Recombineering: Using Drug Cassettes to Knock out Genes *in vivo*

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Purpose

A "gene knockout" or "knockout" is a mutation that inactivates a gene function. These mutations are very useful for classical genetic studies as well as for modern techniques including functional genomics. In the past, knockouts of bacterial genes were often made by transposon mutagenesis. In this case, laborious screens are required to find a knockout in the gene of interest. Knockouts of other organisms have traditionally been made by first using *in vitro* genetic engineering to modify genes contained on plasmids or Bacterial Artificial Chromosomes (BACs) and later moving these modified constructs to the organism of interest by cell culture techniques. Other methods utilizing a combination of genetic engineering and *in vivo* homologous recombination were inefficient at best. Recombineering provides a new way to generate knockout mutations directly on the bacterial chromosome or to modify any plasmid or BAC *in vivo* as a prelude to making knockouts in other organisms. The constructs are designed to the base pair and are not dependent on suitable restriction sites. A drug cassette can be placed anywhere within a gene or the open reading of the gene can be replaced with the drug cassette. Either way, the desired construct is selected for.

Theory

Recombineering is *in vivo* homologous **recombi**nation-mediated genetic engi**neering**. The recombination is mediated by bacteriophage-based recombination systems such as λ Red, RecET, or similar systems. In contrast to classical *in vitro* genetic engineering, recombineering is not limited by the location of restriction sites and the user defines the construct to the base pair. Like genetic engineering, recombineering can be used to make knockout mutations as well as deletions, point mutations, duplications, inversions, fusions and tags. Recombineering is performed by introducing a linear DNA substrate containing the designed change and short homologies to the target DNA into cells expressing the phage-encoded recombination enzymes. These enzymes incorporate the linear DNA into the target, yielding recombinant molecules.

For clarity of this protocol, we concentrate on the most used recombineering system, the bacteriophage λ Red system, which consists of three proteins, Gam, Exo and Beta. The Gam protein inhibits the *E. coli* RecBCD exonuclease, which normally degrades linear dsDNA. Gam is not absolutely required for recombineering but increases the frequency of dsDNA recombination up to 20-fold (Datta et al., 2008). Exo is a 5'→3' double-strand DNA specific exonuclease and is required for dsDNA recombination. The Beta protein, a ssDNA annealing protein, is the central recombinase in recombineering. Importantly, host recombination functions, including the key recombination protein RecA, are not required for recombineering.

The linear DNA substrate is made by PCR amplification of a cassette encoding a drug-resistance gene using bi-partite primers. These primers consist of (from 5'→3') 50 bases of homology to the target region, where the cassette is to be inserted, followed by 20 bases to prime the drug resistant cassette (Figure 1).

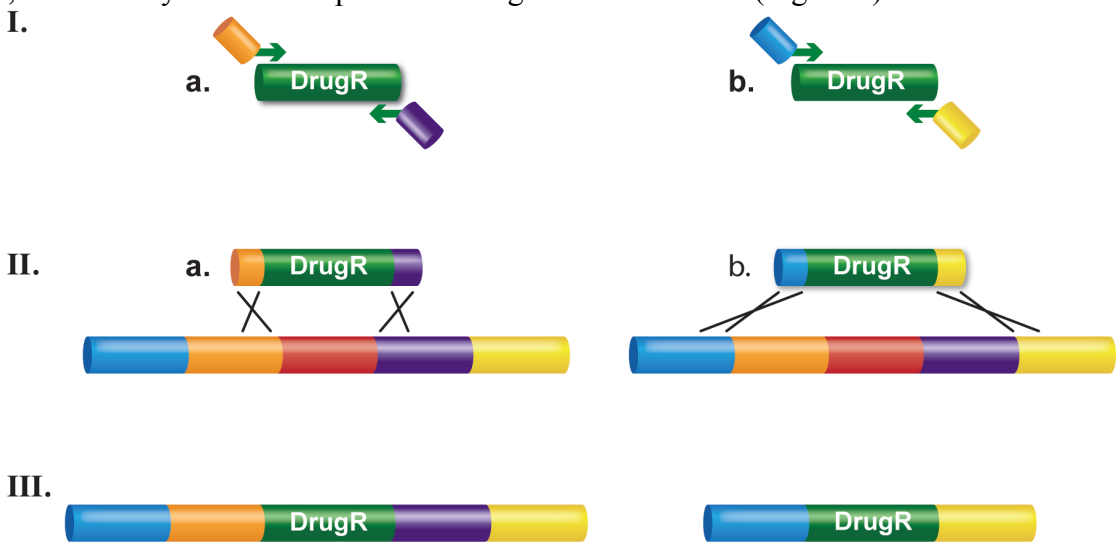

Figure 1. Using dsDNA to generate gene replacement knockouts and deletions. I.) A drug-resistant cassette is made by PCR using two, 70-base hybrid primers. The 5' end of the primer has homology to the target (50-base) and the 3' end of the primer (20-base green arrows) has sequence to prime the drug-resistance template gene (DrugR). Primer design determines the junctions of the final construct. II.) The linear drug-resistant cassette made by PCR is transformed into recombination-competent cells and Red-mediated recombination occurs. Depending on what PCR product was made, (a) or (b), the final recombinant will be a gene knockout/replacement, IIIa, or a more substantial deletion, IIIb, respectively.

The junction sequence created by the primer design determines precisely the final construct. As examples, the cassette can be inserted between two adjacent bases, can be designed to replace the coding sequence of a gene by creating an in-frame, non-polar knockout, or can be used to remove a whole operon and even more. The region deleted can be at least 50kb. The PCR fragment is introduced via electrotransformation into electrocompetent cells that have been induced for the Red system. Having the recombination system highly expressed but under tight regulation is key for achieving optimal recombination frequencies and preventing unwanted rearrangements. The λ Red system under its own regulated promoter is preferred over other systems where the recombination functions are expressed by less tightly

controlled promoters on plasmids. With the Red system, knockouts can be found at a frequency of greater than 10^4 per 10^8 viable cells.

With modifications to the protocol, deletions and point mutations can be made cleanly, without a drug marker or other alteration remaining. This may be accomplished by completing two rounds of dsDNA recombineering using a selection/counter-selection cassette. In the first step, the cassette is inserted and the drug resistance marker is selected. In the second recombination event, the entire cassette is removed by counter-selection. The end result can be a clean deletion, a tag such as GFP or His, or a point mutation created in a gene of interest with no scar left behind. If mutagenic PCR is used to create the linear substrate for the second step, random mutations can be made in the region of interest. The second step can also be done with ssDNA (see other protocol).

Equipment

Thermo cycler for PCR
Agarose gel apparatus
Power supply
Spectrophotometer to read DNA concentration at 260nm and cell density at 600nm
Electroporator (Genepulser II with Pulse Controller II or equivalent)
Constant temperature bacterial incubator set at 30°-32°C. Should contain a roller for liquid culture tubes and shelves for petri plates
32° and 42°C shaking (200rpm) water baths (42°C cannot be an air shaker)
Low-speed centrifuge with Sorvall SA-600 rotor (or equivalent) at 4°C
Refrigerated microcentrifuge at 4°C
Gel imaging system
Insulated ice bucket
Sterile 35 to 50 ml plastic centrifuge tubes
Sterile 50 and 125 ml (or 250) Erlenmeyer flasks, preferably baffled
Micropipettors
Sterile, aerosol-resistant pipettor tips
Pipettes of various sizes
PCR tubes (0.2 ml flat cap tubes)
1.5 ml microfuge tubes
Sterile glass culture tubes with stainless steel closures for culturing bacteria
Spectrophotometer cuvettes
Electrotransformation cuvettes with 0.1cm gap (pre-chilled)
Petri Plates – 100 x 15mm
Optional but highly recommended: DNA analysis software (e.g. Gene Construction Kit by Textco Biosoftware, or Vector NTI by Invitrogen)

Materials

Agarose
DNA molecular weight markers
Ethidium Bromide
PCR cleanup kit such as Qiagen Qiaquick PCR purification kit
Platinum *Taq* DNA Polymerase kit (Invitrogen) or similar DNA polymerase with proof reading ability

PCR grade dNTP set
Double distilled sterile chilled H_2O
Two 70 base primers, desalted but not further purified. See Table 1 for ~20 base drug cassette primer sequences. The ~50 base homology sequence is dependent on the construct being made.
Primers flanking the knockout mutation and within the drug cassette for confirming the construct
Template DNA (See Table 1)
Recombineering-proficient cells. See Table 2 for some options. Plasmids that supply the Red functions are also available. They can be introduced into your strain of choice (Datta, Costantino & Court, 2006; Sharan et al., 2009).

Table 1: Sequence of Primer Pairs for Amplifying Drug-resistance cassettes.

Drug Cassette	Potential template sources[a]	Primer Pair[1]
Ampicillin	pBR322 (New England Biolabs) and derivatives	5' CATTCAAATATGTATCCGCTC 5' AGAGTTGGTAGCTCTTGATC
Kanamycin	pBBR1MCS-2 (Kovach et al., 1994), *Tn5* (Ahmed & Podemski, 1995) Note: this is not the same kanamycin gene as in *Tn903*.	5' TATGGACAGCAAGCGAACCG 5' TCAGAAGAACTCGTCAAGAAG
Chloramphenicol	pACYC184 (New England Biolabs)	5' TGTGACGGAAGATCACTTCG 5' ACCAGCAATAGACATAAGCG
Tetracycline 1: *tetA & tetR*	*Tn10* (Hillen & Schollmeier, 1983) Note: this is not the same tetracycline gene as in pBR322 or pACYC184.	5' CAAGAGGGTCATTATATTTCG 5' ACTCGACATCTTGGTTACCG
Tetracycline 2: *tetA*[2]	*Tn10* (Hillen & Schollmeier, 1983) Note: this is not the same tetracycline gene as in pBR322 or pACYC184.	5' CAAGAGGGTCATTATATTTCG 5' TCCTAATTTTTGTTGACACTCTA
Spectinomycin[3]	pBBR1MCS-5 (Kovach et al., 1994), DH5αPRO (Clontech)	5' ACCGTGGAAACGGATGAAGG 5' AGGGCTTATTATGCACGCTTAA
***cat-sacB* cassette**	pK04/pEL04 (Lee et al., 2001)	5' TGTGACGGAAGATCACTTCG 5' ATCAAAGGGAAAACTGTCCATA
***amp-sacB* cassette**	NC398 (Svenningsen et al., 2005)	5' CATTCAAATATGTATCCGCTC 5' ATCAAAGGGAAAACTGTCCAT

[1] The melting temperature (TM) of these primer pairs is 58°-62°C. Thus, an annealing temp of 54°C will work for all of them. All primer pairs are designed to include a transcriptional promoter. For some genes, the endogenous promoter and shine delgarno sequence are strong enough that the *orf* can be replaced directly with the drug resistance *orf*.
[2] Only TetA is required for tetracycline resistance. This set of *tet* primers makes a smaller cassette but it is unregulated.
[2] Using the spectinomycin cassette to knock out genes can be tricky, with the concentration of Spec needed to allow selection and at the same time prevent

background growth. This concentration must be determined for each construct and in each strain.

Table 2: Useful Recombineering Strains (Modified from Sawitzke et al., 2007)**.**

Strain	Genotype	Special Purpose	Reference
LT521	**MG1655** *gal490 nadA::Tn*10 *pgl*Δ8 [*λ cI857 Δ(cro-bioA)*]	Useful for moving prophage into other backgrounds by P1 transduction using linked *Tn*10.	Lab collection
DY329	**W3110** Δ*lacU169 nadA::Tn*10 *gal490 pgl*Δ8 [*λ cI857 Δ(cro bioA)*]		(Yu et al., 2000)
DY330	**W3110** Δ*lacU169 gal490 pgl*Δ8 [*λ cI857 Δ(cro-bioA)*]		(Yu et al., 2000)
DY331	**W3110** Δ*lacU169* Δ(*srlA-recA*)*301*::*Tn*10 *gal490 pgl*Δ8 [*λ cI857 Δ(cro-bioA)*]	Plasmid recombination.	(Yu et al., 2000)
DY378	**W3110** [*λ cI857 Δ(cro-bioA)*]		(Yu et al., 2000)
DY380	*mcrA* Δ*(mrr-hsdRMS-mcrBC*) *ϕ*80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ*(ara, leu)7697 galU gal490 pgl*Δ8 *rpsL nupG* [*λ cI857ind1* Δ*(cro-bioA)<>tet*] (A derivative of DH10B)	Useful for BAC transformation and manipulations.	(Lee et al., 2001)
SW102	**DY380** Δ*galK*	Use for *galK* selection/counter selection.	(Warming et al., 2005)

Solutions & buffers

Step 3

LB (Luria Broth) pH 7.2

Component	Amount/liter
Bacto-tryptone	10g
NaCl	5g
Yeast Extract	5g

Add water to 1 liter and autoclave for sterility

Tip

Some recipes for LB include 10g of NaCl. We do not recommend this since higher salt reduces cell viability. Be sure to check the specifications if using a commercial supplier.

Step 5

TMG

Component	Final concentration	Stock	Amount/liter
Tris Base	10mM	1 M	10 ml
$MgSO_4$	10 mM	1 M	10 ml
Gelatin	0.01%		100mg

Add water to 1 liter
Adjust to pH 7.4
with HCl.

Autoclave

LB Plates +/- Drug

Add 15g Bacto Agar (Difco) to LB broth to make plates. The concentration of drug needed for selection depends on whether the drug cassette will be in multi-copy (plasmids) or single-copy (BAC, PAC, chromosome).

Table 3. Drug Concentrations for Plates or Broth.

Antibiotic	Single-copy	Multi-copy plasmids
Ampicillin	30	100
Kanamycin	30	50
Chloramphenicol	10	20
Tetracycline	12.5	25
Spectinomycin	30-100*	100
Hygromycin	not determined	50-200

* Using the spectinomycin cassette to knock out genes can be tricky, with the concentration of Spec needed to allow selection and at the same time prevent background growth. This concentration must be determined for each construct and in each strain.

Protocol

Duration

Preparation	none
Protocol	Including waiting two days for oligo orders, from start to confirmed knockout is about 7 days

Tip *This protocol is written assuming the recombineering will be done in E. coli K12. Some paramenters such as growth conditions and electroporator settings may vary with other bacterial species.*

Step 1 Design Construct and Order Primers

Overview *In silico* creation of the final construct in order to design and purchase appropriate PCR primers to make the linear substrate.

Duration 30-60 minutes

1.1 Obtain DNA sequence of the gene or region you wish to knock out. This target sequence must be part of a replicon (BAC, PAC, plasmid,

bacteriophage, chromosome) that will replicate in *E. coli* (or other recombineering-proficient organism).

Tip *DNA analysis software such as Textco's Gene Construction Kit or Invitrogen's Vector NTI greatly simplifies this step.*

1.2 Decide which drug-resistant cassette you wish to use for your knockout.

Tip *Keep in mind that for Step 6, it is helpful if the drug cassette is a different size than the replaced gene.*

1.3 Using the DNA analysis software, paste the sequence of the drug cassette you choose (include promoter and transcriptional terminators as required) into the file containing your gene/region of interest exactly as you want the final construct to appear. Note the two novel DNA junctions you have created by this insertion step.

Tip *Be mindful of the orientation of the drug cassette with respect to the gene. Normally, placing the drug-resistance cassette in the same orientation as the original gene works well.*

Tip *If you are knocking out prokaryotic gene(s), be aware of the possible effects of polarity of your knockout on downstream genes; their expression may be altered. Also, overlapping genes exist in E. coli. Don't delete the ribosome binding site or start codon of a downstream gene. Design carefully. Web sites such as http://ecocyc.com/ can be very helpful.*

1.4 Design the primers. The ~70 base primers will contain, at the 5' end, ~50 bases of homology. This is the sequence just outside the new junctions in the *in silico* construct you have created. In addition, the primer will also contain ~20 bases at the 3' end that will prime synthesis of the chosen drug cassette. This drug cassette priming sequence will usually be that shown in Table 1 unless, for example, you wish to omit the promoter and have drug resistance expressed by the endogenous promoter and ribosome binding site.

Tip *The primers can also include additional short sequences such as His tags, frt or lox sites, or restriction sites.*

Tip *Using the formula of 4°C for a G/C base pair and 2°C for an A/T base pair, the annealing temperature of the primers (include only the drug cassette priming region of the primer) can be set to a suitable value (e.g. 60°- 64°C) by shortening or lengthening the primer length. The two primers should have similar annealing temperatures.*

1.5 Order the two ~70 base primers from IDT or a similar company. 100nMole scale is sufficient (and normally required) for this length. Other than desalting, no additional purification is needed or wanted.

Tip *The four ~20 base primers for confirming the knockouts in Step 6.1 should be designed and ordered now too. These consist of two primers in the flanking DNA and two primers in the drug cassette (see Figure 2).*

Step 2 Set Up and Generate Linear Substrate by PCR

Overview Using the chimeric primers ordered during Step 1 and an appropriate template, the linear recombination substrate will be made and purified.

Duration 5-10 min set up, ~3-4 hours for PCR, and 10 min for cleanup. 1 hour for gel and DNA quantitation.

2.1 Set up PCR reactions using the two ~70 base primers ordered in Step 1. Table 1 (under "Materials") lists potential template sources. A typical reaction is as follows:

Table 4. Typical 50µl PCR reaction mix.

COMPONENT	COMMENTS
38.5 µl H_2O	Sterile and distilled
5 µl 10X buffer	
2 µl MgSO4	50mM
1 µl dNTP mix	Mixture containing 10mM of each dNTP
1 µl primer 1	25pmoles/µl
1 µl primer 2	25pmoles/µl
1 µl template	0.5-1.0 ng/µl
0.5 µl Platinum *Taq*	Invitrogen

Tip *If cells containing a drug cassette in the chromosome are used as a template, 3 µl of an overnight culture can be used. In addition, you can do "touch PCR". In this case, a small amount of a well isolated colony containing the template is collected with an innoculating loop (not wood) and cells are swirled in the PCR mix (made without leaving any volume for template).*

Tip *If a plasmid is used as a PCR template, it must be linearized by restriction. To minimize residual uncut plasmid, use the least amount of linear plasmid possible for the PCR template. Remember that even when cut with a restriction enzyme, some circular plasmid will remain. These will readily transform cells to drug resistance and will show up as "false positives". These will show up in the uninduced control from Step 4.1.*

Table 5. Typical reaction conditions for a ~1.5 kb cassette using Platinum *Taq* are:

STEP	TEMPERATURE (°C)	TIME
1	95	2 min
2	94	30 sec
3	55	30 sec

4	68	1.5 min
5	Go to step 2	29 times
6	68	10 min
7	4	end

Tip *If linear plasmid DNA was used as a template and you are getting residual plasmid transformation from the uninduced control in Step 4.1, cut the PCR product with the modification-dependent restriction enzyme Dpn*I*, which will cut the plasmid DNA but not the unmodified PCR product. Dpn*I *digestion may not totally eliminate plasmid background, however.*

Tip *It is not necessary to gel purify the DNA. In fact, exposure of the PCR product to direct ultraviolet light will damage it, and may result in abnormal recombination frequencies as well as mutations.*

2.2 Clean the PCR product following instructions on a kit such as Qiagen Qiaquick PCR purification kit. Elute in a small amount of TE at a final DNA concentration of 100ng/µl.

2.3 Run a sample of PCR product on a gel with molecular weight markers to confirm size.

2.4 Determine DNA concentration of PCR fragment.

Step 3 Preparing Cells Competent for Recombineering

Overview Cells are prepared to be recombineering-proficient and ready for electrotransformation with the linear substrate made in Step 2.

Duration About 3.5 hours

Preparation The previous day, grow a 5 ml overnight culture of the chosen recombineering cells (Table 2) at 30-32°C. Include the appropriate drug if a plasmid is supplying the Red functions.

Caution *Do not grow recombineering cells at temperatures greater than 34°C.*

Caution *Maintain sterile technique throughout the rest of the protocol.*

3.1 Dilute overnight culture by adding 0.5 ml of the overnight to 35 ml of LB medium with the appropriate drug(s) if needed, in a 125 ml (or 250 ml) baffled Erlenmeyer flask. Dilute the overnight at least 70-fold. Grow cells in a H_2O bath at 32°C with shaking (200rpm) until OD_{600} is from 0.4-0.5 (approximately 2 hrs).

Tip *Cells with different genotypes will grow at different rates. Having the proper OD_{600} is critical – the recombination will not work if the density is too high.*

Tip *Only add drug to the LB if it is needed to maintain a plasmid.*

3.2 Transfer half the culture to a 50 ml (or 125 ml) baffled Erlenmeyer flask and place that flask in a 42ºC H_2O bath to shake at 200rpm; keep the other flask at 32ºC. Shake for 15 min. The culture at 42ºC is now induced for the recombination functions and the 32ºC culture is the uninduced control. Both flasks will be processed identically during the rest of the protocol.

3.3 Immediately after the 15 min induction, rapidly chill both cultures in an ice-water slurry; swirl the flasks gently. Leave on ice for 5-10 min. Label and chill the necessary number of 35-50 ml centrifuge tubes for the induced and uninduced cells.

Tip *Pre-chill the sterile distilled H_2O that will be used for washes. Keep 200 ml bottles of distilled water at 4°C prior to starting the experiment for this purpose and put it on ice as needed. Also chill electrotransformation cuvettes and microcentrifuge tubes for later parts of this step.*

3.4 Transfer both the induced and uninduced cultures to the chilled centrifuge tubes and centrifuge 7 min at ~6500 x *g* (6700 rpm in a Sorvall SA-600 rotor) at 4ºC. Using sterile technique, aspirate or pour off supernatant.

3.5 Add 1 ml ice-cold sterile distilled H_2O to the cell pellet and gently suspend cells with a large disposable pipet tip (do not vortex). After cells are well suspended, add another 30 ml of ice-cold distilled H_2O to each tube, seal, and gently invert to mix, again without vortexing. Centrifuge tubes again as in previous step.

3.6 Promptly decant the 30 ml supernatant very carefully from the soft pellet in each tube and gently suspend each cell pellet in 1 ml ice-cold distilled H_2O.

Tip *As the pellets are very soft, tubes must be removed promptly after centrifugation and care should be taken not to dislodge the pellet. It is ok at this step to leave a small amount of supernatant in the tube.*

3.7 Transfer the suspended cells to pre-chilled microcentrifuge tubes. Centrifuge 30 sec at maximum speed in a 4°C refrigerated microcentrifuge. Carefully aspirate supernatant and suspend cells in 200 µl sterile ice-cold distilled H_2O and keep on ice until used.

Tip *This protocol will prepare enough cells for four electroporations. If more cells are needed, prepare additional flasks.*

Step 4 Electrotransformation of Linear Substrates into the Recombineering-ready Cells

Overview Recombineering-proficient, electrocompetent cells from Step 3 are transformed, via electrotransformation, with the linear substrate from Step 2 to make the knockout.

Duration About 2.5 hours including outgrowth

4.1 In labeled cuvettes on ice, place 50µl of electrocompetent cells. Pipette in 1 µl (~100ng) of salt-free PCR fragment. Next take a 200 µl pipette tip and pipette up and down several times to mix. The cells are now ready for electrotransformation.

Tip *Good mixing of the DNA with the cells is important, however, do not vortex.*

Tip *Using non-aerosol tips will help prevent contamination problems.*

Tip *When modifying a multi-copy plasmid, add 1 µl of the plasmid DNA (~20ng/µl) at this point. Special considerations must be taken when modifying a multi-copy plasmid. See Thomason et. al (2007)for further details.*

Tip *Include the following controls: 1) Induced cells without DNA: if colonies are present on this control plate, either the selection is not working properly, the cells have a high reversion frequency for the property selected, or there is contamination from other bacteria, DNA from the pipettor etc. 2) Uninduced cells plus DNA: this is a control to estimate the number of background colonies due to some contaminating factor in the DNA such as intact plasmid template from the PCR reaction or in the cell prep.*

4.2 Transform the DNA into the cells by electrotransformation. The electroporator should be set to 1.8kV.

Tip *For optimal results, the time constant should be greater than 5 msec, however, we have obtained recombinants with time constants as low as 4.5 msec or so. Lower time constants generally indicate impurities or salts in the cells or the DNA. Occasionally a cuvette may be defective and will arc but arcing is often a sign of too much salt.*

4.3 Immediately after electrotransformation, add 1 ml of room temperature LB medium to the cuvette. Do this before proceeding to the next electrotransformation. After all the electrotransformations are complete, transfer the 1 ml electrotransformation mixes to sterile culture tubes and incubate with shaking (or rolling) at 32ºC for two hours to allow completion of recombination and expression of the drug-resistance gene.

Tip *To ensure that each recombinant is independent, after an outgrowth of 30 minutes, the cells can be plated on filters on LB plates for further outgrowth (Yu et al., 2000).*

Step 5 Selecting for Knockout Mutations

Overview Dilution and plating of cells to select for the knockout mutant.

Duration ~30 minute for dilution and plating of cells then 22-24 hours for colonies to incubate

5.1 Following the outgrowth, make 10-fold serial dilutions of the experimental cultures out to 10^{-6} in a buffered medium lacking a carbon source such as TMG. To select recombinants, spread 0.1 ml of the undiluted culture and of the 10^{-1} and 10^{-2} dilutions on plates selective for the recombinant. See Table 3 (Solutions and Buffers) for drug concentrations to use. Also assay total viable cells by plating 0.1 ml of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions on LB plates. A determination of cell viability allows calculation of a recombinant frequency. If the number of viable cells is too low, less than 10^{7}/ml or so, recombinants may be rare or not found. For the control cultures, both the uninduced (32°C) and the induced (42ºC) to which no DNA was added, plate 0.1 ml of the undiluted culture on a single selective plate.

5.2 Incubate plates at 30-32° until medium-sized colonies appear, normally 22-24 hours.

Step 6 Confirming Knockout Mutations

Overview Using PCR to confirm that the knockout has been made.

Duration 30 min set up, ~3-4 hours for PCR. ~1 hour for gel

6.1 For confirming a knockout by PCR, use two pairs of primers, each pair having one primer in DNA flanking the targeted region and one primer in the drug-resistant cassette, and amplify the two junctions (Figure 2). Another PCR reaction, using the two outside flanking primers, should also be performed to confirm the absence of the gene to be removed, thus ruling out the possibility of a duplication event. As a control, the parent cells should be used as a template. The presence of duplications can indicate that the knockout was made in an essential gene or is polar on one (see Bubunenko, Baker & Court, 2007).

Tip *The frequency of recombination gives an indication as to whether your construction has removed or is polar on an essential gene. Typical knockouts are $10^{4}/10^{8}$ viable but for an essential gene, the frequency is typically >100-fold reduced* (see Bubunenko et al., 2007)..

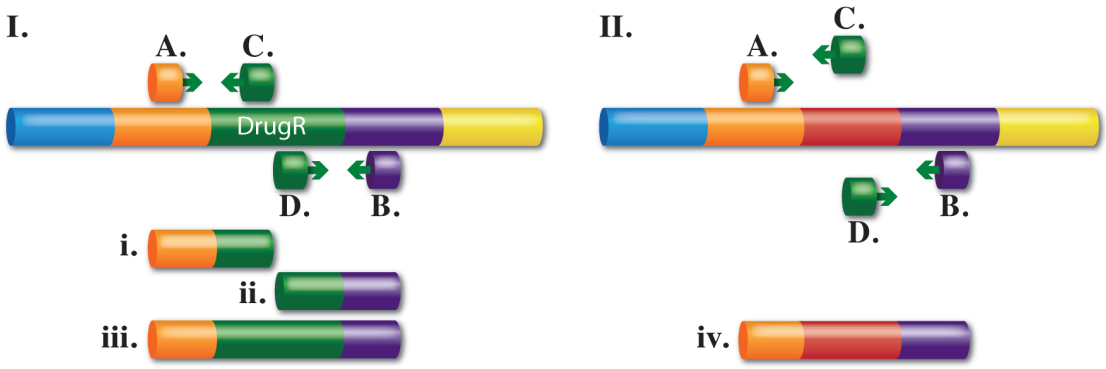

Figure 2. Confirming the knockout mutation. The colors represent different genes. I.) Diagram showing a putative knockout and the primers used to confirm it. Primer set A/C should produce product "i". Primer set D/B should produce product "ii" and primer set A/B should produce product "iii". II) The same primers sets should be used on the parental strain as a control. In this case, however, the only product, "iv", should be with primer set A/B. With careful design, fragments "iii" and "iv" will be of a different size. If not, restriction analysis can often distinguish them. Having all three correct size fragments on the candidate is a good indication the knockout is as designed.

6.2 Run a sample of the PCR products on a gel with molecular weight markers to confirm sizes. If all products are the expected sizes, the knockout is ready to use.

Tip *For troubleshooting and the most up to date information on recombineering, see: http://redrecombineering.ncifcrf.gov/*

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