

Recombineering: Highly Efficient *in vivo* genetic engineering using single-strand oligos

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Purpose

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 10kb) 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 site directed mutagenesis.

Theory

Recombineering is *in vivo* homologous **recombination**-mediated genetic **engineering**. 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 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 and 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.

Many parameters of recombineering using ssDNA have been optimized and oligo design is key for the highest efficiencies. With optimized protocols, we have seen that over 50% of unselected colonies can contain the desired change. Details for oligo optimization include (Sawitzke, Costantino et al. 2011):

- Oligos should be ~70 bases in length with the designed change(s) near the middle of the oligo. Changes should be at least 10 bases from the ends.
- The oligo should correspond in sequence to the lagging-strand of DNA synthesis since using an oligo that corresponds to the leading-strand is ~20-fold less efficient.
- The oligo should be designed to avoid the methyl-directed mismatch repair system (MMR) system, or the recombineering should be done in a cell deficient in this system. Avoiding the MMR system increases the efficiency 100-fold or more. This is the critical parameter for optimization.

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.

When recombination occurs in the bacterial cell, there are usually 4-8 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 non-recombined DNA copies. If cells are spread on plates before outgrowth, recombinant colonies that form will be a mixture of recombinant and parental cells. Spreading cells before outgrowth is the best way to find an un-selected recombinant as the apparent recombination frequency is higher; i.e. more colonies contain a recombinant. In some cases, when a phenotype can be scored directly (i.e. 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. With an extended outgrowth after recombineering, each colony will be clonal but the frequency of recombinant colonies will be reduced by the outgrowth and segregation process.

Recombineering with ssDNA is a powerful technique that can be used in many ways. This protocol outlines using oligos to make non-selected point mutations, either single or closely linked. Figure 1 outlines the steps in this process.

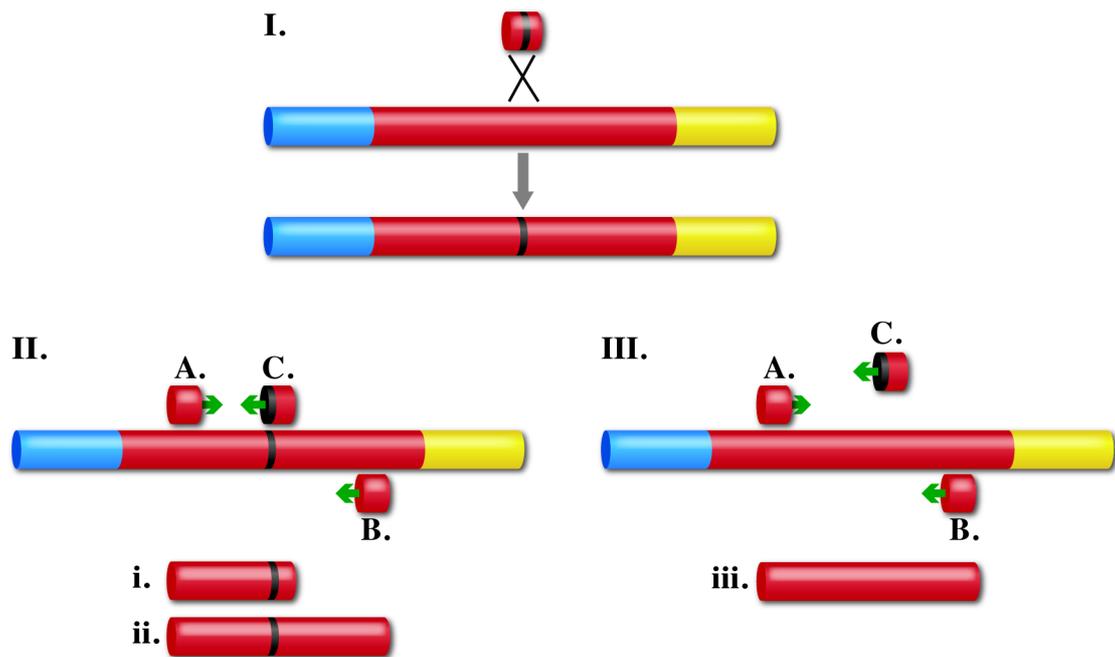


Figure 1. Using recombineering with an oligo to create a sequence change and PCR to screen for it. I. λ Beta inserts a 5 base change (black bar) contained within a ~70 base oligo into a gene on a BAC or in the chromosome in up to 50% of the colonies. The mutations presence can be detected by PCR. II. Using primers “A” and “C”, a PCR product (i) is made 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. Primer “C” is designed to anneal only if the change is present as its 3’ end consists of the designed change. After purified colonies are found that contain the relevant change(s), PCR using primers A and B will be used to make a product to sequence the final construct.

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 the presence of changes using PCR analysis.

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 dsDNA protocol) and the drug marker is selected. In the second recombination event, the entire cassette is removed by counter-selection using an oligo (see Sharan, Thomason et al. 2009).

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 pipetor tips
 Pipettes of various sizes
 PCR tubes (0.2 ml flat cap tubes)
 1.5 ml microfuge tubes (Eppendorf)
 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

Platinum *Taq* DNA Polymerase kit (Invitrogen) or similar DNA polymerase
 with proof reading ability
 PCR grade dNTP set
 Double distilled sterile chilled H₂O
 Ethidium bromide
 Agarose
 DNA molecular weight markers
 Primer for recombineering (see step 1), desalted but not purified further
 Primers for screening and sequencing recombinant candidates
 Recombineering-proficient cells. See Table 1 for some options. Plasmids that
 supply the Red functions are also available and they can be
 transformed into your strain of choice (Datta, Costantino et al. 2006,
 Sharan, 2009 #1486).

Table 1: Useful Recombineering Strains.

Strain	Genotype	Special Purpose	Reference
LT521	MG1655 <i>gal490 nadA::Tn10 pglΔ8</i> [λ <i>cI857</i> Δ (<i>cro-bioA</i>)]	Useful for moving prophage into other backgrounds by P1 transduction using linked <i>Tn10</i> .	Lab collection
DY329	W3110 Δ <i>lacU169 nadA::Tn10 gal490 pglΔ8</i> [λ <i>cI857</i> Δ (<i>cro bioA</i>)]		(Yu, Ellis et al. 2000)
DY330	W3110 Δ <i>lacU169 gal490 pglΔ8</i> [λ <i>cI857</i> Δ (<i>cro-bioA</i>)]		(Yu, Ellis et al. 2000)
DY331	W3110 Δ <i>lacU169</i> Δ (<i>srlA-recA</i>)301:: <i>Tn10</i>	Plasmid recombination.	(Yu, Ellis et al.

	<i>gal490 pglΔ8</i> [λ <i>cI857 Δ(cro-bioA)</i>]		2000)
DY378	W3110 [λ <i>cI857 Δ(cro-bioA)</i>]		(Yu, Ellis et al. 2000)
HME6	W3110 <i>galK_{tyr145UAG} ΔlacU169</i> [λ <i>cI857 Δ(cro-bioA)</i>]	Assay system for oligo recombineering	(Ellis, Yu et al. 2001)
SIMD50	HME6 [λ <i>cI857(int-cIII<>bet)</i>]	Beta only strain. Great for oligo recombineering.	(Datta, Costantino et al. 2008)
SIMD90	SIMD50 <i>mutS<>cat</i>	High frequency oligo recombineering. Defective for MMR.	Lab collection
SIMD61	HME6 [λ <i>cI857(int-cIII<>amp) Δ(cro-bioA)</i>]	No <i>gam</i> gene	(Datta, Costantino et al. 2008)
HME63	W3110 <i>galK_{tyr145UAG} ΔlacU169 mutS<>amp</i> [λ <i>cI857 Δ(cro-bioA)</i>]	Defective for mismatch repair, therefore, gives high level oligo recombineering. Defective for MMR.	(Costantino and Court 2003)
HME68	W3110 <i>galK_{tyr145UAG} ΔlacU169</i> [λ <i>cI857 Δ(cro-bioA)</i>] <i>mutS<>cat</i>	Defective for MMR.	(Thomason, Court et al. 2007)
HME70	W3110 <i>galK_{tyr145UAG} ΔlacU169</i> [λ <i>cI857 Δ(cro-bioA)</i>] <i>mutS<>cat Δ(recA)301::Tn10</i>	Oligo recombineering with plasmids.	(Thomason, Court et al. 2007)
HME71	W3110 <i>galK_{tyr145UAG} ΔlacU169</i> [λ <i>cI857 Δ(cro-bioA)</i>] $\Delta(recA)301::Tn10$	Oligo recombineering with plasmids.	(Thomason, Court et al. 2007)
DY380	<i>mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu)7697 galU gal490 pglΔ8 rpsL nupG</i> [λ <i>cI857ind1 Δ(cro-bioA)<>tet</i>] (A derivative of DH10B)	Useful for BAC transformation and manipulations.	(Lee, Yu et al. 2001)
SW102	DY380 $\Delta galK$	Use for <i>galK</i> selection/counter selection.	(Warming, Costantino et al. 2005)

Solutions & buffers

Step 2

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 4

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

Add 15g Bacto Agar (Difco) to LB broth to make plates.

Table 2. Drug Concentrations to maintain plasmids.

Antibiotic	Multi-copy plasmids
Ampicillin	100
Kanamycin	50
Chloramphenicol	20
Tetracycline	25
Hygromycin	50-200
Spectinomycin	100

Protocol

Duration

Preparation	none
Protocol	Including waiting for oligo orders to arrive, from start to sequenced mutation is about 7-10 days

Tip

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

Step 1 Designing the Oligo for Recombineering

Overview *In silico* creation of the final construct in order to design and order the appropriate oligo to create the desired mutation.

Duration 30-60 minutes

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 organism).

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

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

1.3 Design the 70 base oligo using the sequence you designed in 1.2. 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 non-selected alteration.

- The oligo should correspond in sequence to the DNA strand that is replicated discontinuously, i.e. 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, Yu 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 recombineering should be done in a MMR-deficient strain (see Table 1) or better, the oligo should be designed to avoid MMR.

Designing an oligo 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 6 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 knock out (Sawitzke, Costantino et al. 2011).

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.

Tip Only when a non-essential 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.

3. Changing 4-5 wobble positions in a row, in addition to the designed change, allows 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, Costantino et al. 2011).

Tip As in the previous case, two rounds of this technique can leave one altered base if this is desired.

- 1.4 Design the other required primers. You need two ~20 base primers flanking the region you are modifying to amplify a PCR fragment to sequence (primers "A" and "B" in Figure 1) and confirm the pertinent changes. The fragment made with primers "A" and "B" should be around 400-500bp. In addition, Primer "C" is used to screen for colonies that contain the designed sequence change. The extreme 3' end of Primer "C" must contain all the changes that were present in the oligo used for mutagenesis. Figure 1 shows a 5 base change, but if you make 4 wobble changes and the desired change, this will constitute the 3' end of primer "C". The A/C primer pair should not amplify a parental sequence but will amplify if the mutation was made. A good size for the PCR product made with primers "A" and "C" is 200-300bp.

Tip It simplifies things if you adjust the length of all of these primers such that they have the same annealing temperature, e.g. 60°-64°C. Use the formula of 4°C for a G/C pair and 2°C for an A/T pair.

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 5 changes must be of known sequence so a test oligo "C" can be designed to detect recombinants. Final sequencing will reveal the linked "random" changes.

- 1.5 Order the oligos from IDT or a similar company. 100nMole scale is sufficient (and normally required) for a ~70mer. 25nMole is fine for the shorter primers. Other than desalting, no additional purification is needed.

Tip Avoid snapback oligos.

Step 2 Preparing Cells Competent for Recombineering

Overview The formulation of cells that are recombineering-proficient and ready for electrotransformation with the oligo ordered in Step 1.

Duration About 3.5 hours

Preparation The previous day, grow a 5 ml overnight culture of the chosen recombineering cells (Table 1) 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.*

2.1 Dilute the 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 250 ml (or 125 ml) baffled Erlenmeyer flask. Dilute the overnight at least 70-fold. Grow cells in a H₂O bath at 32°C with shaking (200rpm) until the OD₆₀₀ is from 0.4-0.5 (approximately 2 hrs).

Tip *Cells with different genotypes will grow at different rates. Having the proper OD₆₀₀ 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.*

2.2 Transfer half the culture to a 50 ml baffled Erlenmeyer flask and place that flask in a 42°C H₂O bath to shake at 200rpm; keep the other flask at 32°C. Shake for 15 min. The culture at 42°C is now induced for the recombination functions and the 32°C culture is the uninduced control. Both flasks will be processed identically during the rest of the protocol.

2.3 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 H₂O 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. Also chill electrotransformation cuvettes and microcentrifuge tubes for later parts of this step.*

2.4 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 4°C. Using sterile technique, aspirate or pour off supernatant.

2.5 Add 1 ml ice-cold sterile distilled H₂O 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 H₂O to

each tube, seal, and gently invert to mix, again without vortexing. Centrifuge tubes again as in previous step.

- 2.6 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 H₂O.

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.

- 2.7 Transfer the suspended cells to pre-chilled microcentrifuge tubes. Centrifuge 30 sec at maximum speed in a 4°C refrigerated microcentrifuge. Carefully remove the supernatant and suspend cells in 200 µl sterile ice-cold distilled H₂O 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 3 Electrotransformation of the Oligo into the Recombineering-ready Cells

Overview Recombineering-proficient, electrocompetent cells from Step 2 are transformed, via electrotransformation, with the oligo from Step 1.

Duration About 1-4 hours

- 3.1 In labeled cuvettes on ice, place 50µl of electrocompetent cells. Pipette in 0.5-1 µl (~100ng) of salt-free oligo. 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, never 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.

- 3.2 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.

3.3 Immediately after electrotransformation, add 1 ml of room temperature LB medium to the cuvette. This should be done 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 32°C for 30 min. See 3.4 for further discussion of the appropriate total outgrowth time.

3.4 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 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. However, if a pure recombinant is needed; for example, to express a phenotype such as resistance to a counter-selectable marker, or a colony size phenotype then a >2 hour outgrowth before plating is needed.

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

Step 4 Plating Cells to Screen for Mutations

Overview Dilution and plating of cells to get colonies to screen for the designed mutations.

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

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

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

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 candidates as 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 hour outgrowth in Step 3.4.

Step 5 Screening for the Final Construct

Overview Using PCR to screen for colonies to find those that contain the designed mutation(s).

Duration 3-4 Days

5.1 Figure 1, parts II and III outline how the PCR screen is performed. Well-isolated colonies on the LB plates should be picked, used as a template for “touch PCR”, and patched to a master plate with a sterile loop. Do not use wooden toothpicks as they inhibit PCR. Patch the colony, then using the same loop swirl it in the PCR mix (see below). Use careful bookkeeping as you will need to return to the master plate to retrieve the colonies that produced the correct PCR product. The master plate should be incubated overnight at 30-32°C. The PCR reactions should be set up as follows. Initially, test 40 colonies.

Typical 50µl PCR reaction mix.

COMPONENT	COMMENTS
39.5 µl H ₂ O	Sterile and distilled
5 µl 10X buffer	
2 µl MgSO ₄	50mM
1 µl dNTP mix	Mixture containing 10mM of each dNTP
1 µl primer A	25pmoles/µl
1 µl primer C	25pmoles/µl
0.5 µl Platinum <i>Taq</i>	Invitrogen
template	Swirl inoculating loop with cells

Tip *A master mix of everything but the “template” (enough for 15 reactions) can be made and aliquotted into the required number of tubes.*

Tip *Also 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 primers A and B for a positive controls. There should be no band with the negative control and the same size band (size can be calculated from in silico file) with the two positive controls.*

Tip *If you are not getting any candidates, try using the “other” primer “C” which is on the other DNA strand. The diagnostic primer may be the problem.*

Typical reaction conditions for a 500 bp fragment using Platinum *Taq* are:

STEP	TEMPERATURE (°C)	TIME
1	95	2 min
2	94	30 sec
3	55	30 sec
4	68	30 sec
5	Go to step 2	29 times
6	68	10 min
7	4	end

- 5.2 Run a sample of the PCR products on an agarose gel with DNA molecular weight markers to confirm sizes.
- 5.3 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 here is now pure with either the original or the recombinant sequence.
- Tip* *Alternatively, you can use a Pasteur pipette and take a “core” sample from the master patch. Suspend this in 1ml of LB or TMG, make step-wise 10-fold dilutions and plate 0.1 ml of the 10⁻⁴ and 10⁻⁵ dilutions on LB plates. Incubate at 30-32°C.*
- 5.4 Repeat step 5-1 on 24 isolated colonies of each of the two candidates you are screening. Again, keep a master plate which should be incubated overnight at 30-32°C.
- 5.5 Set up a PCR reaction using primers “A” and “B” on 2-4 candidates that show the correct PCR fragment in step 5-4.
- 5.6 Sequence the PCR product from step 5.5 confirming that the construct in the region where the oligo recombined is exactly as you designed.
- Tip* *Be certain to confirm the sequence across the entire oligo used for the mutagenesis. Errors occur during synthesis of oligos by the company (Oppenheim, Rattray et al. 2004).*
- Tip* *For troubleshooting and the most up to date information on recombineering, see: <http://redrecombineering.ncifcrf.gov/>*

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