Barlow-Hall *in vitro* Evolution Protocol

Barry G. Hall* and Miriam Barlow†

*Bellingham Research Institute, Bellingham, WA Email drbh@mail.rochester.edu
† University of California at Merced, 5200 N. Lake Rd, Merced, CA. Email mbarlow@ucmerced.edu

Background

Although the Barlow-Hall method[1], like DNA shuffling[2] and classical error-prone PCR[3], can be used as a tool for protein engineering, its primary application is for predicting protein evolution in nature. Libraries generated by error-prone PCR amplification of a target gene by the Mutazyme II polymerase are subjected to selection or screening for expression of mutant proteins that have increased activities or expanded substrate ranges. The primary difference between the B-H method and other *in vitro* evolution methods is that the mutation frequency is precisely controlled. Because the mutation frequency can be set by the investigator it is possible to quantify the proportion of the possible single-mutant and double-mutant targets that are present in a library. In nature mutations arise one, and very rarely two, at a time, and the resulting alleles must be fixed into population by selection for an improved protein function. From the proportion of possible single and double mutants present in a library, if no improved proteins are obtained the probability that a successful mutant could arise in nature but has been missed by chance alone can be estimated. The method closely mimics natural evolution[1] and has been used to predict the natural evolution of several antibiotic resistance genes[4-8]. The method also predicts which amino acid substitutions will cause the new phenotype and the order in which they will appear in nature, and at least two such predictions have been confirmed, e.g. TEM-132[4,9] and CTX-M-35 (GenBank AB176534)[8] . The method has also been able to predict the *absence* of evolutionary potential, e.g. that the IMP-1 metallo-β-lactamase does not have the potential to evolve increased activity toward imipenem[7].

The Barlow-Hall method is the subject of US Patent Number: US 6,720,142 B1. Commercial application of the Barlow-Hall method is subject to a license from The University of Rochester Office of Technology Transfer, Rochester, NY.

Materials

Reagents

FailSafe™ PCR kit (Epicentre) **OR** Phusion High-Fidelity Taq polymerase (NEB #F-530S) {highly accurate and efficient amplification}
Restriction enzymes *Nco*I (NEB) and *Bsp*HI (NEB)
T4 ligase
T4 ligase buffer
GeneMorph™ II Random Mutagenesis Kit (Stratagene) consisting of:
Mutazyme™ II polymerase.
40 mM dNTP mix (200µM each final)
10x Mutazyme II reaction buffer
Oligonucleotide F1: 5'-TCATCCGGCTCGTATAATGTGTGGA 3'
Oligonucleotide R1: 5'-ACTCTCTTCCGGGCGCTATCAT-3'
Calf Alkaline Phosphatase (CIP) (NEB)
SOC broth {Appendix III}
L-broth {Appendix III}
plasmid pACSE2 (NcoI cloning site) {see Appendix II}
plasmid pACSE3 (BspHI cloning site, must be prepared from *dam*$^-$ host.) {See Appendix II}
Note: plasmids pACSE2 and pACSE3 my be obtained upon request to M. Barlow.
Electrocompetent *E. coli* strain DH-5α with an electroporation efficiency of **at least** 1 x 10^{10} per µg. (NEB 5-alpha Electrocompetent *E. coli* cat. # C2989K) or the same strain prepared as described in Appendix I.

Equipment

Thermal cycler programmed with the desired amplification protocol
DNA fluorometer or NanoDrop to measure DNA concentrations accurately
QIAquick PCR purification kit (Qiagen)

OR QIAquick gel purification kit (Qiagen)
Electroporator and electroporation cuvettes 1 mm gap

Software
InVitroSimII, a Perl program

Procedure

1 Amplify the target gene

Amplify the target gene using a high-fidelity systems such as the FailSafe system or Phusion High-Fidelity Taq polymerase according to manufacturer's instructions. A 50µl reaction should provide sufficient amplicon product.

The forward primer should include the ATG initiation codon within either an *Nco*I or a *Bsp*HI restriction site and there should be at least 7 bases 5' to that site. The reverse primer should include a restriction site for a second enzyme that does not cut within the target gene but does cut within thepACSE2/pACSE3 cloning cartridge. If possible the second enzyme should work in the same buffer as *Nco*I or *Bsp*HI. The use of two enzymes ensures directional cloning so that the target gene will be in the correct orientation in the plasmid. See Appendix II for cloning cartridge details. Purify the amplicon with a QIAquick PCR purification column **OR** run the PCR product on a gel, cut out the band corresponding to the expected size and purify with a QIAquick gel purification column and determine the DNA concentration with a DNA fluorometer or NanoDrop.

2 Clone the amplicon into pACSE2 (*Nco*I site) or pACSE3 (*Bsp*HI site).

Digest about 1 µg of plasmid and about 1 µg of amplicon with the appropriate restriction enzyme.

Purify the digests with QIAquick PCR columns and determine the DNA concentration with a DNA fluorometer or NanoDrop.

Combine 300 ng digested plasmid with 89 fmol digested amplicon, 4 µl T4 ligase buffer, 2 µlT4 ligase and sterile water to a total volume of 20 µl and allow to ligate overnight at room temperature.

Electroporate into *E. coli* strain DH5αE. {See Appendix I for electroporation procedure}.

Plate serial dilutions of electroporated cells onto L-tetracycline agar {Appendix III} and incubate plates overnight at 37˚. Grow 1 ml cultures from 20 well-isolated overnight in L-tetracycline. Make boiling DNA preps {Appendix IV} from each culture and run 10µl PCR reactions using 1µl of each boiling prep as template. Run the PCR reaction products on a gel along with some digested plasmid and a size standard. Choose two candidates that appear to have the correct insert, prepare plasmid DNA by any standard method, and sequence the insert. Retain a candidate that has the correct sequence.

See Troubleshooting Table

3 Mutate the target gene.

Decide the average number of mutations you want per molecule. Two mutations per molecule is typical when you want to mimic natural evolution, about 14% of amplicons should have zero mutations, 27% 1 mutation, 27% two mutations, and 32% >2 mutations.

First calculate the number of ng of **target** template that are required to produce the desired number of mutations per kb, then apply the equation $ng = e^{\frac{m-12.6}{-1.9}}$, where ng is the number of ng of target required and m is the desirerd number of mutations/kb. Finally, calculate the amount of plasmid required for that amount of template.

Example:

The target is 500 bp that have been cloned into pACSE2 and you want an average of two mutations per molecule. Two mutations per 500 bp = 4 mutations per kb, so applying the above equation you require 92.4 ng target for a 50 µl reaction. pACSE2 is 5201 bp, so the plasmid carrying the target is 5500 bp and the target constitutes 0.909 of the plasmid. You will therefore require 1016 ng of the plasmid for the 50µl reaction.

Set up the following PCR reaction to mutagenize the target gene:

Plasmid volume as required by ng	
10X MutazymeII Buffer	5µl
5 µM oligonucleotide F1	5µl
5 µM oligonucleotide R1	5µl
40 mM dNTP mix	1µl
Mutazyme II enzyme	1µl
H_2O	to give 50 µl final volume

Run thermal cycler programsuggested by StrataGene for GeneMorph.
(95C 2 min., [95C 30 sec., 58C 30 sec., 72C 1 min 10 sec]X30, 72C 10 min, hold at 4C).
Determine the DNA concentration of the amplicon pool with a DNA fluorometer or NanoDrop.
Important! The DNA concentration should be determined accurately. This cannot be done by running serial dilutions on a gel.
Purify the reaction with a QIAquick PCR column **OR** run the PCR product on a gel and cut out the band corresponding to the expected size and purify using QIAquick gel purification columns (Qiagen).
Quantify DNA once again.

4 Prepare digested vector for library construction

Digest 20 µg of pACSE2 or pACSE3 with the same enzymes used to clone the target gene plus CIP (calf alkaline phosphatase). The CIP dephosphorylates the vector and prevents self-ligation of partially digested plasmid. The yield is digested-dephosphorylated plasmid is typically sufficient for preparation of several parallel libraries.

Set up the following reaction*:

pACSE2 or pACSE3	20 µg
Restriction enzyme 1	200 units**
Restriction enzyme 2	200 units**
10X Buffer	40 µl
CIP	100 units
H_2O	to give 400 µl final volume

* This assumes that the two enzymes can be used with the same buffer and incubated at the same temperature. If not, digest with the two enzymes sequentially and include the CIP in the second digest.
**The combined volumes of the restriction enzymes and CIP should not exceed 40µl in order to keep the final glycerol concentration below 5%. If necessary increase the volume of water and 10x buffer.

Incubate the digest for two hours at 37˚, check digestion on an agarose gel and purify over *two* QIAquick columns, eluting each with 50µl of TE. Typical yield is 10µg of digested vector. Determine the DNA concentration, typically about 100 ng/µl. Digested plasmid can be stored in TE at -20˚.

5 Digest the mutagenized amplicon pool

Depending upon the size of the target gene, with an average of two mutations per amplicon and the effective library size of 10^7, the fraction of all possible single and double mutants in a single library ranges from about 75% for a target of about 500 bp to about 30% for a target of 1400 bp. To have 95% confidence of screening all possible single and double mutants thus requires screening three libraries in the former case and nine libraries in the latter case. Multiple independent libraries can be prepared from the same amplicon pool because each ligation consists of about 10^{11} amplicons of which only about 10^7 are sampled in a good library. Each library is thus effectively an independent sample of the pool of alleles.

For **each** library that you require, digest 550 fmol of amplicon with the two appropriate restriction enzymes. When the size of the amplicon is expressed in base pairs calculate the number of nanograms required according to ng = (fmol * size)/1543. For a 1000 bp amplicon this is 357 ng for each library. Purify on a QIAquick column and elute in 50µl. Determine the DNA concentration.

Run an aliquot of the digest on a gel along with appropriate size standards and estimate the fraction of the digest that is amplicon. Depending upon the amount of target template used in the mutagenesis reaction, a significant fraction of the digest may be plasmid (5 kb band). In step 6 correct the amount of digested amplicon by increasing the amount of DNA as necessary to account for the plasmid DNA.

6 Clone the mutagenized amplicons

In separate tubes, for **each** ligation combine, in order:
600 ng of digested dephosphorylated plasmid
534 fmol digested amplicon
Incubate at 56°C for 10 minutes and cool to room temperature
Add remaining:
8 µl 5x T4 DNA ligase buffer
H_2O to give a total of 38µl
2 µl T4ligase
* If vector and/or amplicon concentrations do not permit doing the reaction in 40µl volume, increase the amounts of T4 ligase and ligase buffer accordingly.

Incubate overnight at room temperature (16 to 24 ^{o}C) and EtOH precipitate at follows:

Combine 20 µl ligation mix, 1 µl tRNA [1 µg/µl Yeast/AMBION], 55 µl cold 100% EtOH. Incubate at -20 ^{o}C for at least 15 minutes. Centrifuge 15 min., 15K rpm, at 4 ^{o}C. Remove supernatant, wash the pellet with 70% EtOH, spin again, remove supernatant, air dry the pellet. Resuspend the pellet in 5 µl sterile water.

Purify over a Qiagen min-elute column and elute each in 10µl TE.

7 Electroporation

Work in a cold room using a cold electroporator and cold electroporation cuvettes. For each electroporation have a tube containing 1.0 ml SOC in a dry bath (or water bath) at 37˚ on the

bench in the cold room. Also have a dry bath at 37˚ into which the electroporation cuvettes can be placed immediately after electroporation.

Thaw one 4x aliquot of electrocompetent cells on ice. As soon as it is thawed add the purified ligation from step 5. Mix briefly (but gently) and transfer to four electroporation cuvettes.

Electroporate as described in Appendix I except that the four electorporations are combined with 4 ml SOC in a single tube for expression.
Allow the tet gene to express for 1.5 hour.

8 Determine the library size and expand the transformed population

Add the entire undiluted transformation to 500 ml of L-tetracycline in a tightly sealed 500 ml flask and allow to stand overnight without aeration at 37˚. This expands the library. Plate serial dilutions from this culture in triplicate onto L-tet agar to determine the number of transformants. Incubate overnight at 37˚.

9 The following day:

Determine the absorbance at 600 nm of the expanded library. Plate serial dilutions of the expanded library onto L-tet to determine the population size. For DH5α, when A_{600} =1, there are 1.9 x 10^8 cfus/ml .

Count the colonies from yesterday and calculate the **total** library size.
Freeze aliquots that contain at least 20 x the size of the library at -80˚ for future use.
Pick at least 20 well-isolated colonies from suitable dilution plates and make boiling DNA preps from each (see Appendix IV)
Use the boiling DNA preps as templates in PCR reactions using primers F1 and R1.
Run the PCR reactions on a gel. From the proportion of insert bearing transformants calculate the **effective** library size.

To be useful the effective library size **must** be at least 2 x 10^6, but we typically get 1 x 10^7 or slightly better.

10 Selection

The selection routine will depend upon the new function that is being evolved, but the routine for selecting for new antibiotic resistance can be suitably modified.
1. Dilute a frozen aliquot of a library into 50 ml of L-tet containing 1mM IPTG and grow to an A_{600} of about 0.2. The IPTG induces expression of the cloned target gene.
2. We carry out selection for drug resistance in 50 ml cultures in 100 ml bottles, so we dilute the library in L-broth so that the number of cells per 50 ml is about 10x thelargest library size. (If multiple libraries are being selected for the same function, use the same concentration of cells for the selections even though thelibrary sizes are different in order to eliminate inoculum size effects. This ensures that each culture contains at least one copy of each mutant allele in the library. Since we want to select at a series of different drug concentrations we typically prepare about 500 ml of the diluted culture. A good library will have an effective size of about 10^7, so we will typically have about 10^8 cells in 50 ml, or about 2 x 10^6 per ml. That population density means that it is easy to distinguish growth from non-growth the following day.
3. We distribute 100 ml of the diluted library to the first bottle and 50 ml to each remaining bottle. We then add Drug-X to the first bottle to give the highest concentration to be tested. We mix, then

transfer 50 ml from that bottle to the next bottle, etc. to achieve a set of two-fold dilutions of the drug. From the final bottle we discard 50 ml to ensure that all bottle have the same volume. Alternatively, bacteria can be added to the bottles after the serial dilutions of Drug-X are made. This reduces the possibility of cross contamination between cultures.

4. The bottles are tightly capped then allowed to stand overnight at 37˚. Tightly capping the bottles limits oxygen availability so that the cultures do not become over saturated. We use square bottles rather than flasks because this minimizes the surface area. These standing overnight cultures limit out at about 10^9 /ml.

The culture which grew in the highest concentration of tested drug is transferred (500 μl into 10 ml) into fresh broth with the same concentration of antibiotic and 2X higher. Bacteria from the tube where growth is clearly visible are harvested and plasmid DNA is isolated for subsequent electroporation into DH5αE. The next day 10 single clones are picked for DISC diffusion MIC test.

5. Analyze the phenotypes of the tested clones and send for sequencing at least 1 representative clone for each distinct phenotype.

11 Clonal displacement

The selection described above makes it very likely that after selection the population will consist of a single allele that represents the fittest mutant in the library. Although the initial population might have been 2 x 10^6 cells/ml, and the final population 10^9, the vast majority of that initial population will not have grown at all during selection because most mutants will be either non-functional or no better than the initial unmutagenized allele. Suppose that of 10^7 mutant alleles there were 10 that were fitter than the original allele, and each of those was present in 10 copies. If they were equally fit each would have generated 10^7 descendants resulting from 23 doublings. That is plenty of time for any fitness differences to have taken effect, and it is likely that the fittest mutant will have outgrown the others so that it is >95% of the population - hence the term "clonal displacement". Our experience with antibiotic resistance confirms this expectation. We typically test 20 colonies from the population that grew at the highest drug concentration. Using antibiotic diffusion discs which give a continuous measurement that can discern a finer scale of differences than 2x dilutions can, we very rarely see more than one phenotype in a population after selection.

If it is important to detect as many improved variants as possible one could dilute the culture so that there were on average 0.2 copies of each mutant allele (instead of 10) and do 25 parallel selections. That would improve the chances of detecting additional successful, but not equally fit, alleles.

12 InVitroSimII simulator program

When *in vitro* evolution results in an improved gene - a product with a new substrate specificity or with increased activity - you can demonstrate that the gene has the potential to evolve that new activity in nature. But what happens when *in vitro* evolution fails to produce an improved gene? Can you conclude that the gene lacks the potential for that activity and will not evolve that activity in nature? In the case of antibiotic resistance it may be very important to know whether the potential for resistance to a new drug really does not exist. Can negative evidence permit drawing such a strong inference? After all, the *in vitro* experiment may not have included the necessary mutations just by chance alone.

If, as a practical matter, we accept that unless an improved phenotype can arise as the result of one or two mutations it will not arise in nature, then we can calculate the fraction of all possible single and double mutants are likely to be present in a library of a given size. The calculation is not straight forward because it depends upon the starting sequence of the gene under selection.

Actually, we are not interested in all possible single and double mutations, we are interested only in those mutations that change the protein sequence: single mutations that change one amino acid, double mutations in the same codon that change one amino acid, and double mutations in two codons that change two amino acids.

The program InVitroSimII simulates a random mutation process and keeps track of all of the amino acid replacement molecules. The user provides the starting gene sequence, the average number of mutations per molecule, and the effective library size. The program reports the fraction of single and double mutants (that change the protein sequence) that occurred. Of course, the longer the gene and the smaller the library, the smaller will be the fraction of possible mutants in the library. However, if you know that you screened four libraries each of which included 85% of all the possible single and double mutants, you know that the probability that you missed one possible mutant by chance is 5×10^{-4}. If you failed to detect an improved phenotype among those four libraries then you can be 99.95% confident that an improved phenotype will not evolve in nature.

InVitroSimII compiled for Macintosh OSX and for Windows, and the source code that can be compiled for Unix/Linux systems, can be downloaded at no charge from http://homepage.mac.com/barryghall/Software.html

Acknowledgements

We are grateful to Joanna E. Mroczkowska for suggesting the ethanol precipitation in Step 6 and for the experiments and analysis that led to the equation relating the mutations per kb to amount of target template in step 3ß

Literature Cited

1. Barlow, M. & Hall, B.G. Predicting Evolutionary Potential: *In vitro* Evolution Accurately Reproduces Natural Evolution of the TEM b-Lactamase. *Genetics* **160**, 823-832 (2002).
2. Stemmer, W.P.C. Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* **370**, 389-390 (1994).
3. Vakulenko, S.B., Geryk, B., Kotra, L.P., Mobashery, S. & Lerner, S.A. Selection and characterization of beta-lactam-beta-lactamase inactivator-resistant mutants following PCR mutagenesis of the TEM-1 beta-lactamase gene. *Antimicrob Agents Chemother* **42**, 1542-8. (1998).
4. Barlow, M. & Hall, B.G. Experimental Prediction of the Natural Evolution of Antibiotic Resistance. *Genetics* **163**, 1237-1241 (2003).

5. Barlow, M. & Hall, B.G. Experimental Prediction of the Evolution of Cefepime Resistance from the CMY-2 AmpC b-lactamase. *Genetics* **164**, 23-29 (2003).
6. Salipante, S.J. & Hall, B.G. Determining the limits of the evolutionary potential of an antibiotic resistance gene. *Mol. Biol. Evol.* **20**, 653-659 (2003).
7. Hall, B.G. In vitro evolution predicts that the IMP-1 metallo-β-lactamase does not have the potential to evolve increased activity toward imipenem. *Antimicrob. Agents Chemother.* **48**, 1032-1033 (2004).
8. Welsh, K.J. et al. Experimental prediction of the evolution of ceftazidime resistance in the CTX-M-2 extended-spectrum beta-lactamase. *Antimicrob Agents Chemother* **49**, 1242-4 (2005).
9. Zarnayova, M. et al. Survey of Enterobacteriaceae producing extended-spectrum beta-lactamases in a Slovak hospital: dominance of SHV-2a and characterization of TEM-132. *Antimicrob Agents Chemother* **49**, 3066-9 (2005).

Appendix I Electroporation

In order to achieve large libraries, i.e. close to 10^7, it is necessary to use extremely electrocompetent cell preparations. This protocol describes how to prepare them.

The following materials must be in the cold room the day before preparing the cells

Two sterile 250 ml centrifuge bottles
Rack of tips for 1 ml Pipetteman
Rack of tips for 200 µl Pipetteman
Sterile 10 ml pipettes
2 100 ml bottles of sterile water
A bottle of sterile 10% (v/v) glycerol
A bottle of sterile 20% (v/v) glycerol
Some sterile 50 ml centrifuge tubes

In a -80˚ freezer the day before the preparation

A rack of a sufficient number of closed 0.5 ml microfuge tubes. These should be thick wall tubes, not tubes for PCR. The rack must be a solid plastic or metal rack of the sort that the tubes fit snuggly into each hole that is tapered. It is essential that the walls of the tube are in full contact with the rack, not with air. The rack acts as a heat sink that keeps the tubes close to -80˚ while the cells are being dispensed into the tubes and allows the cells to freeze instantly.

Cell preparation

NOTE!!! The faster the prep is done the greater will be the Electroporation efficiency, so move quickly, without hesitation, but carefully. Be very familiar with the entire procedure before you begin.

Use as light a rotor as is available for the centrifuge steps. The lighter the rotor the more quickly it will stop and the better will be the prep. This matters.

1. The afternoon before the preparation inoculate an overnight culture in 10 ml YENB (see Appendix III) and grow it at 37˚with good aeration. The day of the preparation add 5 ml of the overnight culture to a 2L flask containing 500 ml of YENB. Shake at 37˚ until A_{600} is exactly 0.6 (never over 0.6), this will typically take 120 - 150 minutes. Check A_{600} at no more than 10 minute intervals after A_{600} reaches 0.3 and more frequently as A_{600} gets over 0.5.
2. Chill the flask on ice for about 5 minutes then spin down in sterile 250 ml centrifuge bottles at 1200 x g (3500 RPM in a Sorval SLA rotor) at **4˚** for 5 minutes. *When working with large volumes spin down 250 ml, discard the supe, add another 250 ml and spin again. Do not exceed two spins. Do not increase volume of washes in following steps*. From this point on keep the cells on ice at all times when carrying between the centrifuge and the cold room. Place on ice **instantly** upon removal from the centrifuge. **Do all the work in a cold room**.
3. Pour off supe and hold bottle upside down a few seconds to allow all supe to drain off. Immediately put the bottle back on ice. Resuspend one pellet by adding 10-20 ml of cold sterile water and swirling until entire pellet is suspended. You can pipette the suspension gently up and down to obtain a homogeneous suspension. **Do not vortex! Be gentle!** Add the remainder of 100 ml bottle of cold sterile water. Use about 20 ml of that suspension to suspend the cells in the other bottle and combine the pellets. Centrifuge again at 1200 x g (3500 RPM in a Sorval SLA rotor) at **4˚** for 5 minutes.
4. Repeat step 3
5. Resuspend cells in 10 ml cold 10% glycerol by swirling and centrifuge again at 1200 x g (3500 RPM in a Sorval SLA rotor) at **4˚** for 5 minutes.. While the rotor is slowing down bring the rack of -80˚

microfuge tubes into the cold room. Do not put the rack on ice - the ice will warm up the tubes faster than will the air.

6. Resuspend pellet in 1.0 ml of cold 20% glycerol by pipetting up and down with a 1.0 ml pipetteman.
7. Distribute 40 µl aliquots of cells to the sterile -80˚ 0.5 ml microfuge tubes. This works best by using an electronic pipetteman that allows you to take up 1.0 ml and to repeatedly dispense 40 µl aliquots. Immediately carry the rack to the -80˚ freezer. Do not put the rack on ice - the ice will warm up the tubes faster than will the air.
8. Label a small storage box with the strain name, date, some sort of code to identify this specific preparation, and a reference to the page in your notebook where you recorded the prep. Store the box in the -80˚ freezer
9. After and hour or so quickly transfer the tubes from the rack into the labeled box

Electroporate one sample of the prep with a standard amount of a good standard supercoiled plasmid to determine the Electroporation efficiency. We use a commercial preparation of pUC18 or pUC19 that has been diluted to exactly 10 pg/µl and electroporate with exactly 1 µl as described below. After expression (see **Electroporation** below) plate 10x, 100x and 1000x dilutions onto L-ampicillin.

Electroporation - Do steps 1-5 in the cold room

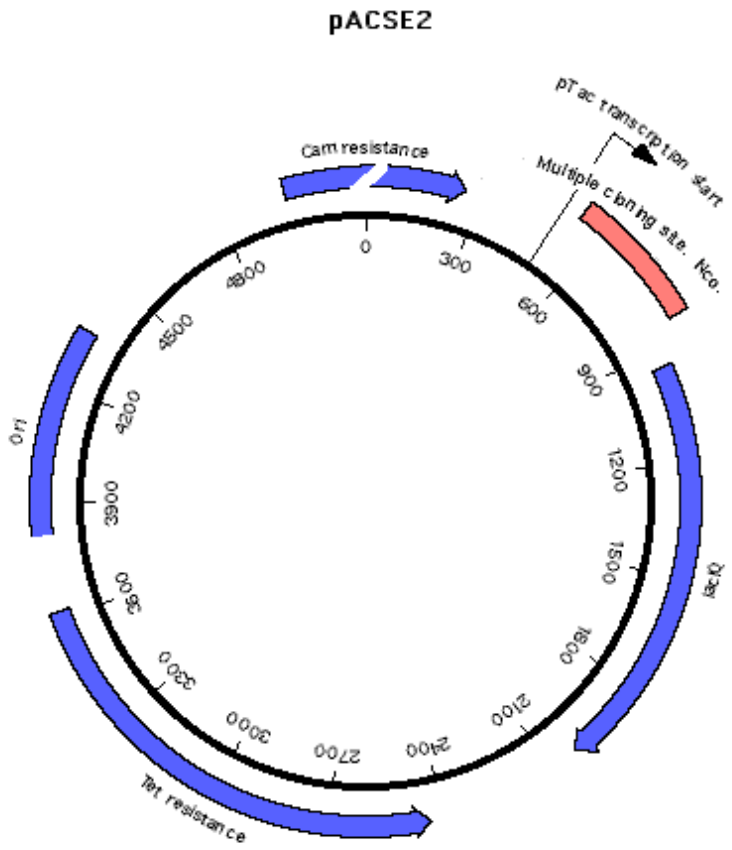
Keep Electroporation cuvettes in the cold room. Use cuvettes with 1.0 mm gap.

1. Thaw tubes of electrocompetent cells on ice in the cold room for a very few minutes. Proceed as soon as the tubes are thawed
2. Add 1 to 5 µl of DNA inTE buffer or water to the cells. Hold on ice 0.5 to 1.0 minutes.
3. Set the Gene Pulser (BioRad) electroporator to 25 µF and 1.6 kV, and the Pulse Controller at 200 Ω. (If using a different machine set as specified by the manufacturer of *E. coli*)
4. Using a cold pipetteman tip, transfer the cell-DNA mixture to a cold cuvette and pulsed.
5. **Immediately** add 1.0 ml of SOC {see Appendix III} at 30˚ to the cuvette and suspend the cells by gently shaking up and down. **Immediately** put the cuvette into a 37˚ dry bath or other device to keep the cells at 37˚
6. After all samples have been electroporated transfer the cells to a sterile 18 x 150 mm capped tube that contains 1.0 ml SOC and aerate at 37˚ for 1.5 hr to allow the antibiotic resistance gene on the plasmid to express. {If it is necessary to express at 30˚ allow two hours}
7. Plate onto selective medium, resuspending in buffer prior to plating if necessary; e.g. when selecting for growth on a particular carbon source or growth in the absence of a particular nutrient.

Appendix II pACSE plasmid vectors

pACSE2 ***used for cloning into the NcoI site***

pACSE2 (5201 bp) is derived from pACYC184 and carries the p15A origin of replication that results in a copy number of 5-10 per cell. It carries a multiple cloning site (MCS) cartridge (bp 534-860), a tetracycline resistance gene, and the lac repressor gene *lacI*^{q}. Genes cloned into the MCS are expressed from the powerful pTAC promoter (bp 347-533) under control of the *lacI*^{q} repressor. The unique NcoI site is at the initiation codon for expression from the pTAC promoter.

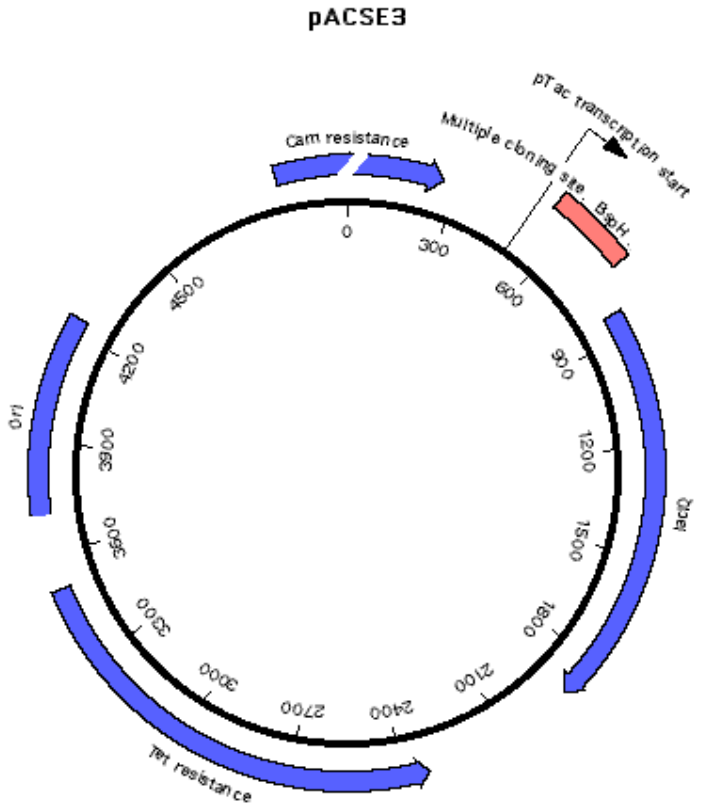


The unique sites within the MCS are:

Alphabetical order		Sequence order	
HpaI	727	**NcoI**	534
KpnI	719	MfeI	633
MfeI	633	KpnI	719
NcoI	534	HpaI	727
NotI	783	StuI	766
NsiI	775	NsiI	775
PstI	794	NotI	783
SacI	844	PstI	794
StuI	766	XhoI	843
XhoI	843	SacI	844

pACSE3 ***used for cloning into the BspHI site***
pACSE3 (5087 bp) was created from pACSE2 by elimination of a BspHI site within the tet gene, converting the NcoI site to a BspHI site, digesting with BspHI and self-ligating to remove the 114 bp 534-648 fragment within the cloning cartridge. The unique BspHI site is at the initiation codon for expression from the pTAC promoter.

The BspHI site is methylated by the *dam* methylase and when methylated is not cut by BspHI. ***pACSE3 must be grown in a dam⁻ strain such as GM19 in order for it to be digested by BspHI***.



The unique sites within the MCS are:

Alphabetical order		Sequence order	
BspHI	534	**BspHI**	534
HpaI	613	KpnI	605
KpnI	605	HpaI	613
NotI	669	StuI	652
NsiI	663	NsiI	663
PstI	680	NotI	669
SacI	730	PstI	680
StuI	652	XhoI	729
XhoI	729	SacI	730

Appendix III Recipes

L-broth
Per liter:
10 g Bacto tryptone (Difco)
5 g Bacto yeast extract (Difco)
10 g NaCl
10 g glucose

YENB
7.5 g Bacto Yeast extract (Difco) + 8 g Bacto Nutrient Broth (Difco) per liter

SOC
2% (w/v) Bacto tryptone (Difco)
0.5% (w/v) Bacto yeast extract (Difco)
10 mM NaCl
2.5 mM KCl
10 mM $MgSo_4$
20 mM glucose

Appendix IV Boiling DNA plasmid preps

Method 1

1. Using a sterile toothpick suspend a medium to large colony in 50 µl water in a thick-wall 0.5 ml microfuge tube. Be sure not to pick up any agar as agar will inhibit downstream reactions such as PCR. Close tube cap tightly
2. Heat at 100˚ in a thermocycler for 5 minutes.
3. Spin one minute in microfuge
4. Use 1-5 µl of supe as template in PCR reaction

Method 2

1. Grow a 1 ml overnight culture in broth
2. Spin down 50 µl of overnight culture
3. Resuspend in 50 µl water or TE
4. Heat at 100˚ in a thermocycler for 5 minutes.
5. Spin one minute in microfuge
6. Use 1-5 µl of supe as template in PCR reaction