CHAPTER TEN

Recombineering: Highly Efficient *in vivo* Genetic Engineering using Single-strand Oligos

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Abstract

Recombineering provides the ability to make rapid, precise, and inexpensive genetic alterations to any DNA sequence, either in the chromosome or cloned onto a vector that replicates in *E. coli* (or other recombineering-proficient bacteria), and to do so in a highly efficient manner. Complicated genetic constructs that are impossible to make with *in vitro* genetic engineering can be created in days with recombineering. Recombineering with single-strand DNA (ssDNA) can be used to create single or multiple clustered point mutations, small or large (up to 10 kb) deletions, and small (10–20 base) insertions such as sequence tags. Using optimized conditions, point mutations can be made with such high frequencies that they can be found without selection. This technology excels at creating both directed and random mutations.

1. THEORY

Recombineering is *in vivo* homologous recombination-mediated genetic engineering. The recombination is mediated by bacteriophage-based

recombination systems such as λ Red, RedET, or similar systems. In contrast to classical *in vitro* genetic engineering, recombineering does not rely on restriction enzymes. Thus with recombineering, the location of restriction sites is not an issue and the user defines the construct to the base pair.

Recombineering is performed by introducing a linear substrate containing the desired change, along with short homologies to the target DNA, into cells expressing the phage-encoded recombination enzymes. These enzymes recombine the linear DNA with the target, yielding recombinant molecules. Single-stranded linear DNA in the form of a ~70 base, commercially available oligonucleotide (oligo) can be used to make point mutations (including mutations that knock out function) as well as deletions (up to 10 kb), and small (up to 20 base) insertions.

1.1. Considerations for Designing Primers for Recombineering Using ssDNA

Many parameters of recombineering using ssDNA have been optimized and oligo design is the key for the highest efficiencies. With optimized protocols, we have seen that over 50% of unselected colonies can contain the desired change. The oligo should be designed keeping the following parameters in mind in order to yield the highest recombination frequencies and make it easier to find the nonselected alteration:

- The oligo should correspond in sequence to the DNA strand that is replicated discontinuously, that is, the lagging-strand. If the leading-strand is chosen, recombination will be reduced up to 20-fold. You may try both strands; one will be more efficient than the other (Ellis et al., 2001).
- The oligo should be ~70 bases in length with the changes near the middle of the oligo. None of the base changes should be less than 10 bases from an end. In some cases, longer oligos may be needed, for example, if a small insertion such as a His-tag is being inserted. Keep in mind that the longer the oligo is, the more likely it is to have errors introduced during synthesis. Further purification of oligos of this length is not typically helpful.
- The oligo should be designed to avoid the methyl-directed mismatch repair (MMR) system, or the recombineering should be done in a cell deficient in this system. Avoidance of the MMR system increases the efficiency 100-fold or more. This is the critical parameter for optimization.

See Table 10.1 for a list of MMR-deficient strains.

Table 10.1 Useful recombineering strains

Strain	Genotype	Special purpose	Reference
LT521	**MG1655** *gal490 nadA::Tn*10 *pgl*Δ8 [λ *c*I*857* 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 *c*I*857* Δ*(cro bioA)*]		Yu et al. (2000)
DY330	**W3110** Δ*lacU169 gal490 pgl*Δ8 [λ *c*I*857* Δ*(cro-bioA)*]		Yu et al. (2000)
DY331	**W3110** Δ*lacU169* Δ*(srlA-recA)301*::*Tn*10 *gal490 pgl*Δ8 [λ *c*I*857* Δ*(cro-bioA)*]	Plasmid recombination	Yu et al. (2000)
DY378	**W3110** [λ *c*I*857* Δ*(cro-bioA)*]		Yu et al. (2000)
HME6	**W3110** $galK_{tyr145UAG}$ Δ*lacU169* [λ *c*I*857* Δ*(cro-bioA)*]	Assay system for oligo recombineering	Ellis et al. (2001)
SIMD50	**HME6** [λ *c*I*857 (int-cIII<>bet)*]	Beta only strain. Great for oligo recombineering	Datta et al. (2008)
SIMD90	**SIMD50** *mutS<>cat*	High-frequency oligo recombineering. Defective for MMR	Lab collection
SIMD61	**HME6** [λ *c*I*857(int-cIII<>amp)* Δ*(cro-bioA)*]	No *gam* gene	Datta et al. (2008)
HME63	**W3110** $galK_{tyr145UAG}$ Δ*lacU169 mutS<>amp* [λ *c*I*857* Δ*(cro-bioA)*]	Defective for MMR, therefore, gives high-level oligo recombineering	Costantino and Court (2003)
HME68	**W3110** $galK_{tyr145UAG}$ Δ*lacU169* [λ *c*I*857* Δ*(cro-bioA)*] *mutS<>cat*	Defective for MMR	Thomason et al. (2007a)
HME70	**W3110** $galK_{tyr145UAG}$ Δ*lacU169* [λ *c*I*857* Δ*(cro-bioA)*] *mutS<>cat* Δ*(srlA-recA)301*::*Tn*10	Oligo recombineering with plasmids. Defective for MMR	Thomason et al. (2007a)

Continued

Table 10.1 Useful recombineering strains—cont'd

Strain	Genotype	Special purpose	Reference
HME71	**W3110** *galK*$_{tyr145UAG}$ Δ*lacU169* [λ *cI857* Δ*(cro-bioA)*] Δ(*srlA-recA*) *301*::*Tn*10	Oligo recombineering with plasmids	Thomason et al. (2007a)
DY380	*mcrA* Δ*(mrr-hsdRMS-mcrBC)* φ80d*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)

Even better, oligos should be designed to avoid MMR. Oligos containing any of the following features will not be recognized by the MMR system:

1. Design the oligo so that, when annealed to the target, it creates a C/C mispair at the target base or six bases away from the target base (Costantino and Court, 2003).
2. Change 5 bases in a row. This is a good way to make a gene knockout (Sawitzke et al., in press).
3. Change 4–5 wobble positions in a row, in addition to the designed change. This allows for high frequency targeted mutagenesis without additional changes to the encoded protein. This method is of general utility and is particularly useful for targeting essential genes with high efficiency in one step (Sawitzke et al., in press).

Whereas recombineering with dsDNA requires both the Red Exo and Beta proteins, only Beta is required for recombination with ssDNA. Thus, either recombineering-proficient cells that produce all three Red functions (Exo, Bet, Gam) or those that produce only the single-strand annealing protein, Beta can be used for ssDNA recombination.

1.2. Considerations for Determining the Duration of Bacterial Outgrowth

When recombination occurs in the bacterial cell, there are usually —four to eight replicating copies of the chromosome. Nevertheless, recombination with an oligo occurs in most instances on one strand of one copy

(Costantino and Court, 2003). During outgrowth of these cells, the chromosome copies segregate from one another, separating recombined from nonrecombined DNA copies. The proper outgrowth time depends on the details of the construct but always requires a minimum of 30 min for recovery from electrotransformation. A 30-min outgrowth period is ideal if there is no selection for the desired genetic alterations. At this time the cells have recovered but no chromosomal segregation has taken place and thus the largest percentage of colonies will contain the relevant construct. In some cases, when a phenotype can be scored directly (e.g., small colony) or a counter-selectable marker is being selected against, sufficient time must be allowed for outgrowth in liquid culture to obtain a pure colony that will exhibit the phenotype. In this case, an extended outgrowth period >2 h is needed before plating. After an extended outgrowth, each colony will be clonal but the frequency of recombinant colonies will be reduced due to the outgrowth and segregation processes.

Recombineering with ssDNA is a powerful technique that can be used in many ways. This protocol outlines using oligos to make nonselected point mutations, either single or closely linked. Figure 10.1 outlines the steps in this process.

With slight modifications to the protocol presented, systems can be set up to select for the mutations and localized mutagenesis can be achieved. For localized mutagenesis, oligos can be purchased containing random bases at several positions, and random colonies can be screened for interesting phenotypes or for the presence of mutations using PCR.

If a selection is needed, this may be accomplished by completing two rounds of recombineering. In the first round, dsDNA recombineering is used to insert a selection/counter-selection cassette (see Recombineering: Using Drug Cassettes to Knock out Genes *in vivo*) and the drug marker is selected. In the second recombination event, the entire cassette is removed by counter-selection using an oligo (Sharan et al., 2009).

2. EQUIPMENT

PCR thermocycler
Gel electrophoresis equipment
UV/Vis spectrophotometer
Electroporator (e.g., Genepulser II with Pulse Controller II, Bio-Rad)
Bacterial incubator (set at 30–32 °C)
Incubator roller (for liquid culture tubes)

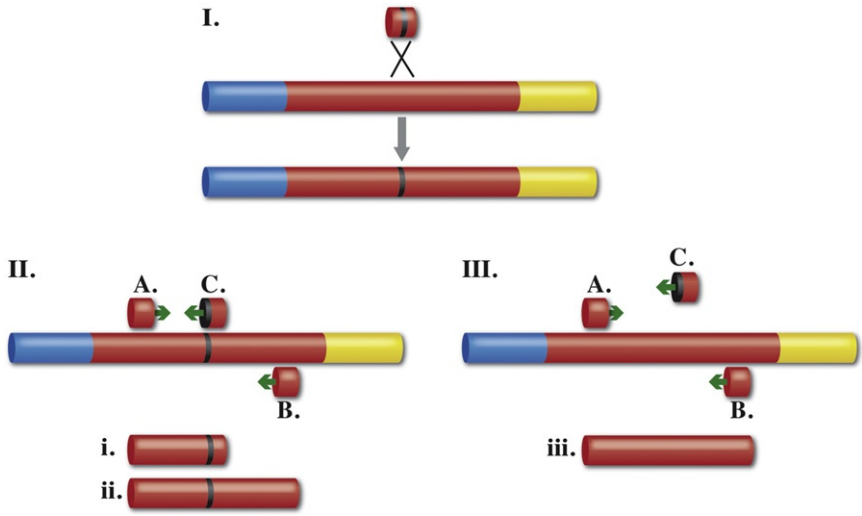

Figure 10.1 Using recombineering with an oligo to create a sequence change and PCR to screen for it. (I) λ Beta inserts a 5 base change (black bar) contained within a ~70 base oligo into a gene on a BAC or in the chromosome in up to 50% of the colonies. The mutations can be detected by PCR. (II) PCR using primers A and C generates a product (i) if the change was incorporated. It is not made if the colony only contains the parental DNA as in (III). Primers A and B flank the change. The 3′ end of primer C is designed to anneal only if the change is present. After purified colonies are found that contain the relevant change(s), PCR using primers A and B will be used to make a product for DNA sequencing to confirm the sequence of the final construct.

Shaking water baths (set at 32 and 42 °C) (42° cannot be an air shaker)
Low-speed centrifuge
Sorvall SA-600 rotor (or equivalent)
Microcentrifuge (refridgerated)
Gel imaging system
Insulated ice bucket
Sterile 35–50 ml polycarbonate centrifuge tubes
Erlenmeyer flasks, preferably baffled (50 and 125 or 250 ml)
Micropipettors
Sterile, aerosol-resistant micropipettor tips
Pipettes
0.2 ml thin-walled PCR tubes
1.5 ml microcentrifuge tubes
Sterile glass culture tubes with stainless steel closures
Spectrophotometer cuvettes

Electrotransformation cuvettes (with 0.1 cm gap)
100 × 15 mm Petri plates
DNA analysis software (e.g., Gene Construction Kit by Textco Biosoftware, or Vector NTI by Invitrogen) (Optional but highly recommended)

3. MATERIALS

Primers (see Theory section and Step 1 for design of primers)
Bacto-tryptone
Sodium chloride (NaCl)
Yeast extract
Tris base
Magnesium sulfate ($MgSO_4$)
Gelatin
Bacto Agar
Agarose
DNA molecular weight markers
Ethidium bromide
Platinum® *Taq* DNA Polymerase High Fidelity kit (Invitrogen) (or similar DNA polymerase with proofreading ability)
dNTP set
Double-distilled sterile chilled H_2O
Recombineering-proficient cells. See Table 10.1 for some options. Plasmids that supply the Red functions are also available. They can be introduced into your strain of choice (Datta et al., 2006; Sharan et al., 2009).

3.1. Solutions & buffers

Step 2 LB (Luria Broth), pH 7.2

Component	Amount
Bacto-tryptone	10 g
NaCl	5 g
Yeast extract	5 g

Add water to 1 l and autoclave

Tip *Some recipes for LB include 10 g 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 4 TMG

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

Add water to 1l. Adjust to pH 7.4 with HCl and autoclave.

LB Plates

Add 15 g Bacto Agar (Difco) to 1 l of LB broth and autoclave. Cool to ~55 °C, add antibiotic as needed, and pour plates.

4. PROTOCOL

4.1. Duration

Preparation	None
Protocol	About 7–10 days

4.2. Preparation

None

4.3. Tip

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

See Fig. 10.2 for the flowchart of the complete protocol.

5. STEP 1 DESIGNING THE OLIGO FOR RECOMBINEERING

5.1. Overview

Create the final construct *in silico* in order to design and order the appropriate oligo to create the desired mutation (see Theory section, Considerations for designing primers for recombineering using ssDNA).

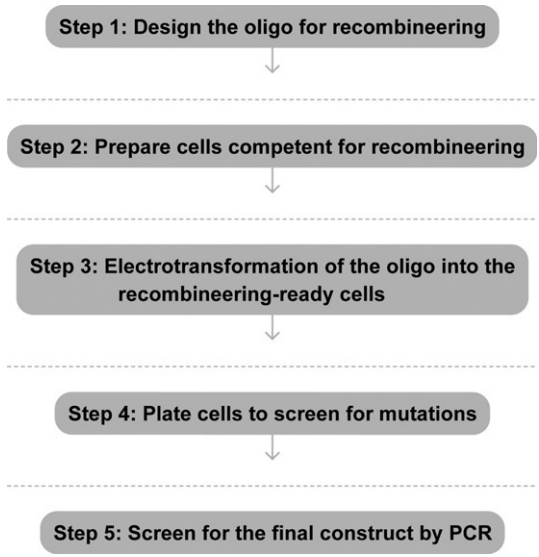

Figure 10.2 Flowchart of the complete protocol.

5.2. Duration

30–60 min

1.1 Obtain the DNA sequence of the gene or region you wish to alter. This target sequence must be part of a replicon (BAC, PAC, plasmid, bacteriophage, chromosome) that will replicate in *E. coli* (or other recombineering-proficient organisms).

1.2 Using DNA analysis software, create *in silico* the final construct that you wish to make.

1.3 Design the 70 base oligo using the sequence of the final construct with the mutation(s) or changes near the middle of the oligo, This is not enough information. Ideally, it should be as we wrote it. If not that, must say something like: "The primer must be designed keeping all the parameters discussed at length in the theory section in mind. Then all that was deleted here must be included in the theory section. This is key to the protocol. keeping in mind all of the parameters discussed in the Theory section.

1.4 Design the primers needed to detect the mutation(s). You will need three ~20 base primers. The first two should be about 400–500 bases apart from one another, flanking the region you are modifying (primers A and B in Fig. 10.1) (see Explanatory chapter: PCR -Primer design). The extreme 3′ end of primer C must contain all of the changes present

in the mutagenic oligo (i.e., the 5 base change seen in Fig. 10.1, or four wobble changes plus the desired change). Primers A and C should be about 200–300 bases apart from each other. Can refer to the chapter on primer design for design of primers A and B if you wish. Need more discussion of design of primer C and why as we had included.

1.5 Order the oligos from IDT or a similar company. We have had the best results from IDT oligos so it should be mentioned. 100 nmol scale is sufficient (and normally required) for a ~70-mer. 25 nmol is fine for the shorter primers. Other than desalting, no additional purification is needed.

5.3. Tip

DNA analysis software such as Gene Construction Kit (Textco) or Vector NTI (Invitrogen) greatly simplifies this process.

5.4. Tip

Good results have been obtained using primers ordered from Integrated DNA Technologies (http://www.idtdna.com/).

5.5. 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. Do not 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.

5.6. Tip

When a nonessential gene is being modified, two rounds of recombineering can be used in order to get the single base change if needed. In the first round, 4–5 bases in addition to the relevant change are altered. In the second round, the 4–5 changes are restored to wild-type sequence leaving only the desired change. Both of these events will occur at high frequencies.

5.7. Tip

The three primers, A, B, and C, should have similar annealing temperatures, 60–64°C. Use the general formula of 4°C for a G/C pair and 2°C for an A/T pair.

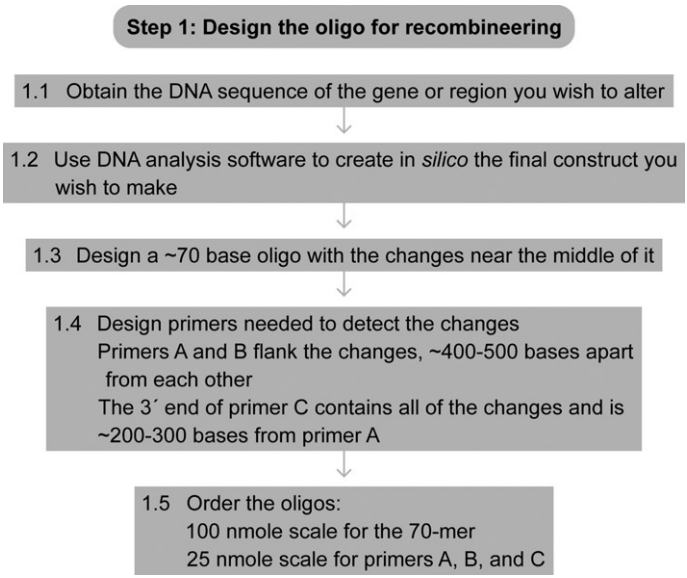

Figure 10.3 Flowchart of Step 1.

5.8. Tip

The A + C primer pair should not amplify the parental DNA but will amplify a DNA molecule containing the mutation. This diagnostic PCR also works if the mutations were put in wobble positions. Just design primer C accordingly. This is due to the fact that primer C is a diagnostic oligo: it contains all of the changes at its 3′-end, thus it will anneal to DNA molecules that have the changes, but it will not anneal to the parental DNA. This is true whether the changes are contiguous or are wobble changes plus the desired change.

5.9. Tip

If you are doing random localized mutagenesis with an oligo, place the random bases to one side of the 5 base changes used to make the recombination a high-frequency event. Those five changes must be of known sequence so a test oligo 'C' can be designed to detect recombinants. DNA sequencing will reveal the linked 'random' changes.

See Fig. 10.3 for the flowchart of Step 1.

6. STEP 2 PREPARING CELLS COMPETENT FOR RECOMBINEERING

6.1. Overview

Make electrocompetent cells that are recombineering-proficient and ready for electrotransformation with the oligo ordered in Step 1 (see Transformation of *E. coli* via electroporation).

6.2. Duration

Overnight, then about 3.5 h

2.1 Grow a 5 ml overnight culture of the chosen recombineering cells (Table 10.1) at 30–32 °C. Include the appropriate drug if a plasmid is supplying the Red functions (see Table 10.2 for antibiotic concentrations).

2.2 Dilute 0.5 ml of the overnight culture into 35 ml of LB medium with the appropriate drug(s) if needed, in a 250-ml (or 125 ml) baffled Erlenmeyer flask. Grow cells in a shaking water bath at 32 °C with shaking (200 rpm) until the $OD_{600} = 0.4–0.5$ (~2 h).

2.3 Transfer half the culture to a 50-ml baffled Erlenmeyer flask, put it in a 42 °C shaking water bath, and shake at 200 rpm for 15 min. Keep the other flask at 32 °C. The culture at 42 °C is induced for the recombination functions while the 32 °C culture serves as the uninduced control. Process both cultures identically for the rest of the protocol.

2.4 Immediately after inducing the cells, rapidly chill both cultures in ice water slurry, swirling the flasks gently. Leave on ice for 5–10 min. Label and chill the necessary number of 35–50 ml centrifuge tubes to pellet the induced and uninduced cells. Prechill the 1.5-ml microcentrifuge tubes, electroporation cuvettes, and sterile distilled water.

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

2.6 Add 1 ml ice-cold sterile distilled H_2O to the cell pellet and gently suspend cells with a large disposable pipette tip (do not vortex). After cells are well suspended, add another 30 ml of ice-cold distilled H_2O to each

Table 10.2 Drug concentrations to maintain plasmids

Antibiotic	Multicopy plasmids ($\mu g\,ml^{-1}$)
Ampicillin	100
Kanamycin	50
Chloramphenicol	20
Tetracycline	25
Hygromycin[a]	200
Spectinomycin	100

[a]Previous protocols have reported using 50 $\mu g\,ml^{-1}$ but we have found with some genetic backgrounds, 200 $\mu g\,ml^{-1}$ is needed for selection.

tube, seal, and gently invert to mix, again without vortexing. Centrifuge at ~6500 × *g*, 4 °C for 7 min.

2.7 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.

2.8 Transfer the suspended cells to prechilled microcentrifuge tubes. Centrifuge 30 s at maximum speed in a microcentrifuge at 4 °C. Carefully remove the supernatant and suspend cells in 200 µl sterile ice-cold distilled H_2O and keep on ice until used.

6.3. Caution

Do not grow recombineering cells at temperatures greater than 34 °C. Maintain sterile technique throughout the protocol.

6.4. Caution

The induction of recombination functions ***must*** *be carried out in a shaking water bath set at 42 °C. Inducing the cultures in a 42 °C shaking incubator will not work.*

6.5. 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.*

6.6. Tip

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

6.7. Tip

Prechill the sterile distilled H_2O *that will be used for washes. Keep 200 ml bottles of distilled water at 4 °C for this purpose and put it on ice as needed.*

6.8. Tip

As the pellets *in Step 2.7 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.*

6.9. Tip

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

See Fig. 10.4 for the flowchart of Step 2.

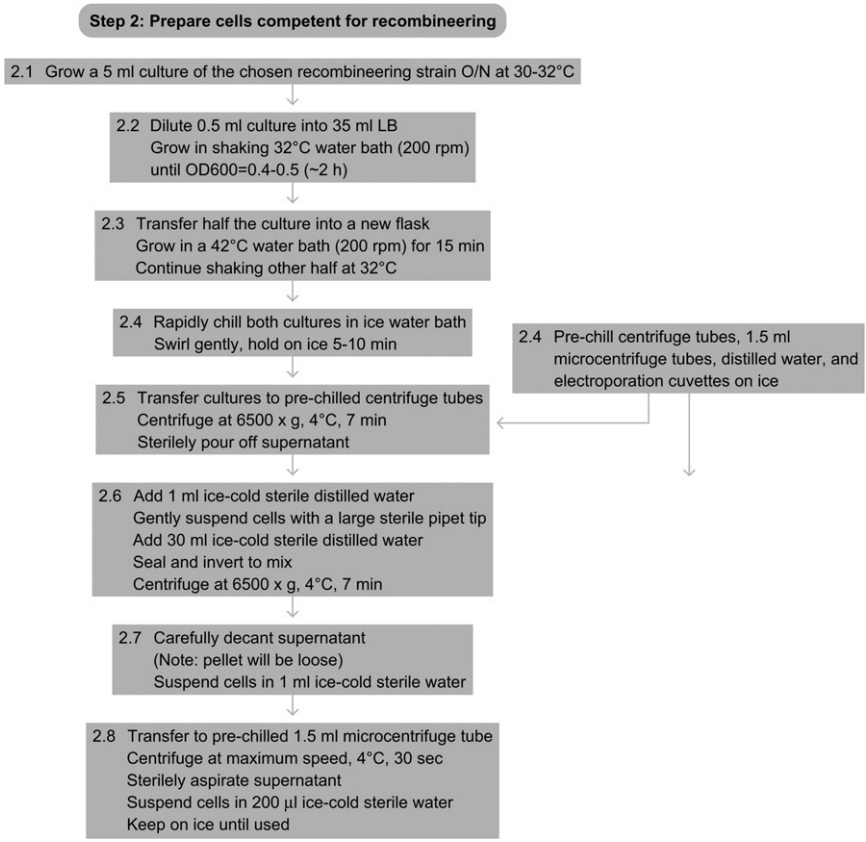

Figure 10.4 Flowchart of Step 2.

7. STEP 3 ELECTROTRANSFORMATION OF THE OLIGO INTO THE RECOMBINEERING-READY CELLS

7.1. Overview

Recombineering-proficient, electrocompetent cells from Step 2 are transformed, via electrotransformation, with the oligo from Step 1 (see Transformation of *E. coli* via electroporation).

7.2. Duration

About 1–4 h

3.1 Place 50 µl of electrocompetent cells in labeled cuvettes on ice. Add 0.5–1 µl (~100 ng) of salt-free oligo. Use a 200 µl pipette tip to pipette

up and down several times to mix. The cells are now ready for electrotransformation.

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

3.3 Immediately add 1 ml of room temperature LB medium to the cuvette and then proceed to the next electrotransformation. After all of the samples have been electroporated, transfer the electrotransformation mixes to sterile culture tubes and incubate with shaking (or rolling) at 32 °C for 30 min (or up to >2 h, see Theory section, Considerations for determining the duration of bacterial outgrowth).

7.3. Tip

Good mixing of the DNA with the cells is important; however, never vortex the samples.

7.4. Tip

Use of nonaerosol barrier tips will help prevent contamination problems.

7.5. Tip

When modifying a multicopy plasmid, add 1 µl of the plasmid DNA (~20 ng µl^{-1}) prior to electroporation. Special considerations must be taken when modifying a multicopy plasmid. See Thomason et al. (2007b) for further details.

7.6. Tip

For optimal results, the time constant should be greater than 5 ms; however, we have obtained recombinants with time constants as low as 4.5 ms 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.

7.7. Tip

For a counter-selection, the outgrowth should be done in 10 ml of LB for >2 h. For more details on outgrowth, see Sawitzke et al. (2007).

See Fig. 10.5 for the flowchart of Step 3.

8. STEP 4 PLATING CELLS TO SCREEN FOR MUTATIONS

8.1. Overview

Dilute and plate cells to get colonies to screen for the designed mutations.

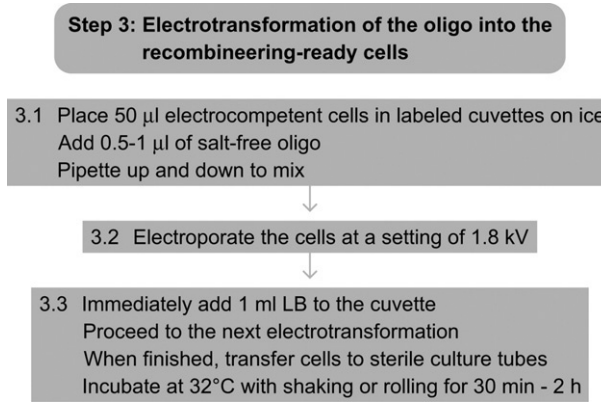

Figure 10.5 Flowchart of Step 3.

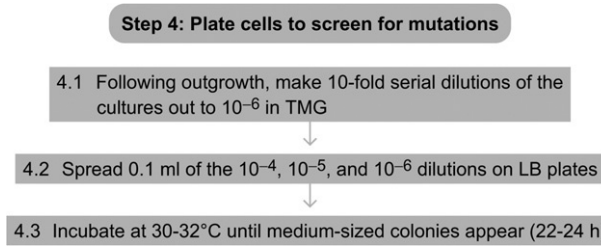

Figure 10.6 Flowchart of Step 4.

8.2. Duration

1 day

4.1 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.

4.2 Spread 0.1 ml of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions on LB plates.

4.3 Incubate plates at 30–32 °C until medium-sized colonies appear, normally 22–24 h.

8.3. Tip

If the mutation you are making might make the cells sick, be certain to wait for small colonies to appear on the LB plates. You may also have to screen through more candidate colonies since there may be a selection against cells that contain your mutation. If you are screening based on this small colony phenotype, be certain you use a >2 h outgrowth in Step 3.3.

See Fig. 10.6 for the flowchart of Step 4.

9. STEP 5 SCREENING FOR THE FINAL CONSTRUCT

9.1. Overview

Use PCR to screen colonies to find those that contain the designed mutation(s) (see Colony PCR). Figure 10.1, parts II and III, outlines how the PCR screen is performed.

9.2. Duration

3–4 days

5.1 Use a sterile loop to pick well-isolated colonies on the LB plates and patch them to a master plate, then using the same loop swirl it in the PCR mix. Incubate the master plate overnight at 30–32 °C. Store the master plate at 4 °C.

5.2 Set up PCR reactions as follows. Initially, screen 40 colonies.
Add to a 0.2 ml PCR tube:
- 39.5 µl sterile, distilled water
- 5 µl 10× Platinum Taq buffer
- 2 µl 50 mM $MgSO_4$
- 1 µl dNTP mix (10 mM each)
- 1 µl primer A (25 pmoles μl^{-1})
- 11 primer C (25 pmoles μl^{-1})
- 0.5 µl Platinum Taq
- Template (see Step 5.2)

5.3 Run PCR with the following cycling conditions for a 500 °bp PCR product:

95 °C	2 min

30 cycles of:

94 °C	30 s
55 °C	30 s
68 °C	30 s

68 °C	10 min
4 °C	Hold

5.4 Run a sample of the PCR products on an agarose gel with DNA molecular weight markers to confirm sizes (see Agarose Gel Electrophoresis).

5.5 Go back to the master plate and pick and streak for single colonies on LB plates two of the patches that gave the correct PCR product with primers A and C. Incubate the plates overnight at 30–32 °C. Each colony is now pure with either the original or the recombinant sequence. Store the plates at 4 °C.

5.6 Repeat steps 5.1–5.4 on 24 isolated colonies from each of the two candidates you are screening. Again, patch colonies onto a new masterplate and incubate it overnight at 30–32 °C. Store the master plate at 4 °C.

5.7 Set up a PCR reaction using primers A and B on —two to four of the candidates that show the correct PCR fragment in Step 5.6.

5.8 Sequence the PCR product from Step 5.7 to confirm that the construct in the region where the oligo recombined is exactly as you designed.

9.3. Tip

Do not use wooden toothpicks as they inhibit PCR.

9.4. Tip

Clearly label PCR tubes and the master plate as you will need to return to the master plate to retrieve the colonies that produced the correct PCR product.

9.5. Tip

A master mix of everything but the template (enough for 45 reactions) can be made and aliquotted into the required number of tubes.

9.6. Tip

Set up a PCR reaction using the parental strain as a template and primers A and C as a negative control. In addition, you should set up two reactions, one with a candidate and one with the parental strain as templates and using primers A and B for positive controls. There should be no band with the negative control and the same-sized band with the two positive controls (size can be calculated from the file generated in silico).

9.7. Tip

If you do not get any positive candidates, try using primer C′, which is on the other DNA strand. The diagnostic primer may be the problem.

9.8. Tip

Alternately, in Step 5.5, you can use a Pasteur pipette and take a 'core' sample from the master patch. Suspend this in 1 ml of LB or TMG, make tenfold serial dilutions and plate 0.1 ml of the 10^{-4} and 10^{-5} dilutions on LB plates. Incubate at 30–32 °C.

9.9. Tip

Be certain to confirm the sequence across the entire oligo used for the mutagenesis. Errors can occur during synthesis of oligos by commercial vendors (Oppenheim et al., 2004).

9.10. Tip

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

See Fig. 10.7 for the flowchart of Step 5.

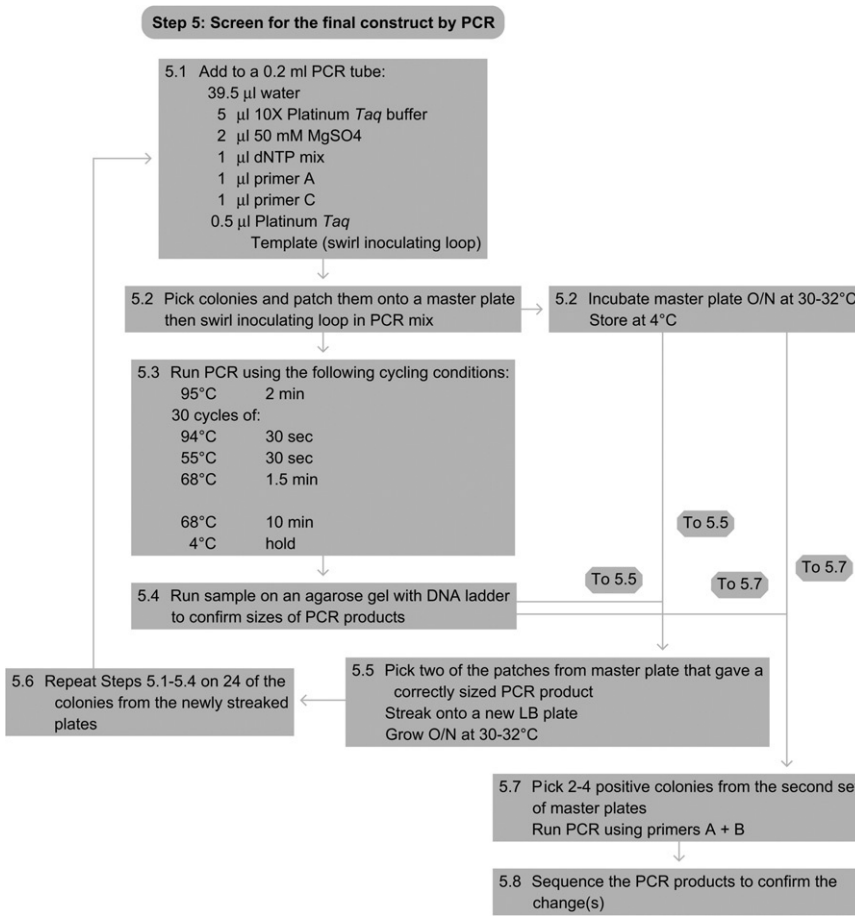

Figure 10.7 Flowchart of Step 5.

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Related Literature

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Referenced Protocols in Methods Navigator

Recombineering: Using Drug Cassettes to Knock out Genes *in vivo*.
Explanatory chapter: PCR -Primer design.
Transformation of *E. coli* via electroporation.
Colony PCR.
Agarose Gel Electrophoresis.