes, is electroporated with a PCR product that contains a drug marker flanked by 50 bases of homology to the target gene (or region) to be deleted. The endpoints of the deletion are dictated by sequences within the PCR primers. Following electroporation, the cells are grown out and plated on antibiotic-selection plates. Gene knockouts can be easily obtained in one day, are verified by PCR analyses, and can be transferred into clean genetic backgrounds by P1 transduction (if so desired).

2. Materials

2.1. Reagents

1. LB medium: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 ml 1 M NaOH. Mix components in 1 l of distilled water and sterilize by autoclaving for 30 min; store at room temperature.

For LB plates, add 15 g agar, autoclave as above, cool for 30 min at room temperature, add antibiotics as needed, and pour into 100 mm × 15 mm petri plates using 25–30 ml per plate.

2. Electroporation washing buffer: 10% glycerol. Dilute 100 ml of glycerol in 900 ml distilled deionized water, autoclave 500 ml in two 1-l flasks, and store at 4°C (6 months).
3. Ampicillin. The stock solution is dissolved at 10 mg/ml in 90% ethanol and stored at –20°C (1 year). Use between 25 and 100 μg/ml in LB plates for growing Amp^R gene replacements; use at 100 μg/ml for growing cells containing pKM208 in culture.
4. Chloramphenicol. The stock solution is dissolved at 20 mg/ml in 90% ethanol and stored at –20°C (1 year). Use at a concentration of 15 μg/ml in LB plates for selecting Cam^R gene replacements.
5. Kanamycin monosulfate. The stock solution is dissolved at 20 mg/ml in water and stored at 4°C (1 month). Use at 20 μg/ml in LB plates for selecting Kan^R gene replacements.
6. Tetracycline. The stock solution is dissolved at 10 mg/ml in 90% ethanol and stored at –20°C (1 year). Use at 3–10 μg/ml in LB plates for selecting Tet^R gene replacements.
7. Isopropylthiogalactopyranoside (IPTG) – Added to cell cultures for induction of the *red* and *gam* functions from pKM208. Dissolve 238 mg of IPTG powder into 10 ml deionized H_2O; filter sterilize, and store at –20°C (6 months).
8. Agarose. Use at 0.75–1.5% for analysis of PCR products.
9. Pfu-Ultra II Fusion HS DNA polymerase (Stratagene, 600670-51). Enzyme used for generating PCR recombineering substrates.
10. Taq DNA polymerase. Enzyme used for colony PCR to check structure of recombinant clones.
11. QIAprep Spin Miniprep kit (Qiagen, 27106). Used for the isolation of plasmids from 5 ml of culture.
12. QIAquick PCR purification kit (Qiagen, 28104). Used for the purification of PCR products to be used as substrates for recombineering.
13. pJW168 – Amp^R, pSC101-derived, Cre recombinase expressing plasmid (21) (Lucigen, 42200-1).
14. EB (elution buffer): 10 mM Tris–HCl, pH 8.5.
15. PBS: Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 ml of distilled water. Adjust pH to 7.4 with HCl; add water to 1 l and autoclave.
16. dNTPs: 2.5 mM each of dATP, dCTP, dGTP, dTTP.

Table 1
Annealing sequences for drug cassettes

Antibiotic (cassette length)	Gene(s)	Primer pair (5′ to 3′)[a]	Drug concentration (μg/ml)
Kanamycin (944 bp)	Tn*903* (*aph*) *type I*	CACGTTGTGTCTCAAAATCTC TACAACCAATTAACCAATTCTG	20
Kanamycin (949 bp)	Tn5 (*aph*) type II	TATGGACAGCAAGCGAACCG TCAGAAGAACTCGTCAAGAAG	20
Chloramphenicol (822 bp)	Tn*9 cat*	TGAGACGTTGATCGGCACGT ATTCAGGCGTAGCACCAGGC	15
Ampicillin (975 bp)	Tn*3 bla*	CGCGGAACCCCTATTTGTTT GGTCTGACAGTTACCAATGC	50
Tetracycline (1,996 bp)	Tn*10 tetRA*	CTCGACATCTTGGTTACCGT CGCGGAATAACATCATTTGG	7
Gentamicin (616 bp)	Tn*1696 aacC*	CGAATCCATGTGGGAGTTTA TTAGGTGGCGGTACTTGGGT	10

[a]These sequences should be placed on the 3′ ends of the primers used to generate the recombineering substrate

17. Dimethyl sulfoxide (DMSO), molecular biology grade.
18. Sterile distilled water.
19. Primers (as defined in Table 1).
20. Recombineering plasmid: Plasmid pKM208 expresses the λ *red* and *gam* functions under control of the Ptac promoter (6) and can be obtained from *addgene.com*. The plasmid contains a temperature-sensitive origin of replication (cells containing the plasmid should be grown at 30°C). The plasmid also contains the *lacI* repressor under control of its own promoter (to keep *red* and *gam* expression turned off in the uninduced state), and the *bla* gene, which confers resistance to ampicillin (see Note 1).

2.2. Equipment

1. Thermocycler (e.g., Minicycler PTC-200, MJ Research).
2. Two incubators set at 30°C and 37°C for growth of recombineering strains and recombinant colonies, respectively.
3. Two shaking water baths set at 30°C and 42°C for growth of *E. coli* recombineering cultures.

4. Spectrophotometer and cuvettes for measuring optical densities of bacterial cultures.
5. Biorad Gene Pulser Xcell Electroporation system (#165-2660) or BioRad MicroPulser Electroporator (#165-2100).
6. Electroporation cuvettes – sterile, 0.1 cm gap, package of 50 (Bio-Rad, 165-2089).
7. Centrifugation tubes – 40 ml (Nalgene, Oak Ridge Centrifuge Tubes, 3119-0050).
8. Pipets to deliver up to 1 ml (P-1000), 200 µl (P-200), or 20 µl (P-20) of liquid or culture.

3. Methods

3.1. Preparation of Targeting Substrate by PCR

1. The standard targeting substrate for recombineering is a PCR product that contains a drug marker flanked by upstream and downstream regions of the target site. Primers for the PCR are typically 70 bases in length and are designed so that 20 bases on the 3′ ends will anneal to and amplify a drug cassette of one's choice. See Table 1 for sequences and templates used for amplifying a variety of drug cassettes used in recombineering. The 50 bases on the 5′ ends of the primers contain the upstream sequence and the reverse complement of the downstream sequence, respectively of the target site (see Fig. 1).
2. A high fidelity polymerase such as Pfu UltraII fusion polymerase should be used to generate the targeting substrate. Alternative High Fidelity polymerases for this step include Platinum High Fidelity Taq polymerase (Invitrogen 11304-011), or Roche Expand High Fidelity polymerase (Roche, 04-738-250-001).
3. Prepare PCR reaction as follows: 31 µl sterile distilled water, 5 µl 10× PCR buffer (supplied by manufacturer), 5 µl 2.5 mM dNTPs, 2 µl primer A (20 µM), 2 µl primer B (20 µM), 2 µl DMSO, 2 µl template DNA (~10 ng), 1 µl High Fidelity Pfu UltraII fusion polymerase (see Fig. 1 and Note 2).
4. Perform standard PCR. We typically use the following program for 0.8–1-kb amplicons; (step 1) 95°C, 1 min; (step 2) 94°C, 30 s; (step 3) 58°C, 30 s; (step 4) 72°C, 1 min; (step 5) repeat last three steps 29 times; (step 6) 72°C, 5 min; (step 7) hold at 4°C. The extension times (step 4) should be increased for products expected to be longer than 1 kb, though check the elongation properties of the polymerase as reported by the manufacturer.
5. When completed, load 3 µl of the PCR on a 0.75% agarose gel to check for correct size and purity of the recombination

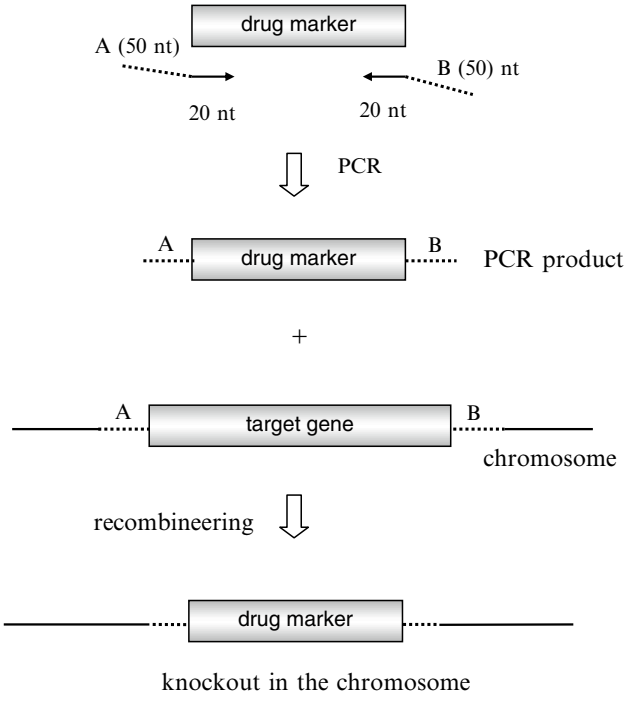

Fig. 1. Generation of recombineering substrate by PCR. The first primer contains sequence from its 5′ ends that is identical to the upstream region of the target gene (*dotted line* marked A). The second primer contains from its 5′ end the reverse complement of the sequence in the downstream region of the target gene (*dotted line* marked B). The last 20 bases of the primers anneal to and amplify the drug marker (see Table 1 for these sequences). The product of this PCR is ~1–2 kb amplicon (depending on the drug marker) which contains 50 base pair ends that are homologous to the target region. After filter cleaning and elution in a low salt buffer (EB) or water, the PCR product is electroporated into recombineering-proficient *E. coli* cells. After a growth period, the recombinant is selected on an antibiotic-selection plate.

substrate. If present as a single species, clean the PCR product with PCR-quick clean kit (Qiagen) or similar type of PCR purification kit. Elute the DNA in 30–50 μl of EB buffer or deionized water (see Note 3). If side products are present, gel-purify the recombineering substrate on a 0.75% agarose gel. If the recombineering substrate is not found, repeat PCR with 2–4°C decrease in annealing temperature and/or remove DMSO from the PCR. If band still not present (and known PCR control is working), redesign and order new primers (see Note 4).

3.2. Preparation of Recombineering-Proficient Electrocompetent *E. coli* Cells

1. Transform the *E. coli* strain of interest with Red-recombineering plasmid pKM208 (Amp^R). Plate transformation at 30°C on LB plates containing 100 μg/ml ampicillin overnight (see Note 5). Inoculate a fresh colony into 5 ml LB containing 100 μg/ml ampicillin and roll overnight at 30°C.

2. In a 125 ml flask, inoculate 20 ml of LB containing 100 μg/ml ampicillin with 100 μl of the 5 ml overnight culture containing pKM208. Grow cells in a shaking water bath with aeration at 30°C to an OD of 0.2 (~10^7 cells/ml) and add 200 μl of 0.1 M IPTG (final concentration is 1 mM). Continue to grow cells at 30°C.
3. At an OD between 0.4 and 0.6 (~10^8 cells/ml), place culture in a water bath prewarmed to 42°C. Aerate by shaking for an additional 15 min (see Note 6).
4. Place culture in an ice-water bath and swirl moderately for 10 min.
5. Pour culture into prechilled centrifugation tubes (Nalgene, Oak Ridge Centrifuge tubes, 3119-0050) and collect cells by centrifugation at 3,800 × *g* in SS-34 rotor. Alternatively, use sterile 50 ml Falcon tubes in swinging bucket bench top centrifuge at 3,800 × *g*. Handle tubes gently so as not to disturb the cell pellet. Pour off supernatant slowly and resuspend the cells in 2 ml of ice-cold 10% glycerol. Resuspend the cells with P-1000 pipet by gently pipeting cells back and forth (easier done in this smaller volume). Add 18 ml of ice-cold 10% glycerol, mix culture by inverting tube four to five times, and recentrifuge.
6. Resuspend the cells in 1 ml ice-cold 10% glycerol and transfer to a prechilled 1.5 ml Eppendorf tube. Spin cells in refrigerated microcentrifuge at 10k for 1 min at 4°C. Gently pour off supernatant and remove last ~200 μl with P-200 pipet, being careful as not to disturb pellet. *Repeat this step once more* (see Note 7).
7. Resuspend the pellet in 100–150 μl of ice-cold 10% glycerol with P-200 pipet by gently pipeting back and forth. Make sure no clumps are present. Place cells on ice and use within 30 min (see Note 8). This amount of cells is good for two to three trials using 50 μl of electrocompetent cells per electroporation. If more samples need to be done, the process can be scaled up by growing more cells in additional 125 ml flasks (see Note 9).

3.3. Electroporation of Recombineering-Proficient Cells with PCR Fragments

1. Prechill the electroporation cuvettes (0.1 cm) by placing in an ice-water bath for 10 min. In a prechilled sterile Eppendorf tube, mix 50 μl of electrocompetent cells with 0.1–0.5 μg of PCR substrate. Ideally, use 1–3 μl of DNA per 50 μl of electrocompetent cells. Do not exceed 5 μl of DNA per 50 μl of cells as this amount of substrate increases the possibility of arcing. Arcing occurs when the charge is dissipated as a spark outside the electroporation chamber, and no pulse is detected by the electroporation device (see Note 10).
2. Assemble the Gene Pulser II to Pulse Controller II (Bio-Rad). Select preset protocol for transformation of *E. coli* cells using

0.1 cm cuvette. If using alternate electroporation set-up, set voltage to 1,800 V, use 25 μF capacitance and 200 Ω resistance.

3. Transfer the DNA-cell mixture to a prechilled cuvette, replace the cap, and incubate on ice for 1 min. Quickly (but thoroughly) dry the cuvette with miniwipes, place the cuvette into the electroporation chamber, and release charge. The time constant should be close to 5 ms. A value much less than 5 ms for the time constant indicates impurities (i.e., salt) in the DNA sample or electrocompetent cell preparation.
4. Using the P-1000 pipet, immediately add 0.5 ml of LB to cuvette. Pipet back and forth a few times and transfer cells to 2.5 ml LB in sterile culture tube. It is not necessary to include ampicillin or IPTG in the outgrowth medium, as the Red and Gam proteins are already at their optimal levels.
5. Perform appropriate controls (see Note 11).

3.4. Outgrowth and Selection of Recombinants

1. The electroporated cells are further grown by rolling or shaking for 90–120 min at 37°C. This is an important step as it allows the cells to recover from the electrophoretic shock and express adequate amounts of the drug resistance marker gene prior to exposure to the selection plate.
2. After outgrowth, spread 0.2 and 0.5 ml aliquots of the culture on LB antibiotic-selection plates. Incubate the plates at 37°C overnight (no need to grow at 30°C, as Red-expression is no longer desired). Also plate 100 μl of 10^{-4} and 10^{-5} dilutions of the culture on LB plates to determine the total number of cells present. Percent recombineering frequency can be expressed as the fraction of drug-resistant colonies divided by total cell titer × 100. This number is often normalized to the number of recombinants per 10^8 of viable cells (see Note 12).
3. Allow the rest of the culture to grow overnight at 37°C. If no colonies appear on the plates after overnight growth, spread the rest of the culture on additional drug selection plates and incubate at 37°C overnight. Some recombinants take longer to appear than others.
4. Use drug concentrations in the plates that will select for the drug marker at single copy in the chromosome. These concentrations are lower relative to the same markers present on multicopy plasmids. Drug concentrations in the selection plates we have employed include the following: chloramphenicol, 10–15 μg/ml; kanamycin, 20 μg/ml; tetracycline, 3–7 μg/ml; gentamycin, 10 μg/ml; and ampicillin, 25–50 μg/ml.
5. If no colonies are found on the drug-selection plates, try troubleshooting (see Note 13).

3.5. Verification of Recombinants and Curing of Red-Producing Plasmid pKM208

1. Restreak candidate gene knockout strains on to fresh antibiotic-selection plates and incubate at 37°C overnight. Spontaneous mutants arising on the drug plates typically do not restreak as well on these plates as true gene replacement candidates.
2. Colony PCR can be used to verify the structure of the recombinant. A high-fidelity polymerase is not required (or recommended) for these PCRs. Use a standard Taq polymerase, which works well for colony PCRs. One should use primers that are positioned ~100 bp upstream and downstream of the sequences used for targeting the gene replacement, as well as primers reading out of the drug marker cassette. These primers (see Fig. 2) can be used to verify the 5′ junction of the knockout (primers 1 and 2), the 3′ end of the knockout (primers 3 and 4), as well as any overall differences in size of the gene replacement (primers 1 and 4). A third set of primers should be used to amplify a 500–700-bp region of the target gene or region, which should appear when wild-type cells are used as a template by colony PCR, but absent when the recombinant cells are used (see Note 14).
3. Design primers #2, #3, #5, and #6 (see Fig. 2) to give PCR products in the 500–700 bp range. These products are easy to generate by PCR and can be readily distinguished from PCR artifacts that might occur at 300 bp and below. It is also

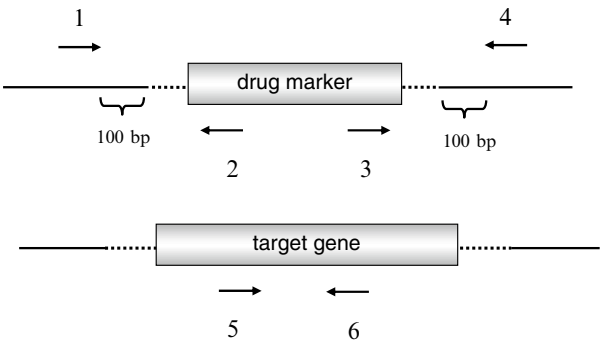

Fig. 2. Primers for gene knockout verification. To verify the 5′ junction, use a primer containing sequences ~100 bp upstream of the sequence used to generate the PCR recombineering substrate (primer #1) and a primer in the in the drug cassette reading leftward (primer #2). To verify the 3′ junction, use a primer containing sequences ~100 bp downstream of the sequence used to generate the PCR recombineering substrate (primer #4) and a primer in the in the drug cassette reading rightward (primer #3). If the size of the gene or region deleted is different from that of the drug cassette, then a PCR using primers #1 and #4 will generate a band diagnostic for the knockout. If the size of the parental and recombinant PCR product is the same, then restriction analysis can usually be used to reveal the presence of the knockout. Finally, a PCR to verify the absence of the wild-type locus in the recombinant should be performed using primers #5 and #6 (see Note 10).

a good idea to run a computer simulation of the PCR before ordering the primers, to avoid the generation of primer dimers that might interfere with detection of the diagnostic band. Amplify is a free Mac software program that can be used to simulate and test PCR reactions in silico (http://engels.genetics.wisc.edu/amplify). Alternatively, for Vector NTI program users, check primers with Oligo analyses programs Thermodynamic Properties and Oligo Duplexes.

4. The λ Red + Gam producing plasmid pKM208 contains a temperature-sensitive origin of replication, where optimal growth occurs at 30°C and restrictive growth occurs at 42°C. Thus, the recombinants can be cured of pKM208 following construction of the knockout by growth of the cells at 42°C. In some cases, streaking out two consecutive times at 42°C is required for promoting loss of the plasmid. Verification of plasmid loss can be found by sensitivity to ampicillin (100 μg/ml), followed by electrophoresis of 10 μl of a minilysate of the cell culture and noting the absence of pKM208 (8,731 bp).
5. If no recombinants are found at this point, perform troubleshooting (see Note 13).

3.6. Generation of Unmarked Gene Knockouts

A procedure for generating a gene knockout and removing the antibiotic resistance takes advantage of the phage P1 Cre-mediated site-specific recombination system (22). The *loxP* sequence (ATAA CTTCGTATA(N)$_8$TATACGAAGTTAT) is a target sites for the Cre recombinase (23). A Cre-promoted recombination event will delete the DNA between directly repeated two *loxP* sites, leaving behind one *loxP* site in the recombinant (24). The use of the Cre-*loxP* system for creating unmarked gene knockouts was developed by Sauer and Henderson (25). The removal of the drug marker after Red-mediated gene deletion is done in a similar manner as described above, with two exceptions. First, the drug marker in the PCR template plasmid should be flanked by *loxP* site-specific recombination sites. Secondly, after recovery of the marked gene deletion, a plasmid expressing the P1 Cre recombinase (pJW168) can be used to delete the drug marker from the chromosome (21). This plasmid, like pKM208, contains a temperature-sensitive origin of replication and can be easily evicted. This system is easy to employ, occurs at high frequency, and allows multiple alterations of the chromosome to occur without the need for multiple drug markers. The only concern is that there is a scar left over (the *loxP* sequence in place of drug marker). Repeated use of this procedure could leave multiple scars in the chromosome, which themselves might become substrates of unintended Cre-promoted recombination.

1. Generate a PCR recombineering substrate as described above in Subheading 3.1, but use as a template drug marker that is flanked by *loxP* target sites (21).

2. After selection, verification, and curing of the recombinant strain of the Red-producing Amp^R pKM208 plasmid, the cell is transformed with Amp^R pJW168 that expresses the Cre recombinase (21). A colony is picked and grown overnight in LB containing 100 μg/ml ampicillin at 30°C in the presence of IPTG (to induce *cre*).
3. The overnight culture is diluted 10^{-4}, 10^{-5}, and 10^{-6}-fold in PBS (or LB) and portions of the dilutions (100–200 μl) are spread on LB plates.
4. Single colonies are streaked as short patches (~0.5 cm) first on to LB plates containing the antibiotic encoded by the evicted drug marker, and then on LB plates with no drugs. This screen identifies recombinant clones that have lost the antibiotic drug cassette by Cre-mediated excision. This step is usually very efficient and drug-free recombinants are easily found.

4. Notes

1. Plasmids have been described that express the λ *red* and *gam* genes from the Ptac promoter (pKM208 – www.addgene.com) (6), the P_{BAD} promoter (pKD46 – http://cgsc.biology.yale.edu) (3), or the phage lambda P_L promoter (pSIM6 – court@ncifcrf.gov) (26). The protocol presented above describes the use of pKM208, where expression of the *red* and *gam* genes is induced by the addition of IPTG. The protocol is the same when using these other Red and Gam-producing plasmids, with the exception of the induction steps: for pKD46, *red* and *gam* are induced by the addition of 10 mM arabinose; for pSIM6, *red* and *gam* are induced by a 15-min incubation at 42°C. All these plasmids carry the same temperature-sensitive origin of replication and the *bla* gene conferring resistance to ampicillin. Options for recombineering plasmids containing drug markers other than ampicillin are available from D. Court (26) htpp://web.ncifcrf.gov/research/brb/recombineering-information.aspx; and at addgene.com (Murphy lab). To use *bla* as a gene knockout marker (Table 1), an alternative Red-producing plasmid containing a different drug marker is needed.
2. The choice of template used for generating the recombineering substrate is crucial. Intact circular plasmids should not be used as templates. While they are used at low amounts in a typical PCR (~10 ng), the template plasmid will still be present in a purified PCR product and will transform *E. coli* at high efficiency giving rise to false-positive recombinants on antibiotic-selecting plates. To prevent these false positives from arising, one can (1) gel purify the PCR product, (2)

treat the PCR product with *Dpn*I, which will digest the template plasmid but not the unmethylated PCR amplicon, (3) perform colony PCR with a strain containing the drug marker in the chromosome, (4) use drug markers cloned into conditionally replicating vectors such as R6K *oriR*γ origin vectors that require engineered pir+ host strains that provide the *trans*-acting Π protein for replication (3), or (5) use as a PCR template, a gel-purified fragment of the marker-containing plasmid that is free of its origin of replication. The last option is quite useful, as 1 μg of this fragment can serve as a successful template for 100 PCRs.

3. In some cases (e.g., when dealing with enteropathogenic strains of *E. coli*), the use of higher concentrations of the PCR substrate will give a better chance of recovering a recombinant. To this end, the 50 μl of cleaned PCR product can be concentrated by ethanol precipitation and resuspended in 10 μl of EB (10 mM Tris–HCl, pH 8.0). To do this, dilute 50 μl of DNA to 350 μl with precipitation buffer (20 mM Tris–HCl, 10 mM NaCl, 2 mM EDTA, 0.5M ammonium acetate, pH 6.5), add 3 μl of 10 mg/ml of tRNA (as carrier), and fill the 1.5 ml Eppendorf tube with ethanol. Vortex the mixture well, freeze at –20°C for 30 min, and spin out the precipitate at high speed in a microcentrifuge for 5 min. Remove the supernatant, dry the pellet with one wash of cold ethanol, let the pellet dry, and resuspend the DNA in 10 μl of EB. We have found that samples prepared in this way allow higher amounts of DNA to be electroporated without causing sparking (i.e., arcing, no pulse delivered to sample due to dissipation of the charge outside the cuvette, usually the result of residual salt in the sample).
4. The lack of PCR products (in general) is usually indicative of problems with one or more components of the reaction, or errors in the cycling program. But remember, the primer annealing sequences in Table 1 and their templates have been used repeatedly in successful PCRs, so a problem in generating a substrate for gene replacement (with all control reactions with known reagents working properly) most likely indicates problems with one of the primers. If so, do not spend much effort in trying to optimize the PCR, just order new primers.
5. If no transformants with pKM208 are found, try plating cells on decreasing concentrations of ampicillin (25–50 μg/ml). Once established, cells containing the plasmid should be grown in LB containing 100 μg/ml ampicillin.
6. This heat shock step is optional. It has proved useful for obtaining recombinants in pathogenic strains such as enterohemorrhagic *E. coli* and enteropathogenic *E. coli*. In *E. coli* K-12, the stimulation due to the heat shock is variable depending on the loci being deleted. The reason for this observation is not known.

7. Multiple glycerol washes are necessary to thoroughly remove salts from the cell preparation to increase resistance thus preventing arcing during electroporation.
8. For long-term storage, flash freeze the samples by swirling in an dry ice-ethanol bath, then store the cells at –80°C. The fold-less transformation is variable (depending on the initial competence), but generally expect about a five- to tenfold drop in transformation efficiency over a 6-month period. This step is useful when the total number of recombinants is not critical. However, when high transformation efficiencies are required, one should use freshly prepared cells.
9. The electroporation can also be scaled down to 25 μL of cells (just above the minimum volume required for a 0.1 cm cuvette) to allow processing of more samples.
10. If a spark occurs, chances are that the sample did not receive the appropriate charge to generate pores in the membrane to promote DNA uptake. However, we have seen examples where a spark has been observed, and upon plating the cells, recombinants were in fact recovered.
11. Perform appropriate controls. The most important control is to electroporate the host cells with no DNA. Spreading of the cells for this "blank" on antibiotic-selecting plates should give no colonies. The presence of colonies is indicative of host cell line contamination. Sometimes, this control gives rise to small colonies on the drug plates indicative of spontaneous resistance. These colonies generally should be fewer in number (relative to plates that were spread with cells containing DNA) and should not grow well upon restreaking onto fresh antibiotic-selection plates. For a positive control, knock out a gene that has been done before (*lacZ* for example), just in case there is something peculiar about the knockout being attempted.
12. When comparing the recombination rates of different strains, it is advisable to include a small amount (10–50 ng) of an intact plasmid as an electroporation control. This plasmid can be mixed directly with the PCR substrate and co-electroporated into *E. coli*. Even the same cell preparation can exhibit various transformation efficiencies when electroporated side-by-side on the same day. The plasmid should possess a different drug marker relative to the Red-producing plasmid and the recombination substrate, and the recombineering frequencies are reported as recombinants per competent cell (recombinant titer/plasmid transformant titer). Recombineering with linear dsDNA substrates is usually on the order of 10^{-4} to 10^{-5} per viable cell. One can typically expect 50 ng of an intact plasmid to transform about 10% of the cell population following electroporation. Thus, the range of recombinant titer/plasmid titer is expected to be 10^{-3} to 10^{-4}. However, these numbers

can vary depending on the purity of the DNA samples and the electrocompetence of the cells. In addition, while recombineering with small homology substrates (50 bp flanks) works in a variety of strains that are deficient for host recombination (e.g., *recA* strains), the total number of recombinants may be reduced due to lower strain viability, relative to wild type, following electroporation.

13. Troubleshooting. If no colonies or recombinant clones are found, examine this list of possible reasons/solutions:

    No Colonies

    (a) Design the primers so that the drug marker reads in the same direction as neighboring genes. If one direction does not work, try the other.

    (b) Clean PCR substrate by ethanol precipitation (see Note 3).

    (c) Problem with PCR product. Generate more or order new oligos.

    (d) Make sure cells are electrocompetent by transforming with an intact plasmid (e.g., pBR322). One should obtain at least 10^7 transformants per microgram of DNA.

    (e) Measure total numbers of survivors on LB plates. Less than 10^6 cells/ml following electroporation indicates that the cells were not grown to high enough density, were lost during centrifugation steps, or are not surviving the electroporation shock. In this last case, check for salt contamination in PCR sample or in the washed cell preparation.

    (f) Increase cell outgrowth postelectroporation to a longer period of time (2 h or more), or even overnight.

    (g) Recombineering strain was grown at 37°C, a temperature too high to maintain the recombineering plasmid (pKM208 requires growth at 30°C).

    (h) Make minilysate preparation from recombineering strain; verify the presence of pKM208 (8731 bp).

    (i) Forgot to add inducer IPTG, or added it too late.

    Colonies obtained but not recombinant targeted knockout.

    (j) Make sure the PCR substrate is free of intact plasmid (see Note 2).

    (k) Check negative control electroporation without DNA (see Note 11) to ensure cell line is not contaminated with plasmid.

14. Verification of the absence of the wild-type loci by PCR analysis is important, as one can (on occasion) find PCR products representative of the replaced target gene (including junctions between the 3′ and 5′ regions of the drug marker and

adjacent chromosomal regions of the target gene), but still find an intact target gene present on the chromosome. This anomalous event might happen when recombineering occurs in a strain that is transiently duplicated for the targeted loci, thus allowing both deleted and wild-type versions of the gene to be present in the same chromosome. Such events can mistakenly identify an essential gene as nonessential.

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